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The Carbohydrate Moiety of Band-3 Glycoprotein of Human Erythrocyte Membranes

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Band-3 glycoprotein was purified from human blood-group-A erythrocyte membranes by selective solubilization and gel chromatography on Sepharose 6B in the presence of sodium dodecyl sulphate. The purified glycoprotein was subjected to hydrazinolysis in order to release the carbohydrate moiety. The released oligosaccharides were *N*-acetylated and applied to a column of DEAE-cellulose. Most of the band-3 oligosaccharides obtained were found to be free of sialic acids. When this neutral fraction was subjected to gel chromatography on a column of Sephadex G-50, two broad peaks were observed indicating that the band-3 glycoprotein was heterogeneous in the size of the oligosaccharide moieties. All fractions from gel chromatography were found to contain galactose, mannose, *N*-acetylglucosamine and fucose. The higher-molecular-weight (mol.wt. 3000–8000) peak consisted of fucose, mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in a molar proportion of 1.6:3.0:8.4:10.5:0.2. Most of these oligosaccharides were digested with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase after α-L-fucosidase treatment to give a small oligosaccharide with the structure αMan_2–βMan–βGlcNAc–GlcNAc. Methylation studies and limited degradation by nitrous acid deamination showed that the oligosaccharides contained the repeating disaccharide Galβ1→4GlcNAcβ1→3, with branching points at C-6 of some of the galactose residues. These results indicate that a major portion of the band-3 oligosaccharide has a common core structure, with heterogeneity in the numbers of the repeating disaccharides, and contains fucose residues both in the peripheral portion and in the core portion. Haemagglutination tests were also carried out to determine the blood-group specificities of the glycoprotein and the results demonstrated the presence of both blood-group-H and I antigenic activities.

Band-3 glycoprotein, also known as protein E, is one of the predominant intrinsic proteins of the human erythrocyte membrane (Fairbanks *et al.*, 1971; Bretscher, 1971; Jenkins & Tanner, 1975). This glycoprotein has a molecular weight of approx. 90000–100000 and has recently attracted the interest of numerous investigators because of its many biological functions, such as those related to membrane transport (Cabantchik & Rothstein, 1974; Brown *et al.*, 1975), lectin receptors (Findlay, 1974; Adair & Kornfeld, 1974; Yokoyama *et al.*, 1978) and blood-group-I activity (Childs *et al.*, 1978). Heterogeneity of band-3 glycoprotein with respect to the degree of glycosylation and the type of sugar chains has been suggested (Yu & Steck, 1975; Drickamer, 1978) since it migrates as a diffuse band in sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Concerning the sugar moiety of this glycoprotein, Drickamer (1978) reported that the carbohydrate appeared to be attached to a single site in the *C*-terminal third of the molecule and was heterogeneous in size. Genetic variants of band-3 glycoprotein were found in blood group i and En(a–) erythrocytes by Fukuda *et al.* (1979) and by Gahmberg *et al.* (1976). The oligosaccharide from band-3 glycoprotein from En(a–) erythrocytes had a higher molecular weight than that from normal erythrocytes. On the other hand band-3 glycoprotein prepared from blood-group-i erythrocytes was found to have oligosaccharides with a lower molecular weight and a lower degree of branching than those from blood-group I erythrocytes.

Abbreviation used: GlcNAcol, *N*-acetylglucosaminitol.

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In this paper, we describe the isolation of oligosaccharrides from the band-3 glycoprotein and also the partial elucidation of their chemical structures.

Materials and Methods

Analytical procedures

Analyses of glycoprotein were performed as described below. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out according to Fairbanks *et al.* (1971), except that 7.5% (w/v) polyacrylamide gels were used to distinguish band-3 glycoprotein from the periodate/Schiff-1 glycoprotein. Amino acid analysis was performed with a Hitachi 034 amino acid analyser, after hydrolysis for 24 and 48 h in 6 M-HCl at 110°C. Tryptophan was determined on unhydrolysed glycoprotein samples by the spectrophotometric method of Goodwin & Morton (1946). Half-cystine was determined as cysteic acid after performic acid oxidation by the method of Moore (1963). Neutral sugars were determined by g.l.c. after reduction to the respective alditols, followed by acetylation by the method of Spiro (1966), hydrolysis being performed with 0.5 M-H_2SO_4 at 100°C for 8 h. Hexosamines were determined with an amino acid analyser by the method of Spiro (1966). Hydrolysis for this assay was carried out with 4 M-HCl at 100°C for 6 h.

The carbohydrate composition of the oligosaccharides was analysed by using a gas-liquid chromatograph equipped with a column (0.3 cm × 100 cm) of 0.05% (w/w) ECNSS-M; the carbohydrates were chromatographed as alditol acetates after hydrolysis of the oligosaccharide with 2 M-HCl at 100°C for 3 h.

Neutral sugars and sialic acids were assayed by the phenol/H_2SO_4 reaction (Dubois *et al.*, 1956) and by the periodate/resorcinol method (Jourdian *et al.*, 1971) respectively.

