Structure of a Complex-Type Sugar Chain of Human Glycophorin A†

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ABSTRACT: Tryptic glycopeptide T1 of human glycophorin A [Tomita, M., & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2964-2968] was subjected to hydrazinolysis. After N-acetylation, the complex-type oligosaccharides were isolated by gel filtration. The major neutral oligosaccharide (2000 molecular weight) was purified by a combination of ion-exchange and gel-permeation chromatography. Treatments with endo- and exo-glycosidases, periodate oxidation, and methylation analysis indicated that the major neutral oligosaccharide has the following structure: Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc. This oligosaccharide was retained by *Ricinus communis* agglutinin–Sepharose and retarded by Sepharose 4B coupled with erythroagglutinating E4 isolectin from *Phaseolus vulgaris*. Retention by concanavalin A–Sepharose was observed only after treatment of the oligosaccharide with β-galactosidase.

The sialoglycoprotein of the human erythrocyte membrane has been extensively studied in several different laboratories (Marchesi et al., 1976). The major component, designated as glycophorin A, corresponds to the major $NaIO_4$–Schiff positive bands (PAS-1 and -2; Steck, 1974) on sodium dodecyl sulfate–polyacrylamide gels. Glycophorin A is one of the major intrinsic transmembrane proteins of the human erythrocyte (Bretscher, 1971; Furthmayr et al., 1975). Tomita and co-workers demonstrated that glycophorin A was composed of 131 amino acids and an average of 16 oligosaccharide chains, 15 of which are linked to serine or threonine residues, the other being attached to asparagine (Tomita & Marchesi, 1975; Tomita et al., 1978). The major part of serine- or threonine-linked sugar chains released by alkaline hydrolysis was a tetrasaccharide with the structure AcNeuα2→3Galβ1→3(AcNeuα2→6)GalNAc (Thomas & Winzler, 1969). Two different structures have been proposed for asparagine-linked sugar chains (Kornfeld & Kornfeld, 1970, 1971; Thomas & Winzler, 1971), but neither of the structures is conclusive.

We have previously shown that an oligosaccharide prepared from the band-3 glycoprotein of human erythrocyte membrane has a unique structure containing →3Galβ1→4GlcNAcβ1→ repeating units (Tsuji et al., 1980). We now describe in this paper the isolation and characterization of an asparagine-linked sugar chain from a tryptic fragment, T1, of human glycophorin A and its structure. We also demonstrate that this oligosaccharide can be bound by several different lectins.

Experimental Procedures

Isolation of a Tryptic Glycopeptide, T1, from Human Erythrocytes. Glycopeptides of glycophorin were prepared by digestion of outdated and pooled human erythrocytes (type OMN) with trypsin (TRL grade, Worthington) and purified by DEAE-cellulose ion-exchange chromatography according to Winzler et al. (1967). The sialoglycopeptides thus separated were further fractionated by gel filtration on a column of Sephadex G-150 (Pharmacia) according to Tomita et al. (1978). The fractions corresponding to T1 of glycophorin A were pooled and used for this study.

Analytical Procedures. Neutural sugars and amino sugars in sialoglycopeptides were analyzed according to Spiro (1972). Sialic acid was determined by $NaIO_4$–resorcinol (Jourdian et al., 1971). Amino acid analysis was performed with a Hitachi 034 amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for 24 h. Oligosaccharides were hydrolyzed in 2 N HCl at 100 °C for 3 h, and the released monosaccharides were converted to alditol acetates and analyzed by gas–liquid chromatography on a column (0.3 × 100 cm) of 0.5% (w/w) ECNSS-M on Gas Chrom Q (80–100 mesh). Total neutral sugars were assayed by the phenol–H_2SO_4 reaction (Dubois et al., 1956).

Isolation and Fractionation of Oligosaccharides from the Tryptic Fragment of Glycophorin A. Sialoglycopeptides T1 (150 mg) were subjected to hydrazinolysis to release asparagine-linked sugar chains (Fukuda et al., 1976). The oligosaccharides released were dissolved in 2 mL of 4.5 M sodium acetate solution and N-acetylated at room temperature by the additions of 5 portions of 1.0 mL of acetic anhydride at 10-min intervals. After 1 h with occasional mixing, the reaction mixture was applied to a column (1.5 × 95 cm) of Bio-Gel P-4 (−400 mesh) (Bio-Rad) and eluted with distilled water at room temperature. The oligosaccharides from the asparagine-linked sugar chain were further fractionated by ion-exchange chromatography on a column of DEAE-Sephacel (Pharmacia). The column (1.2 × 15 cm) was equilibrated with 4 mM pyridine–1 mM acetate buffer (pH 6.0), and the oligosaccharides were eluted with the same buffer and then with a gradient from 4 mM/1 mM to 400 mM/100 mM of pyridine/acetate buffer. The neutral oligosaccharides were further fractionated on a column of Bio-Gel P-4 (−400 mesh; 1.5 × 200 cm) as described by Sabbagh & Fagerson (1973).

Radioisotope Labeling of Oligosaccharide. Tritiated borohydride reduction was performed according to Takasaki & Kobata (1978). About 100 nmol of oligosaccharide was reduced with 5 μmol (0.25 mCi) of NaB^3H_4 (New England Nuclear) in 300 μL of 0.01 M NaOH at 25 °C for 4 h, 10 mg of $NaBH_4$ was then added, and the reaction was allowed to continue at 25 °C for another 2 h. The reaction was stopped by adding one drop of glacial acetic acid. After the mixture was passed through a small column of Dowex 50W-X8 (H^+ form), the boric acid was removed by repeated evaporation with methanol. Radioactive contaminants from NaB^3H_4 were removed by descending paper chromatography for 16 h on Whatman No. 1 paper in 1-butanol–ethanol–H_2O (16:1:4) (Yamashita et al., 1978).