Isolation of band-3 glycoprotein

Blood-group-A human-erythrocyte membranes were prepared from 200 ml of packed cells by using 10 mM-Tris/HCl buffer, pH 7.4, as a haemolysing solution. The membranes were then suspended in 2 l of an aqueous solution containing 1 mM-EDTA and 5 mM-2-mercaptoethanol, adjusted to pH 7.5 with 1 M-NaOH. After incubation overnight at 4°C, the membranes were collected by centrifugation at 30000 × *g* for 30 min and were suspended in 240 ml of ice-cold distilled water after which the suspension was adjusted to pH 12.0 with 1 M-NaOH. After incubation at 4°C for 30 min with occasional stirring, the suspension was centrifuged at 100000 × *g* for 30 min and the pellet was solubilized in 10 mM-Tris/HCl buffer, pH 8.0, containing 1% sodium dodecyl sulphate, 1 mM-EDTA, and 40 mM-2-mercaptoethanol. The solution was then subjected to gel chromatography on a column (2.8 cm × 90 cm or 6 cm × 90 cm) of Sepharose 6B that had been previously equilibrated with 40 mM- Tris/acetate buffer, pH 7.4, containing 1% sodium dodecyl sulphate and 2 mM-EDTA. The fractions corresponding to band-3 glycoprotein were pooled and dialysed successively against ethanol and then distilled water. The precipitated materials were collected by centrifugation, solubilized in 10 mM-Tris/HCl buffer, pH 8.0, containing 1% sodium dodecyl sulphate, 1 mM-EDTA and 40 mM-2-mercaptoethanol following which the whole procedure was repeated.

Isolation of oligosaccharides from band-3 glycoprotein

To release oligosaccharides from band-3 glycoprotein, hydrazinolysis was carried out according to the method described previously (Fukuda *et al.*, 1976). Dried purified band-3 glycoprotein (200 mg) was heated at 105°C for 4 h in a sealed evacuated tube with 2 ml of freshly distilled anhydrous hydrazine containing 20 mg of hydrazine sulphate. After evaporation of the hydrazine *in vacuo*, the residue was dried over conc. H_2SO_4 in a vacuum desiccator and then dissolved in a small amount of 4.5 M-sodium acetate. After centrifugation at 30000 × *g* for 10 min the supernatant was *N*-acetylated according to the method of Spiro (1966). *N*-Acetylated oligosaccharides were isolated by subjecting the reaction mixture to gel chromatography on a column (1.5 cm × 100 cm) of Sephadex G-50 at room temperature.

Fractionation of oligosaccharides from band-3 glycoprotein

DEAE-cellulose (DEAE-Sephacel) and Sephadex G-50 were purchased from Pharmacia (Uppsala, Sweden). *Ricinus communis* agglutinin, purified according to Tomita *et al.* (1972), was coupled to CNBr-activated Sepharose 4B as described previously by Matsumoto & Osawa (1972).

The *N*-acetylated oligosaccharide fraction was applied to a column (1.5 cm × 12 cm) of DEAE-cellulose. The column was first washed with 2 mM-Tris/HCl, pH 7.4, and then the sample was eluted with the same buffer containing 200 mM-NaCl. The neutral oligosaccharide fraction was separated by gel chromatography on a column (1.5 cm × 100 cm) of Sephadex G-50. Elution was performed with water at a flow rate of 5 ml/h. Further fractionation was performed by affinity chromatography on an agglutinin–Sepharose column (1.5 cm × 23 cm). After elution with 10 mM-sodium phosphate/0.15 M-NaCl, pH 7.3, the oligosaccharides remaining bound to the affinity adsorbent were eluted with

0.2 M-sodium borate buffer, pH 8.0 (Svensson *et al.*, 1970; Kennedy & Rosevear, 1973). The fractions obtained by the affinity chromatography were then rechromatographed on a Sephadex G-50 column under the same conditions as described above.

Radioactive labelling of oligosaccharides

Tritium labelling of oligosaccharides with NaB^3H_4 was performed according to Mizuochi *et al.* (1978). About 100 μg of oligosaccharides were reduced with 5 μmol (1.25 mCi) of NaB^3H_4 in 300 μl of 10 mM-NaOH at 25°C for 4 h. Unlabelled $NaBH_4$ (10 mg) was then added and the reaction was allowed to continue at 25°C for another 2 h. The reaction was stopped by acidifying the reaction mixture by passing it through Dowex 50W X8 (H^+ form). The remaining borate was removed by repeated evaporation with methanol. To remove any likely radioactive contaminants from the NaB^3H_4, descending paper chromatography was carried out for 16 h on Whatmann 3MM paper with ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.) as the solvent (Koeltzow *et al.*, 1968).

Treatment with glycosidases

β-Galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase were purified from jack-bean meal by the method of Li & Li (1972). α-L-Fucosidase and α-*N*-acetylgalactosaminidase from *Charonia lampas* were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). β-Mannosidase from snails and β-L-fucosidase from *Bacillus fulminans* were supplied by Dr. T. Okuyama (Seikagaku Kogyo Co.) and Dr. K. Furukawa (Gunma University School of Medicine, Maebashi, Japan), respectively.

Oligosaccharides were incubated with glycosidases in 0.05–0.2 ml of the appropriate buffers under a toluene layer for 3–40 h followed by heating in a boiling water bath for 3 min.

For digestions with β-galactosidase, β-*N*-acetylhexosaminidase and α-mannosidase, 0.05 M-sodium acetate buffer, pH 4.0, was used. For digestions with α-L-fucosidase (*C. lampas*) and α-*N*-acetylgalactosaminidase, 0.1 M-sodium citrate/phosphate buffer, pH 4.0, containing 0.5 M-NaCl, was used. For digestion with the α-L-fucosidase (*B. fulminans*) 0.01 M-sodium phosphate buffer, pH 6.6, was used.

Gel permeation chromatography

Gel permeation chromatography was performed using a high-performance liquid chromatograph (Tri-rotor, Jasco, Tokyo) equipped with a column (0.8 cm × 100 cm) of Bio Gel P-4 (−400 mesh) at a flow rate of 0.3 ml/min. During operation, the column was maintained at 55°C by circulating warm water in a jacket, as described by Sabbagh & Fagerson (1973). Oligomers of glucose and *N*-acetylglucosamine were used as standards. Sugars were monitored by a refractive index detector (Shodex RI SE-11, Showa Denko, Tokyo) and a u.v. spectrophotometer (Uvidec 100-II, Jasco).

Methylation analysis

Oligosaccharides were methylated by the method of Hakomori (1964). The permethylated product was purified on a small column of Sephadex LH-20 and subjected to acetolysis, hydrolysis, reduction and acetylation as described by Stellner *et al.* (1973). Alditol acetates of partially methylated sugars were analysed by g.l.c. on a column (0.3 cm × 200 cm) of Gas-chrom Q coated with 3% (w/w) OV-225 or 1% (w/w) OV-1. Each peak was identified by gas chromatography–mass spectrometry (Shimadzu-LKB 9000) with a column (0.3 cm × 200 cm) of 1% (w/w) OV-1. Conditions for the mass spectrometry were as follows: ion source temperature, 270°C; separator temperature, 260°C; ionizing potential, 70 eV; and trap current, 60 μA.

For the methylation study of the product of the nitrous acid deamination, the unhydrolysed permethylated sample was analysed by gas chromatography–mass spectrometry on a column (0.3 cm × 200 cm) of 1% (w/w) OV-1. The column temperature was varied between 140–300°C at the rate of 10°C/min.

Nitrous acid deamination

Nitrous acid deamination was performed according to the method of Hase & Matsushima (1969). *N*-Deacetylated oligosaccharides obtained by hydrazinolysis after reduction with $NaBH_4$ were deaminated with 0.2% (w/v) sodium nitrite in 0.45 M-sodium acetate buffer, pH 3.6, at 25°C for 5 h. The deaminated products were fractionated by gel permeation chromatography on a column of Bio-Gel P-4 after reduction with NaB^2H_4 or NaB^3H_4. The carbohydrate composition of each fraction was analysed by g.l.c. on a column (0.3 cm × 100 cm) of 0.05% (w/w) ECNSS-M after conversion to alditol acetate derivatives; *myo*-inositol was used as an internal standard.

Standard galactosyl 2,5-anhydromannose and 2,5-anhydromannose were prepared from Galβ1 → 4GlcNAcβ1 → 2Man [synthesized according to Kaifu & Osawa (1976)] and from glucosamine hydrochloride, respectively.

Results

Isolation of band-3 glycoprotein

The procedure used for the isolation of band-3 glycoprotein, using Sepharose 6B chromatography, resulted in two major peaks (Fig. 1). The first peak was found to consist mainly of band-3 glycoprotein, as identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, while the second peak

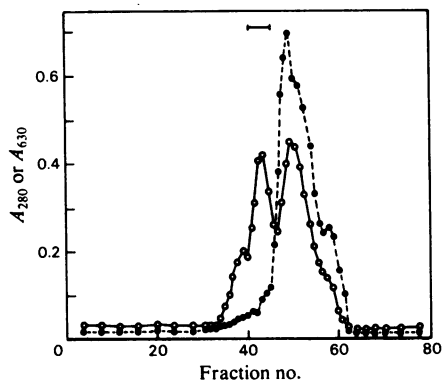

Fig. 1. *Gel chromatography of alkali-treated human erythrocyte membranes on Sepharose 6B*
Experimental details are described in the text. The solubilized membranes were applied to a column (2.8 cm × 90 cm) of sepharose 6B, and elution was carried out with 40 mM-Tris/acetate buffer, pH 7.4, containing 1% sodium dodecyl sulphate and 2 mM-EDTA. Fractions (5 ml) were collected and analysed for proteins (○, A_{280}) and for sialic acids (●, A_{630}).

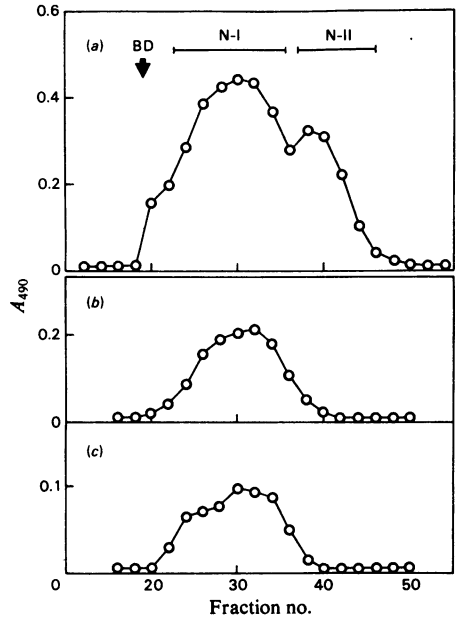

Fig. 2. *Gel chromatography of the neutral oligosaccharide fraction on Sephadex G-50*
Experimental details are described in the text. Fractions (4 ml) were collected and analysed for neutral sugars. (*a*) Neutral oligosaccharide fraction passed through a DEAE-cellulose column. (*b*) *Ricinus* agglutinin–Sepharose-bound oligosaccharide fraction [N-I-R(+)]. (*c*) *Ricinus* agglutinin–Sepharose-unretarded oligosaccharide fraction [N-I-R(−)]. BD, the elution position of Blue Dextran.