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Glycosidase Treatment of Tritium-Labeled Oligosaccharides. β-Galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal (Li & Li, 1972). α-L-Fucosidase from *Charonia lampas* and endo-β-*N*-acetylglucosaminidase D from *Diprococcus pneumonea* were purchased from Seikagaku Kogyo. Snail β-mannosidase (Sugahara & Yamashina, 1972) was kindly supplied by Dr. A. Shibata, Seikagaku Kogyo. NaB^3H_4-reduced oligosaccharides (1–4 × 10^4 cpm, about 0.5–2 nmol) were digested at 37 °C with glycosidases (0.2–4.0 unit) in 0.1 mL of the appropriate buffers under a toluene layer for 18–24 h followed by heating at 100 °C for 3 min to terminate the reaction. In the case of β-*N*-acetylhexosaminidase digestion, oligosaccharides were incubated with 40 units of the enzyme in 0.1 mL of the buffer for 96 h. Then the reaction mixture was passed through small columns of Dowex 50W-X8 (H^+ form) and Bio-Rad AG-3 (OH^- form). Digestions with β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were carried out in 0.05 M sodium acetate buffer (pH 4.0). For digestions with α-L-fucosidase and β-mannosidase, 0.1 M sodium citrate–0.1 M sodium phosphate buffer (pH 4.0) containing 0.5 M NaCl was used. Endo-β-*N*-acetylglucosaminidase digestion was carried out in 0.1 M sodium citrate–0.1 M sodium phosphate buffer (pH 6.0).

High-Performance Gel-Permeation Chromatography. In order to analyze purity and molecular size of oligosaccharides, gel-permeation chromatography was performed by using a high-pressure liquid chromatograph (Tri-Rotar, Jasco) equipped with two columns (0.8 × 50 cm) of Bio-Gel P-4 (–400 mesh). Elution was performed with Millipore (0.45 μm) filtered and twice-distilled water at a flow rate of 0.3 mL/min. During this operation, the columns were maintained at 55 °C by circulating warm water in jackets. Reduced oligosaccharides prepared by hydrazinolysis of the unit B glycopeptide from porcine thyroglobulin with the structures Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcol and Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcol and their glycosidase-digested derivatives were used as standards. Oligosaccharides with a tritium label at their reducing end were also used. Details on the structures of these oligosaccharides, a part of which we have previously described (Kondo et al., 1977), will be published elsewhere. A mixture of oligomers of *N*-acetylglucosamine was obtained by a partial hydrolysis of chitin according to Rupley (1964) and was also used as a standard. *N*-Acetylglucosamine oligomers were useful as internal standards when tritrium-labeled oligosaccharides are analyzed. The elution of oligosaccharides was monitored by a variable-wavelength ultraviolet spectrophotometer (Uvidec 100-II, Jasco). Oligomers of glucose were also used as standards (Nishigaki et al., 1978). The elution was monitored by a refractive index detector (Shodex SE-11, Showa Denko). Fractions of 0.3 mL or 0.45 mL were collected, and aliquots were counted in a liquid-scintillation counter.

High-Voltage Paper Electrophoresis. High-voltage paper electrophoretic analyses of NaB^3H_4-reduced monosaccharides were carried out with a Fujiox high-voltage electrophoresis unit (Fujiriken) on Whatman No. 1 paper in 0.06 M sodium borate buffer (pH 9.6) according to Takasaki & Kobata (1978).

Methylation Analysis. Oligosaccharides after reduction with NaB^2H_4 were methylated by the method of Hakomori (1964). The permethylated products were purified on a small column of Sephadex LH-20 (Pharmacia) and subjected to acetolysis, hydrolysis, reduction, and acetylation as described by Stellner et al. (1973). Alditol acetates of partially methylated sugars were analyzed by gas–liquid chromatography on a column (0.3 × 200 cm) of 3% (w/w) OV-225 or 1% (w/w) OV-1 on Gas Chrom Q (80–100 mesh). Gas chromatographic peaks were identified by a mass spectrometer (Shimadzu-LKB 9000, Shimadzu). The conditions for the mass spectrometry were the following: ion-source temperature, 270 °C; separation temperature, 200 °C; ionizing potential, 70 eV; trap current, 60 μA.

$NaIO_4$ Oxidation. NaB^3H_4-reduced oligosaccharides were oxidized in 0.2 mL of 0.05 M sodium acetate buffer (pH 4.0) containing 0.075 M $NaIO_4$ at 4 °C for 24, 48, and 96 h in the dark. The reaction was terminated by adding 10 μL of ethylene glycol. After 1 h at room temperature, 1 mL of 0.1 M sodium borate buffer (pH 8.0) containing 0.1 M $NaBH_4$ was added, and the mixture was incubated at 25 °C for 4 h. After decomposition of the borohydride with glacial acetic acid, the reaction mixture was passed through a small column of Dowex 50W-X8 (H^+ form), and boric acid was removed by repeated evaporation with methanol. The sample was then hydrolyzed in 0.05 M H_2SO_4 at 80 °C for 1 h followed by passage through a small column of Bio-Rad AG-1 (formate form). The effluent was evaporated to dryness, and the product was analyzed by gel-permeation chromatography on a column of Bio-Gel P-4.