was found to be the periodate/Schiff-positive glycoproteins together with bands 4.5 and 7. After rechromatography on a column of Sepharose 6B, the fraction corresponding to the first peak in Fig. 1 was found to be pure band-3 glycoprotein as identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis when stained for either proteins with Coomassie Brilliant Blue or carbohydrates with periodate-Schiff reagent.

The results of the amino-acid and carbohydrate analyses of the purified band-3 glycoprotein show good agreement with the results of other investigators (Ho & Guidotti, 1975; Furthmayr *et al.*, 1976).

Fractionation of oligosaccharides from band-3 glycoprotein

When *N*-acetylated oligosaccharides released from band-3 glycoprotein by hydrazinolysis were subjected to ion-exchange chromatography on DEAE-cellulose, about 85% of the oligosaccharides could be recovered without absorption. This indicates that most of band-3 oligosaccharides were free of sialic acids.

The neutral oligosaccharides, separated by Sephadex G-50 gel chromatography (Fig. 2*a*) were found to be heterogeneous with respect to their molecular weight since two broad peaks were obtained. This result is in good agreement with that reported by Drickamer (1978). When the two peaks of oligosaccharides, designated as N-I and N-II, were separately pooled, the molecular weight ranges were found to be 3000–8000 and 1500–2500, respectively.

The N-I- oligosaccharide fraction was further fractionated by means of affinity chromatography using *Ricinus* agglutinin–Sepharose 4B as an affinity adsorbent. The N-I oligosaccharides were separated into two fractions by this procedure. About 65% of these oligosaccharides were bound to *Ricinus* agglutinin–Sepharose 4B and were eluted by 0.2 M-sodium borate buffer, pH 8.0. These fractions are designated N-I-R(−) and N-I-R(+) respectively. N-I-R(−) and N-I-R(+) were rechromatographed on Sephadex G-50 (Fig. 2*b*,*c*). These oligosaccharide fractions were still found to be somewhat heterogeneous in size.

Characterization of N-I-R(+) and N-I-R(−) oligosaccharides

The results of the carbohydrate composition analysis of some of the fractions rechromatographed on Sephadex G-50 are shown in Table 1. The results revealed the presence of galactose, mannose, *N*-acetylglucosamine and fucose in all fractions and a small amount of

Table 1. *Carbohydrate compositions of oligosaccharides N-I-R(+), N-I-R(−) and N-II*
The fractions are as shown in Fig. 2. Molar ratios are expressed in relation to mannose taken as 3.0.

Oligosaccharide and fractions	Molar ratio				
	Fucose	Mannose	Galactose	*N*-Acetylglucosamine	*N*-Acetylgalactosamine
N-I-R(+) 24	2.9	3.0	16.1	18.3	0.1
26	2.5	3.0	14.0	16.6	0.1
28	1.8	3.0	11.9	13.6	0.0
30	1.5	3.0	10.0	12.5	0.0
32	1.2	3.0	8.0	9.9	0.0
34	1.0	3.0	7.6	9.5	0.0
36	1.0	3.0	6.9	8.3	0.0
Unfractionated	1.3	3.0	9.5	11.5	0.0
N-I-R(−) 27	2.5	3.0	9.5	11.0	0.7
30	1.9	3.0	8.0	9.5	0.3
33	2.0	3.0	5.5	7.3	0.2
36	2.0	3.0	5.1	7.0	0.1
Unfractionated	2.1	3.0	6.6	8.5	0.3
N-I	1.6	3.0	8.5	10.5	0.2
N-II	0.6	3.0	2.8	5.6	0.0

N-acetylgalactosamine in the higher-molecular weight fractions. The oligosaccharides of a higher molecular weight had a higher galactose and *N*-acetylglucosamine content than those of a lower molecular weight. The carbohydrate compositions of N-I-R(−) and N-I-R(+) were found to be: fucose, 0–3 mol/mol; galactose, *n* mol/mol; mannose; 3 mol/mol; *N*-acetylglucosamine $(n+2)$ mol/mol. Fractions from N-I-R(−) contained a greater amount of fucose and *N*-acetylgalactosamine than those from N-I-R(+).