Lectin–Sepharose Affinity Chromatography of Oligosaccharides. In order to elucidate the interaction of oligosaccharides with lectins, the following lectins coupled to Sepharose 4B were used. Concanavalin A (Con A)[1]–Sepharose was from Pharmacia. *Ricinus communis* agglutinin (RCA) was purified by the method of Tomita et al. (1972) and coupled to Sepharose 4B (RCA–Sepharose) as described previously (Matsumoto & Osawa, 1972). Erythroagglutinating *Phaseolus vulgaris* agglutinin (E-PHA)–Sepharose and leucoagglutinating *P. vulgaris* agglutinin (L-PHA)–Sepharose were from E. Y. Laboratory. NaB^3H_4-reduced oligosaccharides were applied to a small column of lectin–Sepharose (0.5 × 1.5 cm for Con A–Sepharose or RCA–Sepharose and 0.25 × 3.5 cm for E-PHA–Sepharose or L-PHA–Sepharose), and the columns were eluted with 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Absorbed radioactive oligosaccharides were eluted with 0.2 M sodium borate buffer (pH 8.0) (Svensson et al., 1970; Kennedy & Rosevear, 1973). In the case of Con A–Sepharose, the column was further washed with 0.2 M methyl α-D-mannoside in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Results

Isolation of T1 Glycopeptide of Glycophorin A. Tryptic fragments from intact human erythrocytes were isolated according to Winzler et al. (1967). Gel filtration of these fragments on Sephadex G-150 yielded two peaks corresponding to two sialoglycopeptides obtained from purified glycophorin A by trypsin digestion, namely, T1 and T2 (Tomita et al., 1978), in good agreement with the result reported by Furthmayr (1978). We pooled the fractions corresponding to T1. Analytical results showed amino acid and carbohydrate compositions were almost identical with those of T1 derived from

[1] Abbreviations used: Con A, concanavalin A; GlcNAcol, *N*-acetylglucosaminitol; E-PHA, erythroagglutinating *Phaseolus vulgaris* agglutinin; L-PHA, leucoagglutinating *Phaseolus vulgaris* agglutinin; RCA, *Ricinus communis* agglutinin.

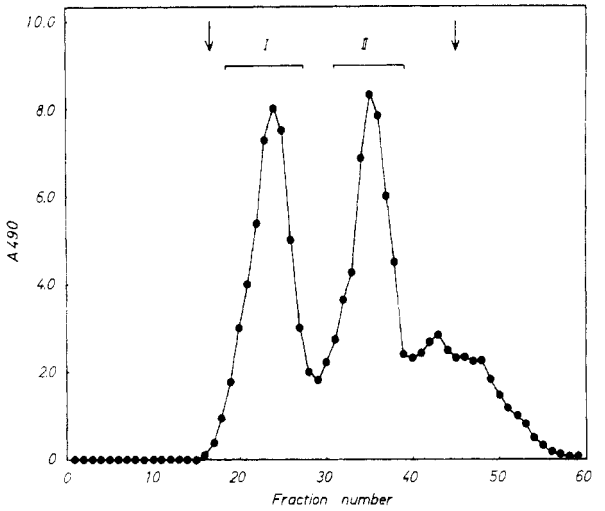

FIGURE 1: Gel chromatography of hydrazinolysate of sialoglycopeptide T1 after N-acetylation. The products were chromatographed in distilled water on a column of Bio-Gel P-4 (−400 mesh, 1.5 × 95 cm) at a flow rate of 15 mL/h. Each 3.6-mL fraction was collected and assayed for neutral sugars. Fractions were pooled as noted and lyophilized.

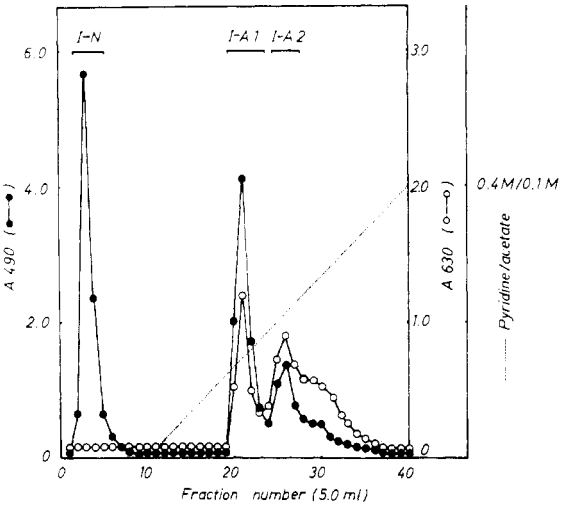

FIGURE 2: Ion-exchange chromatography of oligosaccharide from Bio-Gel P-4 column. DEAE-Sephacel column (formate form) (1.2 × 15 cm) was washed with 4 mM pyridine–1 mM acetate buffer, pH 6.0, after eluting the column with the same buffer; elution was carried out with a 150 mL at linear gradient of 4 mM pyridine–1 mM acetate (pH 6.0) to 400 mM pyridine–100 mM acetate (pH 5.5). Each 5.0-mL fraction was analyzed for neutral sugar (●) and sialic acid (○). Fractions were pooled as noted and lyophilized.

Table I: Carbohydrate Compositions of Oligosaccharide Fractions

carbohydrate	I[a]	II[b]	I-N-1[a]	I-N-2[a]	I-N-3[a]
fucose	1.2	0.1	1.7	1.6	1.0
mannose	3.0	0.1	3.0	3.0	3.0
galactose	3.3	1.0	5.3	3.7	2.2
N-acetylglucosamine	6.1	0.2	8.4	6.9	4.9
N-acetylgalactosamine	0.0	0.9	0.0	0.0	0.0
sialic acid	0.9	1.4	0.0	0.0	0.0

[a] Molar ratios normalized to 3.0 mol of mannose. [b] Molar ratios normalized to 1.0 mol of galactose.

purified glycophorin A as analyzed by Tomita et al. (1978).

Oligosaccharide from T1 Glycopeptide. When N-acetylated products of hydrazinolysis were subjected to gel filtration on Bio-Gel P-4, two major peaks of oligosaccharides were detected (Figure 1). As shown in Table I, the carbohydrate analysis showed that fraction I consisted of complex-type sugar chains containing mannose, galactose, fucose, glucosamine, and sialic acid. Fraction II was mainly composed of serine- or threonine-linked sugar chains. Ion-exchange chromatography of fraction I on a column of DEAE-cellulose resulted in the separation of one neutral and two sialic acid containing oligosaccharides, as shown in Figure 2 The relative proportions of I-N, I-A1, and I-A2 were not always reproducible in separate experiments, presumably depending on the degree of desialization during storage of erythrocytes. I-N, I-A1, and I-A2 had virtually identical carbohydrate compositions except that the proportion of sialic acid in I-A2 was twice as much as in I-A1. This indicated that I-A1 and I-A2 were possibly a monosialylated and a disialylated oligosaccharide, respectively. In some experiments, a small peak of a more acidic oligosaccharide, which appeared to be trisialylated, was also observed. Fraction I-N was further fractionated on a Bio-Gel P-4 column. As shown in Figure 3, I-N gave one major peak and two additional peaks with higher molecular weights. The results of the carbohydrate analysis showed that the oligosaccharides of higher molecular weights had a higher content of galactose and glucosamine. The major oligosaccharide I-N-3 contained fucose, galactose, mannose, and *N*-acetylglucosamine in a molar ratio of 1:2:3:5.

Determination of the Size of Oligosaccharide I-N-3. In order to determine the size and also to check the purity, I-N-3

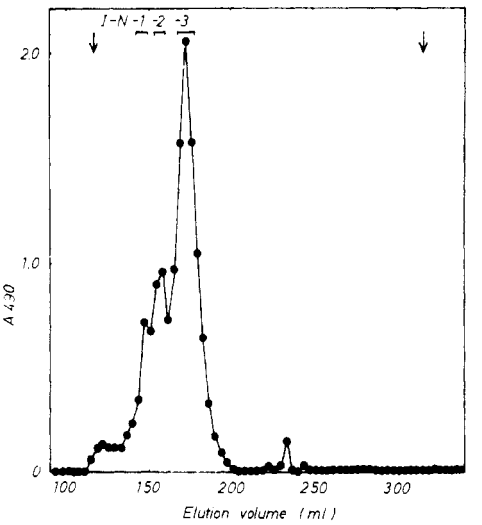

FIGURE 3: Gel chromatography of neutral oligosaccharide fraction on Bio-Gel P-4 according to Sabbagh & Fagerson (1973). Two columns (1.5 × 100 cm) maintained at 55 °C were eluted with distilled water at a flow rate of 15 mL/h. Each 3.6-mL fraction was collected and analyzed for neutral sugar. Arrows indicate the eluting positions of blue dextran and glucose. Fractions were pooled as noted and lyophilized.

was subjected to high-performance gel-permeation chromatography. When the eluate was monitored by the absorbance at 210 nm, a single synmetric peak was observed between the elution positions of the authentic Gal→GlcNAc→(Gal→GlcNAc→)Man→(Gal→GlcNAc→Man→)Man→GlcNAc→(Fuc→)GlcNAcol and Gal→GlcNAc→Man→(Gal→GlcNAc→Man→)Man→GlcNAc→(Fuc→)GlcNAcol, as shown in Figure 4. The mobility corresponded to 7.4 *N*-acetylglucosamine residues. This result suggests that oligosaccharide I-N-3 is a single molecular species having a molecular weight of about 2000.

Analysis with Glycosidases Utilizing Tritium-Labeled Oligosaccharide. When NaB^3H_4-reduced oligosaccharide I-N-3 was digested with α-L-fucosidase or β-galactosidase, the decrease in size corresponding to one fucose or two galactose residues was detected by high-performance gel-permeation

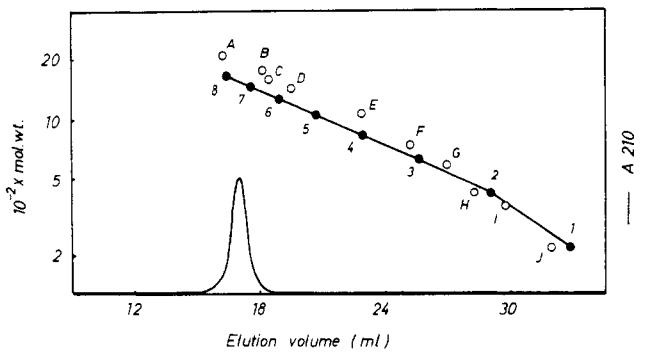

FIGURE 4: Molecular size analysis of major neutral oligosaccharide on high-performance gel-permeation chromatography. Closed circles (1–8) indicate the eluting positions and calculated molecular weights of chitin oligosaccharides. (A)-(I) indicate the eluting positions and calculated molecular weights of authentic standard oligosaccharide alcohols from porcine thyroglobulin: (A) Gal→GlcNAc→(Gal→-GlcNAc→)Man→(Gal→GlcNAc→Man)Man→GlcNAc→(Fuc→)GlcNAcol; (B) Gal→GlcNAc→Man→(Gal→GlcNAc→Man→)-Man→GlcNAc→(Fuc→)GlcNAcol; (C) GlcNAc→(GlcNAc→)-Man→(GlcNAc→Man→)Man→GlcNAc→(Fuc→)GlcNAcol; (D) GlcNAc→Man→(GlcNAc→Man)Man→GlcNAc→(Fuc→)-GlcNAcol; (E) Man→(Man→)Man→GlcNAc→(Fuc→)GlcNAcol; (F) Man→GlcNAc→(Fuc→)GlcNAcol; (G) GlcNAc→(Fuc→)-GlcNAcol; (H) GlcNAc→GlcNAcol; (I) Fuc→GlcNAcol; (J) GlcNAcol. Experimental details are in the text.