Treatment of N-I-R(+) and N-I-R(−) oligosaccharides with glycosidases

The results of incubation of NaB^3H_4-reduced N-I-R(−) and N-I-R(+) with various glycosidases and chromatography on a column of Sephadex G-50 are shown in Fig. 3. Most of N-I-R(+) and a part of N-I-R(−) could be digested with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase after treatment with α-L-fucosidase (*C. lampas*) to give small oligosaccharides (Fig. 3, *f* and *m*). The other part of the oligosaccharides were resistant to these treatments and were degradable only by treatment with α-*N*-acetylgalactosaminidase, followed by digestion with β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 3, *g* and *n*). Only parts of N-I-R(+) and N-I-R(−) could be digested to small oligosaccharides with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 3, *e* and *l*). No significant decrease in the sizes of the oligosaccharides was, however, detected in gel permeation chromatography on Sephadex G-50 after treatment with α-L-fucosidase (*C. lampas*) in combination with β-galactosidase (Fig. 3, *c* and *j*) or β-*N*-acetylhexosaminidase (Fig. 3, *d* and *k*). This suggests that a major portion of the band-3 oligosaccharides have a common structure with heterogeneity in the number of repeating βGal-βGlcNAc disaccharides and that fucose and *N*-acetylgalactosamine residues are present in the peripheral portions of some of the oligosaccharides, particularly in the N-I-R(−) fraction.

In order to determine the sequence of the core oligosaccharides obtained from N-I-R(−) and N-I-R(+) by the glycosidase digestion, the ^3H-labelled core oligosaccharides were subjected to sequential glycosidase digestion and the reaction mixtures were analysed by gel-permeation chromatography on a Bio-Gel P-4 column according to the method of Mizuochi *et al.* (1978). Identical results were obtained for both core oligosaccharides and only the result obtained for the core oligosaccharide of N-I-R(−) is documented below. The elution position of the core oligosaccharide after gel permeation chromatography was found to be the same as that of standard Man_3–GlcNAc–GlcNAcol (Fig. 4*b*). When the core oligosaccharide was incubated with α-mannosidase, two mannose residues were removed from the oligosaccharide and the major peak obtained had the same elution position as standard Man–GlcNAc–GlcNAcol (Fig. 4*c*). This α-mannosidase digest could be converted to a major peak with the same elution position as GlcNAc–GlcNAcol by β-mannosidase digestion (Fig. 4*d*). This could be digested with β-*N*-acetylhexosaminidase to give a major product with an identical elution position to that of *N*-acetylglucosaminitol (Fig. 4*e*).

This peak was identified as *N*-acetylglucosaminitol by high voltage paper electrophoresis in sodium borate buffer by the method of Takasaki &

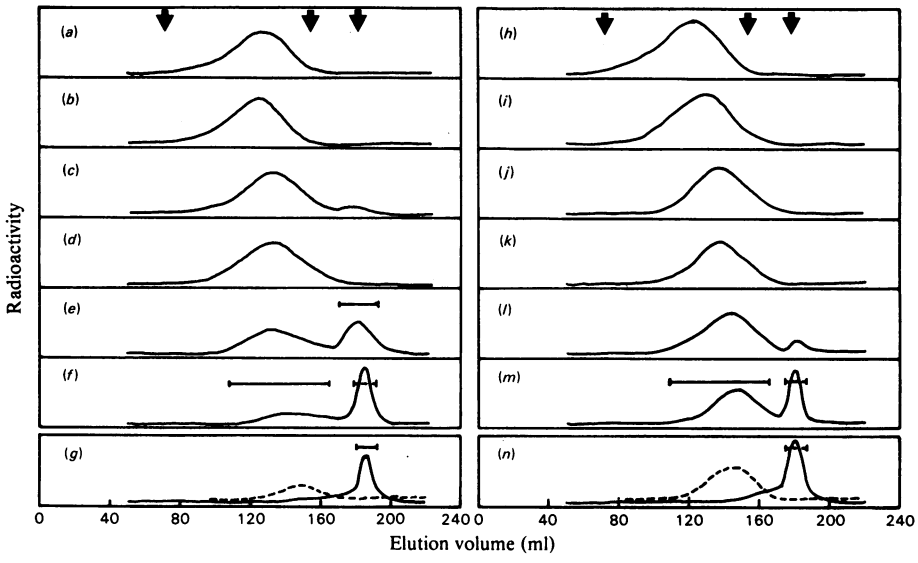

Fig. 3. *Gel permeation chromatography on Sephadex G-50 column of N-I-R(+) and N-I-R(−) after treatment with glycosidases*

Experimental details are described in the text. NaB^3H_4-reduced N-I-R(+) (*a–g*) and N-I-R(−) (*h–n*) were digested with various exoglycosidases, applied to a column (1.5 cm × 100 cm) of Sephadex G-50 and eluted with water at a flow rate of 5 ml/h. (*a*), (*h*) NaB^3H_4-reduced N-I-R(+) or N-I-R(−); (*b*), (*i*) product of α-L-fucosidase (*C. lampas*) treatment of (*a*) or (*h*); (*c*), (*j*) product of β-galactosidase treatment of (*b*) or (*i*); (*d*), (*k*) product of β-*N*-acetylhexosaminidase treatment of (*b*) or (*i*); (*e*), (*l*) product of treatment with β-galactosidase plus β-*N*-acetylhexosaminidase of (*a*) or (*h*); (*f*), (*m*) product of treatment with β-galactosidase plus β-*N*-acetylhexosaminidase of (*b*) or (*i*); (*g*), (*n*) product of treatment with β-galactosidase plus β-*N*-acetylhexosaminidase after a treatment of the higher molecular weight fraction of (*f*) or (*m*) with α-*N*-acetylgalactosaminidase. Control experiments (– – – –) were carried out without α-*N*-acetylgalactosaminidase treatment. The arrows in these Figures indicate the elution positions of standard saccharides: from left to right Blue Dextran, $(Fuc)(Gal)_2(Man)_3(GlcNAc)_4$ and $(Fuc)(Man)_3(GlcNAc)_2$.

Kobata (1978). The minor peaks shown were probably caused by a side reaction product of hydrazinolysis derived from *N*-acetylglucosamine at the reducing end of the oligosaccharide (Fukuda *et al.*, 1976). When the minor peak at the final stage (Fig. 4*e*) was analysed by high-voltage paper electrophoresis it was found to have a lower mobility than any known standard sugar alcohols. These results indicate that sequence of the core structure of N-I-R(−) is αMan_2–βMan–βGlcNAc–GlcNAcol.

When the small oligosaccharide fraction obtained by the treatment of N-I-R(+) only with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 3*e*) was analysed by Bio Gel P-4 column chromatography, it was divided into two peaks whose elution positions were identical with those of Man_3–GlcNAc–GlcNAcol and Man_3–GlcNAc–(Fuc–)-GlcNAcol; the two species were equal in abundance. With α-mannosidase treatment two mannose residues were removed from both species, and the elution positions of the products after gel permeation chromatography were found to be identical with those of standard Man–GlcNAc–GlcNAcol and Man–GlcNAc–(Fuc–)GlcNAcol respectively. When the product whose elution position was identical with Man–GlcNAc–(Fuc–)GlcNAcol was digested with α-L-fucosidase (*C. lampas*), a peak with the same elution position as Man–GlcNAc–GlNAcol was observed. The results therefore suggest that a part of N-I-R(+) contained a fucosyl residue in the core portion.

It has previously been suggested that fucosyl residues were present both in the peripheral portions and in the core portions of the oligosaccharides, and we therefore carried out α-L-fucosidase treatments with two enzymes that had different aglycone specificity in order to elucidate any possible heterogeneity in the fucosyl linkages. We used N-I oligosaccharides treated with α-*N*-acetylgalactosaminidase as substrates to eliminate the contribution from the small amount of *N*-acetylgalactosamine. Fucose released was measured by g.l.c. with a column (0.3 cm × 200 cm) of 3% (w/w) OV-225 after conversion to peracetylated fucitol using

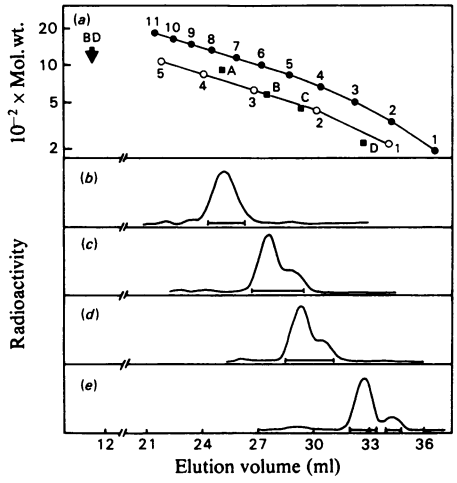

Fig. 4. *Sequential glycosidase digestion of the core oligosaccharide obtained by the glycosidase treatment of N-I-R(−)*

Experimental details are described in the text. The NaB^3H_4-reduced core oligosaccharide (the small oligosaccharide shown in *m* and *n* of Fig. 3) was subjected to gel permeation chromatography on a Bio Gel P-4 column after glycosidase treatments. (*b*) Core oligosaccharide; (*c*) product of α-mannosidase treatment of (*b*); (*d*) product of β-mannosidase treatment of (*c*); (*e*) product of β-*N*-acetylhexosaminidase treatment of (*d*). The top panel (*a*) indicates elution positions of standard sugars and their molecular weights: ●, glucose oligomers; ○, *N*-acetylglucosamine oligomers; ■, oligosaccharides prepared from the unit-B glycopeptide of porcine thyroglobulin; A, $(Man)_3(GlcNAc)(GlcNAcol)$; B, (Man)(GlcNAc)-(GlcNAcol); C, (GlcNAc)(GlcNAcol); D, GlcNAcol; BD, the elution position of blue dextran.

2-deoxyglucose as an internal standard. Most of the fucose residues (72%) were liberated from N-I by treatment with α-L-fucosidase (*B. fulminans*) which specifically hydrolysed Fuc(α1→2)Gal (Kochibe, 1973), while a treatment with α-L-fucosidase (*C. lampas*), which has a broad aglycone specificity (Nishigaki *et al.*, 1974), released all fucose residues. This result suggests that at least two kinds of fucosyl linkage are present in N-I and one of these linkages is Fuc(α1→2)Gal.

Methylation study

Similar results were obtained with oligosaccharides N-I-R(−), N-I-R(+) and N-I, except for the fucose and *N*-acetylgalactosamine derivatives.

Most of the *N*-acetylglucosamine residues were substituted at C-4 (Table 2), whereas galactose residues were found as non-reducing terminal groups in the chain substituted at C-3 or at the branching point substituted at C-3 and C-6. This result is consistent with that reported by Fukuda *et al.* (1979). N-I oligosaccharides must therefore be highly branched, with oligosaccharide units originating from every third galactose residue.