chromatography (Figure 5a,b). No change of the size was observed after repeated treatments with β-*N*-acetylhexosaminidase (data not shown). When β-galactosidase-treated I-N-3 was digested with β-*N*-acetylhexosaminidase, the product comigrated with Man→(Man→)Man→GlcNAc→(Fuc→)GlcNAcol, and a decrease in size corresponding to 2.1 *N*-acetylglucosamine residues was observed on a Bio-Gel P-4 column (Figure 5c). However, the decrease in size after β-*N*-acetylhexosaminidase treatment of authentic GlcNAc→-(GlcNAc→)Man→(GlcNAc→Man→)Man→GlcNAc→-(Fuc→)GlcNAcol was found to correspond to 2.5 *N*-acetylglucosamine residues, on a Bio-Gel P-4 column, as shown in Figures 4 and 5. Therefore, the structure of the β-galactosidase-treated I-N-3 can be assumed to be different from that of the authentic sample described above. Furthermore, the β-galactosidase- and β-*N*-acetylhexosaminidase-treated I-N-3 released two mannosyl residues by digestion with α-mannosidase (Figure 5d), and subsequent β-mannosidase digestion gave a radioactive peak at the position corresponding to that of GlcNAc→(Fuc→)GlcNAcol (Figure 5e). I-N-3 which had previously been digested with β-galactosidase and then β-*N*-acetylhexosaminidase was further treated with endo-β-*N*-acetylglucosaminidase D from *Diprococcus pneumonea*. This yielded a oligosaccharide which comigrated with authentic Fuc→GlcNAcol (Figure 5f). Digestion of this material with α-L-fucosidase resulted in a radioactive product which comigrated with GlcNAcol (Figure 5g). This final product was identified as *N*-acetylglucosaminitol, when analyzed by high-voltage paper electrophoresis in borate buffer. These results showed that the sugar sequence of oligosaccharide I-N-3 was $(Gal)_2$-$(GlcNAc)_3$-$(Man)_3$-GlcNAc-(Fuc-)GlcNAc.

Methylation Analysis. The results of methylation analysis of oligosaccharide I-N-3 are shown in Table II. Despite the fact that no decrease in size was detected after β-*N*-acetylhexosaminidase treatment, the presence of 1 mol of nonreducing terminal *N*-acetylglucosamine was detected. In good agreement with the results from enzymatic degradation, the reducing terminal was found to be *N*-acetylglucosamine substituted at positions 4 and 6, half of which was detected as 1,3,5-tri-*O*-methyl-2-deoxy-2-(acetylacetamido)-4,6-di-*O*-acetylglucitol (Hase & Lietschel, 1976). Three of five glu-

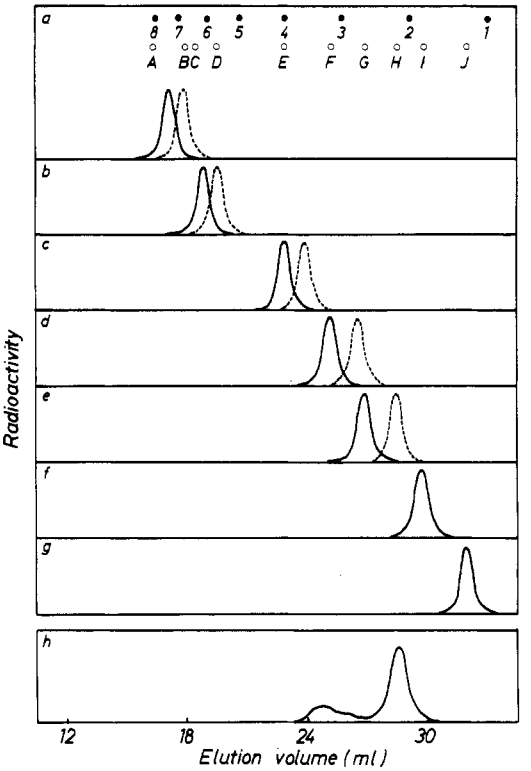

FIGURE 5: Elution profile of NaB^3H_4-labeled oligosaccharide I-N-3 after glycosidase digestion or $NaIO_4$-oxidation mild acid hydrolysis. Experimental details are described in the text. After glycosidase digestion or $NaIO_4$-oxidation mild acid hydrolysis, NaB^3H_4-reduced oligosaccharides were analyzed on high-performance gel-permeation chromatography. Each 0.3- or 0.45-mL fraction was collected and counted in a liquid-scintillation counter. Closed circles and open circles [(1–8) and (A–J)] as in Figure 4. The dotted line indicated the elution profiles of NaB^3H_4-reduced oligosaccharides first treated with α-L-fucosidase and subjected to sequential glycosidase digestion. (a) Original oligosaccharide; (b) product of β-galactosidase treatment of (a); (c) product of β-*N*-acetylhexosaminidase treatment of (b); (d) product of α-mannosidase treatment of (c); (e) product of β-mannosidase treatment of (d); (f) product of endo-β-*N*-acetylglucosaminidase D treatment of (c); (g) product of α-L-fucosidase treatment of (f); (h) product of $NaIO_4$ oxidation (48 h)–mild acid hydrolysis of the original oligosaccharide.