Nitrous acid deamination

After reduction with $NaBH_4$ the *N*-deacetylated N–I oligosaccharide was deaminated with nitrous acid to depolymerize the molecule at the positions occupied by glucosamine, producing fragments terminating at the reducing end with 2,5-anhydromannose. Deaminated products were fractionated by gel permeation chromatography on a Bio Gel P-4 column after reduction with NaB^2H_4 or NaB^3H_4 (Fig. 5). More than 90% of the total radioactivity was recovered in four fractions; the

Table 2. *Methylation analyses of oligosaccharides N-I, N-I-R(+) and N-I-R(−)*

Molar ratios are expressed in relation to total amount of mannose derivatives taken as 3.0.

	Methyl groups		Molar ratios in oligosaccharides		
Sugar	Number	Position	N-I	N-I-R(+)	N-I-R(−)
Fucose	3	2,3,4	1.0	1.2	1.8
Galactose	4	2,3,4,6	1.6	1.9	0.9
	3	2,4,6	4.1	4.3	3.9
		2,3,4	0.6	0.5	0.5
		3,4,6	0.4	0.3	0.4
	2	2,4	2.0	2.4	1.8
		4,6	0.2	0.0	0.2
Mannose	3	3,4,6	1.8	1.7	1.7
	2	2,4	1.0	1.0	0.9
		3,6	0.2	0.3	0.4
N-Acetylglucosamine	4	1,3,5,6	0.2	0.3	0.0
	3	1,3,5	0.5	0.4	0.7
		3,4,6	0.2	0.0	0.0
	2	3,6	9.3	10.6	8.0
N-Acetylgalactosamine	3	3,4,6	0.2	0.0	0.3

compositions of fractions I, II and III are shown in Table 3.

Fraction III, which was the largest peak, was identified as galactosyl 2,5-anhydromannitol since it eluted at the same position as authentic galactosyl 2,5-anhydromannitol and was found to be composed of galactose and 2,5-anhydromannitol in the molar ratio of 1:1. This was confirmed by an analysis by gas chromatography–mass spectrometry of permethylated fraction III (reduced by NaB^2H_4). The retention time of permethylated fraction III was identical with that of methylated galactosyl 2,5-anhydromannitol. Furthermore, a similar mass spectrum to that of the methylated authentic standard was obtained (relative intensities in parentheses): *m/e* 45 (52), 46 (24), 71 (29), 75 (22), 88 (100), 101 (93), 155 (5), 158 (30), 187 (8), 190 (25), 219 (4), 250 (52). Fraction I was eluted in the tetrasaccharide region on gel permeation chromatography and contained approx. three residues of mannose/residue of 2,5-anhydromannitol, as well as a very small amount of contaminating galactose and fucose. This product represents the mannose residues attached to *N*-acetylglucosamine at the core portion. Fraction II contained fucose, galactose and 2,5-anhydromannitol at a molar proportion of 0.9 : 1.1 : 1.0, and after elution through a Bio Gel P-4 column was found in the trisaccharide region. The fucosyl linkage that was hydrolysable with α-L-fucosidase (*B. fulminans*) was present in N-I. These results strongly suggest that fraction II is Fuc–Gal–2,5-anhydromannitol. Fraction IV, which corresponds to a monosaccharide fraction, contained 2,5-anhydrotalitol, 2,5-anhydromannitol and 2-deoxyglucitol, which probably arose from the terminal galactosamine residues, terminal glucosamine residues and glucosaminitol residues at the reducing end respectively.

These results strongly indicates that Galβ1→4GlcNAcβ1→3 repeating units, heterogeneous in number, exist at the peripheral portion of N-I oligosaccharides and that the core portion consists of three mannose and two *N*-acetylglucosamine residues as illustrated in Fig. 6.

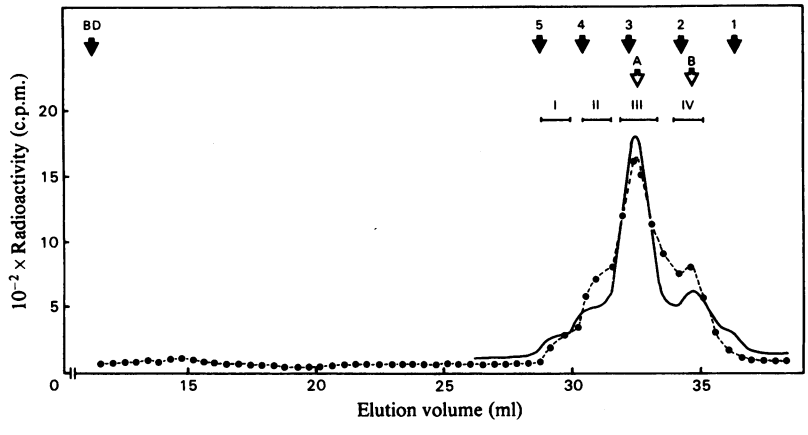

Fig. 5. *Gel permeation chromatography on Bio Gel P-4 column of deaminated products of N-deacetylated N-I*
Experimental details are described in the text. Deaminated products were fractionated by gel permeation chromatography after reduction with NaB^2H_4 or NaB^3H_4. Sugars were monitored by their refractive indices (——) and radioactivity (●). The black arrows indicate elution positions of glucose oligomers, the numbers indicating the number of glucose units, and white arrows indicate elution position of galactosyl 2,5-anhydromannitol (A) and 2,5-anhydromannitol (B). BD, the elution position of Blue Dextran.

Table 3. *Carbohydrate compositions of the products of nitrous acid deamination*
The fractions are as shown in Fig. 5.

	Molar ratio to 2,5-anhydromannitol of			
Fraction	Fucose	2,5-Anhydromannitol	Mannose	Galactose
I	0.2	1.0	2.7	0.2
II	0.9	1.0	0.0	1.1
III	0.0	1.0	0.0	1.0

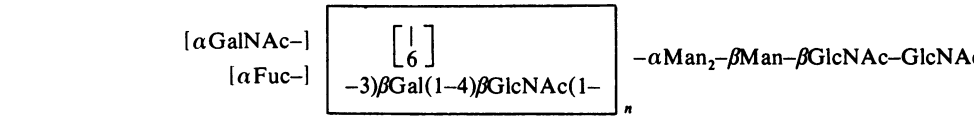

Fig. 6. *Proposed general structure of N-I oligosaccharides*

Discussion

It has been suggested previously that the carbohydrate moiety of the band-3 glycoprotein species has heterogeneity because it characteristically migrates as a rather broad band during electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. In this investigation, oligosaccharides were separated from band-3 glycoprotein by hydrazinolysis and were fractionated by ion-exchange chromatography, gel permeation chromatography and affinity chromatography using *Ricinus* agglutinin–Sepharose 4B. After this analysis an elucidation of the structure of the oligosaccharide fraction designated N-I was carried out. Fraction N-I, containing around 68% of the oligosaccharides separated from band-3 glycoprotein, was found to be fairly heterogeneous in size. The molecular weight range of N-I was estimated to be in the region of 3000–8000 as determined by its elution obtained from a Sephadex G-50 column. The estimated molecular weights are in good agreement with those from the carbohydrate composition. The carbohydrate composition of the oligosaccharides, listed in Table 1, shows that galactose and *N*-acetylglucosamine are the predominant sugar constituents. Most exist as repeating *O*-β-galactosyl(1→4)*N*-acetylglucosamine(1→3) sugar sequences, as shown in Fig. 6. Furthermore, the presence of the αMan_2–βMan–βGlcNAc–GlcNAc sugar sequence at the core portion is interesting, since this structure is found in many glycopeptides known as 'complex-type' sugar chains. The results also demonstrated that parts of the N-I oligosaccharides had α-L-fucosyl residues in their core portions, similar to those of the 'complex-type' sugar chains. The N-I fraction was further separated into two fractions on *Ricinus* agglutinin–Sepharose 4B columns. These two fractions were designated as N-I-R(–) and N-I-R(+) respectively. The results of the structural analysis, however, showed that the basic structures were rather similar. It is possible that, in addition to the terminal galactose residues in the N-I-R(–) oligosaccharides, there could be a greater amount of terminal fucose and *N*-acetylgalactosamine residues than in the N-I-R(+) oligosaccharides. This would account for the lower affinity of N-I-R(–) for *Ricinus* agglutinin-Sepharose 4B, which has a specificity for galactose, and would explain why two fractions were obtained.

Recently it has been reported that band-3 glycoprotein is a blood-group-I carrier (Childs *et al.*, 1978) and that the Galβ1→4GlcNAcβ1→3-(Galβ1→4GlcNAcβ1→6)Gal structure, presumably included in N-I at the peripheral portion, is required for blood-group I activity (Watanabe *et al.*, 1979). We were also able to detect blood-group-I activity of N-I by the heamagglutination inhibition test. There is no possibility that N-I is contaminated with oligosaccharides from blood-group-i erythrocytes, however, since there is practically zero incidence of i activity in Japanese blood (Nakajima, 1977) as used in this work. Furthermore, blood-group-H(O) and A activities were also checked by this method using the *Ulex europeus* haemagglutinin I and anti-A sera, respectively. N-I showed distinct haemagglutination inhibitory activity against anti-H(O) haemagglutinin, but did not show such activity against anti-A sera at a concentration of 60μg/ml. This result suggests that N-I oligosaccharides are not major determinants of blood-group-A specificity. A small amount of *N*-acetylgalactosamine residues in the peripheral portion possibly forms part of the blood-group-A determinant, the activity of which may be too weak to be detected.

It has been reported in a recent study that high-molecular weight glycopeptides with the repeating structure Galβ1→4GlcNAcβ1→3, similar to the N-I-oligosaccharides of the present study and termed 'poly(glycosyl)peptides' (Krusius *et al.*, 1978) or 'erythroglycan' (Järnefelt *et al.*, 1978) were isolated from delipidated human erythrocyte membranes. In the present study the molecular weight range of N-I was found to be somewhat lower than that of the glycopeptides [4000–13000 for 'poly(glycosyl) peptides' or 7000–11000 for 'erythroglycan']. Due to the lack of good molecular weight standards, however, it is difficult to estimate accurately the molecular weights of such large oligosaccharides or glycopeptides. One possibility is that the N-I oligosaccharides are portions of these 'poly-(glycosyl)peptides' or of 'erythroglycan'.

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