cosamine residues were substituted at position 4. All fucose and galactose constituted nonreducing terminals. Two of the three mannosyl residues were detected as 3,4,6-tri-*O*-methyl derivatives, and another was 2-mono-*O*-methylmannitol. No di-*O*-methyl derivatives of mannitol were detected. NaB^2H_4-reduced oligosaccharide I-N-3 (about 300 nmol) was treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase (12.5 units each in 1 mL of incubation mixture) and separated on a column of Bio-Gel P-4. The oligosaccharide which was eluted at the position of the peak shown in Figure 5c was collected and subjected to the methylation analysis. Because of limited amounts of the sample available, the results shown in Table II are qualitative. However, the data indicate the absence of 2-mono-*O*-methylmannitol and simultaneous appearance of 2,4-di-*O*-methyl and 2,3,4,6-tetra-*O*-methyl derivatives, showing after removal of *N*-acetylglucosamine trisubstituted mannose was converted to disubstituted mannose.

$NaIO_4$ Oxidation Analysis. The Smith degradation of oligosaccharide I-N-3 resulted in a radioactive material which comigrated with Man→GlcNAc→GlcNAcol on a column of Bio-Gel P-4 (Figure 5h). It was confirmed that this product had the structure Manβ1→4GlcNAcβ1→4GlcNAcol by sequential digestion with β-mannosidase and β-*N*-acetylhexosaminidase (data not shown). However, minor additional peaks

Table II: Methylation Analysis of Oligosaccharides

linkage in oligosaccharides	methylated sugar	oligosaccharides: I-N-3	oligosaccharides: β-galactosidase- and β-N-acetylhexosaminidase-treated I-N-3
Fuc1→	2,3,4-tri-*O*-methylfucitol	0.9	–[a]
Gal1→	2,3,4,6-tetra-*O*-methylgalactitol	1.7	–
Man1→	2,3,4,6-tetra-*O*-methylmannitol	0.0	+[b]
→2Man1→	3,4,6-tri-*O*-methylmannitol	2.2	–
→6 →3Man1→	2,4-di-*O*-methylmannitol	0.0	+
→6 →4Man1→ →3	2-mono-*O*-methylmannitol	0.7	–
→6 →4GlcNAc	1,3,5-tri-*O*-methyl-2-(methylacetamido)glucitol	0.4	+
	1,3,5-tri-*O*-methyl-2-(acetylacetamido)glucitol	0.4	+
GlcNAc1→	3,4,6-tri-*O*-methyl-2-(methylacetamido)glucitol	1.0	–
→4GlcNAc1→	3,6-di-*O*-methyl-2-(methylacetamido)glucitol	3.2	+

[a] Not detected. [b] Detected.

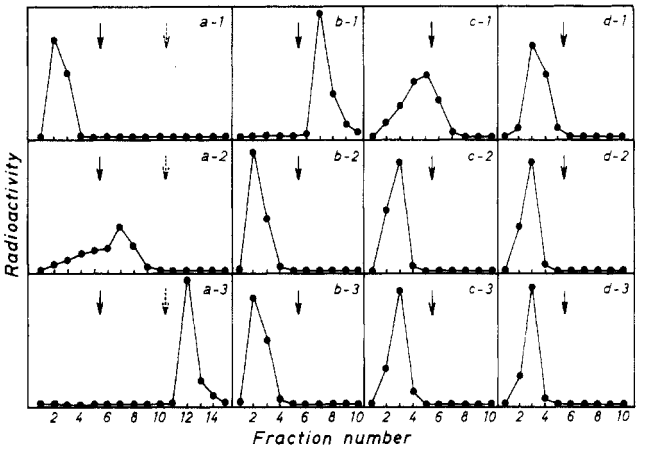

FIGURE 6: Behavior of NaB^3H_4-reduced oligosaccharide I-N-3 and its glycosidase-treated derivatives on columns of lectin–Sepharose. Oligosaccharides (2×10^3 cpm; about 0.1 nmol) were applied to the columns, incubated at room temperature for 30 min, and then eluted with 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Fractions 6–10 were eluted with 0.2 M sodium borate buffer (pH 8.0). Fractions 11–15 from Con A–Sepharose were eluted with 0.2 M methyl α-mannoside in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Elutions were performed at a flow rate of 1 fraction/10 min; (a) Con A–Sepharose (0.5 × 1.5 cm), each 0.4-mL fraction was collected; (b) RCA–Sepharose (0.5 × 1.5 cm), each 0.2-mL fraction was collected; (c) E-PHA–Sepharose (0.25 × 3.5 cm), each 0.2-mL fraction was collected; (d) L-PHA–Sepharose (0.25 × 3.5 cm), each 0.2-mL was collected. (a-1–d-1) Intact oligosaccharide; (a-2–d-2) β-galactosidase-treated oligosaccharide; (a-3–d-3) β-galactosidase- and β-*N*-acetylhexosaminidase-treated oligosaccharide.

were also detected (Figure 6). These peaks were presumably byproducts caused by $NaIO_4$ oxidation since they were also detected in the case of the Smith degradation of the authentic Gal→GlcNAc→Man→(Gal→GlcNAc→Man)Man→-GlcNAc→(Fuc→)GlcNAcol.

Interaction of Oligosaccharide with Various Lectins Conjugated to Sepharose 4B. Interactions of NaB^3H_4-labeled oligosaccharide I-N-3, its β-galactosidase-treated derivative, and its β-galactosidase- and β-*N*-acetylhexosaminidase-treated derivative with Con A, RCA, E-PHA, and L-PHA were studied by use of immobilized lectins. As shown in Figure 7, although the original oligosaccharide was absorbed by RCA–Sepharose and retarded by E-PHA–Sepharose, no or very little association was detected with both Con A–Sepharose and L-PHA–Sepharose. After β-galactosidase treatment, association with E-PHA–Sepharose and RCA–Sepharose was

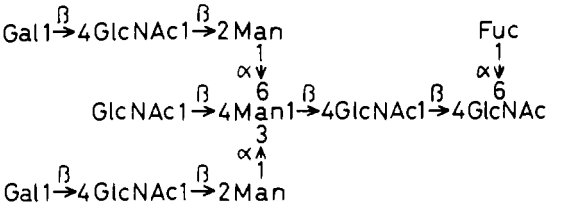

FIGURE 7: Proposed structure of oligosaccharide I-N-3.

diminished. On the other hand, the β-galactosidase-treated oligosaccharide was strongly retarded by Con A–Sepharose. The association with Con A–Sepharose was further increased after removal of *N*-acetylglucosamine residues.

Discussion

Two different structures were proposed previously for the asparagine-linked sugar chain of human glycophorin (Kornfeld & Kornfeld, 1970, 1971; Thomas & Winzler, 1971). One difficulty was presumably the extraordinary resistance of this glycoprotein to pronase treatment which prevented the isolation of a pure glycopeptide with a single sugar chain. In this study, oligosaccharides were isolated by hydrazinolysis. Although serine- or threonine-linked sugar chains were also released by this treatment, these were easily separated from complex-type sugar chains by gel filtration.

In this study, we prepared a tryptic fragment of glycophorin A by digesting intact erythrocytes (Winzler et al., 1967) and purified a sialoglycopeptide, T1, by ion-exchange chromatography (Winzler et al., 1967) and gel filtration as described by Tomita et al. (1978). Furthmayr (1978) and Tomita et al. (1978) suggested that the fragment prepared by digestion of intact erythrocytes or the whole glycophorin fraction was contaminated by small amounts of glycophorin B or C derived sialoglycopeptides. Most of these contaminating glycopeptides were found to be removed by ion-exchange chromatography on DEAE-cellulose. In fact, we confirmed that the T1 fragment prepared by digestion of intact erythrocytes is virtually identical with that prepared from purified glycophorin A in amino acid and carbohydrate compositions (data not shown). However, the possibility that the T1 fragment is still contaminated with small amounts of glycopeptides from the other erythrocyte glycoproteins cannot completely be excluded. The presence of minor neutral oligosaccharides (I-N-1 and I-N-2) in an I-N fraction must then be ascribed either to the contamination with oligosaccharides from the other erythrocyte glycoproteins or to possible heterogeneity of the complex-type

oligosaccharide chain on glycophorin A.

The purity of the major oligosaccharide (I-N-3) used for the structural analysis is based primarily on the molecular size, since differences in the molecular size of only a single neutral sugar residue are detectable by the gel-permeation chromatography system employed by us. This system is a modification of the system described by Sabbagh & Fagerson (1973), with an additional advantage of rapid analysis. The fact that after descending paper chromatography, glycosidase treatment, or affinity chromatography by use of immobilized lectins the NaB^3H_4-reduced oligosaccharide always behaves as a single molecular species strongly supports the homogeneity of the original oligosaccharide.

In the table of methylation analysis, values were normalized by using the results from authentic oligosaccharides of porcine thyroglobulin. Because there is no trisubstituted mannosyl residue in the complex-type sugar chain of porcine thyroglobulin, the value for 2-mono-*O*-methylmannitol in Table II is tentative. It is probable that the trisubstituted mannose is more resistant to hydrolysis.

β-*N*-Acetylhexosaminidase digestion of β-galactosidase-treated NaB^3H_4-reduced oligosaccharide I-N-3 resulted in an oligosaccharide which comigrated with authentic Man→-(Man→)Man→GlcNAc→(Fuc→)GlcNAcol. The decrease in molecular size was less than that from GlcNAc→-(GlcNAc→)Man→(GlcNAc→Man→)Man→GlcNAc→-(Fuc→)GlcNAcol and larger than that from GlcNAc→-Man→(GlcNAc→Man→)Man→GlcNAc→(Fuc→)GlcNAcol (Figure 5c), indicating the presence of two or three β-*N*-acetylglucosaminyl residues in a structure different from both of these oligosaccharides.

On the basis of these data, the most probable structure proposed for oligosaccharide I-N-3 is as shown in Figure 7. The *N*-acetylglucosamine residue which is directly linked to a β-mannosyl residue is not substituted by galactose because all three outer *N*-acetylglucosamines were removed by the Smith degradation. This structure is similar to one of the carbohydrate units of immunoglobulin A as described by Baenziger & Kornfeld (1974) which, however, lacks fucose. Baenziger & Kornfeld (1974) suggested that the terminal *N*-acetylglucosamine residue linked to β-mannosyl residue was resistant to cleavage by β-*N*-acetylhexosaminidase and that this linkage was only cleaved in the presence of α-mannosidase. The difference between their results and ours presumably depends on the ratio of enzyme to substrate since no release of *N*-acetylglucosamine was observed when the oligosaccharide was incubated with a low concentration (0.2 unit/0.1 mL) of the enzyme.

After treatment with neuraminidase (*Arthrobacter ureafaciens*, Nakarai Chemical Co.), oligosaccharide I-A1 and I-A2 gave similar elution profiles on Bio-Gel P-4 gel chromatography as I-N, having a major peak at the identical eluting position (data not shown). This material after being subjected to sequential glycosidase digestion gave results identical with those of I-N-3. These findings indicate that the structures of the major acidic oligosaccharides I-A1 and I-A2 except for sialylation are identical with I-N-3.

Baenziger & Fiete (1979) recently demonstrated that the glycopeptide from IgA, after desialization, was not bound by Con A–Sepharose. Removal of two galactose residues resulted in binding by Con A–Sepharose. Our results are consistent with theirs, indicating a structural similarity between I-N-3 and the sugar chain of IgA. We also found that oligosaccharide I-N-3 had high affinity for RCA–Sepharose and E-PHA–Sepharose. Thus, this sugar chain could be one of the major binding sites for these lectins on human erythrocytes. Interactions of a variety of other lectins with this oligosaccharide and its glycosidase-treated derivatives are under investigation.

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Ordered Conformation of Polypeptides and Proteins in Acidic Dodecyl Sulfate Solution†

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ABSTRACT: The conformation of some polypeptides and proteins in sodium dodecyl sulfate ($NaDodSO_4$) solutions was studied by circular dichroism. The type and extent of induced structure depend on their helix- and β-forming potential. Anionic side groups in segments of helix or β form tend to destabilize the ordered structure unless they are protonated. β-Endorphin has one Glu inside a predicted helical segment; its helicity in a $NaDodSO_4$ solution is enhanced at pH below 4. α-Melanocyte-stimulating hormone having a Glu in a β segment undergoes a pH-induced coil to β transition in 1.25 mM $NaDodSO_4$ (excess surfactant will disrupt the β form). Reduced somatostatin assumes a β form in 2 mM $NaDodSO_4$ and a partial helix in 25 mM $NaDodSO_4$, both of which are unchanged in acidic pH because it lacks -COOH groups. The unordered gastrin with five consecutive Glu's becomes helical in a $NaDodSO_4$ solution at pH 4. Neurotensin with one Glu has no structure-forming potential and is unordered in both neutral and acidic $NaDodSO_4$ solutions. This charge effect also manifests in segments of ordered structure for polypeptides and proteins such as glucagon, cytochrome *c*, parvalbumin, ribonuclease A, and lysozyme. The effect is especially predominant in tropomyosin that is rich in clusters of anionic side groups. Its more than 90% helicity is reduced to about one-half in a neutral $NaDodSO_4$ solution, but most of it can be restored by lowering the pH to 2-4.

$NaDodSO_4$[1] is known to alter the helicity of many proteins in aqueous solution (Reynolds & Tanford, 1970; Visser & Blout, 1971; Jirgensons, 1976; Mattice et al., 1976; Su & Jirgensons, 1977). Proteins of low helicity often become more ordered and those having more than 50% helix less ordered. Some proteins that are rich in β form can actually be converted into helices in excess $NaDodSO_4$ solutions; notable examples are concanavalin A (Kay, 1970) and elastase (Visser & Blout, 1971). These findings make it difficult to interpret the change in conformation of proteins in surfactant solutions.

Previously, we reported that the conformation of oligo- and polypeptides in surfactant solutions depends on the amino acid sequence which in turn dictates the structure-forming potentials (Wu & Yang, 1978; Yang & Wu, 1978). In a $NaDodSO_4$ solution the anionic surfactant first binds to the cationic side groups of polypeptides. Additional surfactant ions then cluster around the polypeptide chain, segments of which are induced to adopt an ordered structure. At low molar surfactant/peptide ratio a β form can exist if a segment has the β-forming potential, but excess surfactant usually disrupts the β form and may convert it into a helix if the segment also has the helix-forming potential. Polypeptides without any structure-forming potential remain unordered regardless of the surfactant concentration used. We report herein the charge effect on the conformation of oligopeptides and proteins in $NaDodSO_4$ solution. Anionic Glu and Asp residues can destabilize the induced conformation if they are located in the structure-forming segments of the polypeptide chain. While these like-charged residues will repel each other, thus weakening an ordered structure, it is the electrostatic repulsion between the negatively charged Glu and Asp and $DodSO_4^-$ that will hinder the clustering of the latter anions onto the polypeptide chain. Thus, even a lone charged Glu or Asp strategically located can interfere with the formation of an induced helix or β segment. The pH effect on the conformation of protein-surfactant complexes has been studied by Jirgensons (1976) and Su & Jirgensons (1977), but the quantitative aspect of such effect has not yet been established.

Experimental Section

Materials. Synthetic α-MSH (Lot C 0126), bovine neurotensin (Lot A 1024), and somatostatin (Lot D 1226) were purchased from Beckman and horse heart cytochrome *c* and bovine pancreas RNase A from Schwarz/Mann. Synthetic human β-endorphin was obtained from Peninsula Laboratories and egg white lysozyme from Sigma Chemical Co. Parvalbumin B from carp muscle was prepared by the method of Pecherer et al. (1971) and tropomyosin by the method of Cummins & Perry (1973).

Polypeptides and proteins were dissolved in water, except that tropomyosin was in buffers of 0.1 ionic strength (Conway, 1952). The concentrations of parvalbumin and tropomyosin were determined by micro-Kjeldahl analysis, using 15.8% and 16.6% as the nitrogen contents, respectively. Because of limited supplies the concentrations of α-melanocyte-stimulating hormone, neurotensin, and somatostatin were based on the

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; α-MSH, α-melanocyte-stimulating hormone; RNase, ribonuclease; CD, circular dichroism.

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