Glycocalicin, a predominant glycoprotein on the human platelet surface, has been purified from a platelet suspension by sonication, ammonium sulfate precipitation and acid treatment followed by chromatography on columns of wheat germ agglutinin-Sepharose and Sephacryl S-300. Ser/Thr-linked (O-linked) oligosaccharides were released by alkaline borohydride treatment, and fractionated by high performance liquid chromatography with an anion-exchange resin. The structure of a major oligosaccharide aldito1 separated by high performance liquid chromatography was investigated by a combination of compositional analyses, methylation and glycosidase treatments, and proposed to be a hexaasaccharide aldito1, NeuAcα2-3Galβ1-4GlcNAcβ1-6(NeuAcα2-3Galβ1-3)N-acetylgalactosaminitol. We also found some sugar units which appeared to be intermediates in the biosynthetic pathway of the major hexaasaccharide.

Glycocalicin is a predominant glycoprotein present on the human platelet surface (1, 2). This glycoprotein seems to be loosely bound to platelet membranes and can be recovered easily in a soluble subcellular fraction after sonication of platelets. It has previously been suggested that glycocalicin is identical or closely related to so-called glycoprotein Ib of platelet membranes, and recent studies have indicated that glycocalicin is derived from glycoprotein Ib which contains additional peptides when compared with glycoprotein (3, 4). Furthermore, glycocalicin and glycoprotein Ib have been proposed as platelet receptors for both ristocetin/von Willebrand factor-induced and thrombin-induced platelet activation (5). The platelets from patients with the Bernard-Soulier syndrome are deficient in both glycoprotein Ib and glycocalicin (6, 7), and fail to respond to these stimuli (8). Glycocalicin has an apparent Mr of 150,000, as estimated by sodium dodecyl sulfate SDS-polyacrylamide gel electrophoresis, and contains approximately 60% (w/w) carbohydrate (9). Recently, studies concerning the molecular architecture of platelet plasma membranes have been carried out, but very little is known about carbohydrates present on the platelet cell surface. In this study, we attempted to elucidate the carbohydrate structure of glycocalicin which is a well characterized surface glycoprotein of human platelets.

**EXPERIMENTAL PROCEDURES**

**Purification of Glycocalicin from Human Platelets**—Platelets were isolated from platelet concentrates by differential centrifugation according to the method of Anderson and Gahmberg (10). Glycocalicin was extracted from washed human platelets and purified mainly by the method of Okumura et al. (9), except that chromatographies on columns of WGA-Sepharose and Sephacryl S-300 were employed in the present study.

WGA was purified as described previously (11), and coupled to carboxyl-Sepharose 4B by the method of Matsumoto et al. (12) using water-soluble carbodiimide. The crude glycocalicin fraction was obtained by successive steps of sonication, ammonium sulfate fractionation, and acid treatment as described previously (9). This fraction was applied to a WGA-Sepharose column (1.5 × 8 cm) which was equilibrated with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.85% NaCl after dialysis of the fraction against the same buffer. The column was washed with the same buffer until the absorbance at 280 nm became approximately 0.01, and then the bound glycoproteins including glycocalicin were eluted with the buffer containing 0.25 M N-acetylglucosamine. This eluate was then chromatographed on a Sephacryl S-300 column (1.2 × 65 cm), and elution was carried out with 0.01 M sodium phosphate buffer, pH 7.3, containing 0.5 M NaCl.

**Analytical Methods**—Polyacrylamide gel electrophoresis in the presence of SDS was carried out with the system of Laemmli (13) using 8% polyacrylamide slab gel.

The amino acid composition was determined with a Hitachi Uvidec 100-11 (Hitachi Ltd., Tokyo, Japan), after hydrolysis in 6 M HCl for 24 h at 110 °C. Static acids were assayed by the periodate-resorcinol method (14).

N-Acetyleneuraminic acid was identified by gas-liquid chromatography on a column (0.3 × 200 cm) of Gas-chrom Q coated with 2% OV-1, after methanolation followed by trimethylsilylation according to the method of Yu and Ledeen (15).

**Isolation of Sugar Chains from Glycocalicin**—Ser/Thr-linked sugar chains were released from glycocalicin by alkaline borohydride treatment. Glycocalicin was incubated with 1 M NaBH₄ or NaBH₃ (348 mCi/mmol, New England Nuclear) in 0.1 M NaOH aqueous solution at 37 °C for 24 h. The amounts of glycocalicin subjected to β-elimination were 1 mg for NaBH₄, and 0.1 mg for NaBH₃, respectively. After neutralization by dropwise addition of acetic acid to pH 5.0, the reaction mixture was applied to a Sephadex G-50 column which was equilibrated with 20 mM/10 mM pyridine/acetic acid in order to recover the oligosaccharide aldito1s liberated.

**High Performance Liquid Chromatography**—A Jasco Tri-rotor high pressure liquid chromatograph (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a variable wavelength ultraviolet spectrophotometer (UV/Spec 100-H, Jasco) was used. Two HPLC systems were employed in the present study. One was ion exchange chromatography using a column (4.6 × 250 mm) of Hitachi Custom Resin #2830 as an anion-exchange resin. The elution was performed with a concave gradient of an aqueous sodium chloride solution at a flow rate of 1.0 mL/min. The column temperature was maintained at 55 °C. This system was found to be effective for separation of acidic oligosaccharides (16).
The other system was gel permeation chromatography with two serial columns (7.2 × 500 nm) of Bio-Gel P-4 (400 mesh) as described previously (17). The columns were eluted with distilled water at a flow rate of 0.3 ml/min and maintained at 55 °C. This system was used for determination of the molecular weights of oligosaccharides. Mixtures of oligomers of glucose and N-acetylglucosamine obtained by partial hydrolysis of dextran and chitin, respectively, were used as standards.

Carbohydrate Composition and Methylation Analysis—The carbohydrate composition of an oligosaccharide was analyzed with a gas-liquid chromatograph equipped with a column (0.5 × 100 cm) of 0.8% ECNSS-M, after conversion to the respective alditol acetates according to the method of Spiro (18), hydrolysis being performed with 2 M HCl at 100 °C for 2.5 h.

Methylation of oligosaccharide alditols was performed according to Hakomori (19), and the permethylated sugars were purified on a small column of silica gel (20). Alditol acetates of partially methylated sugars were prepared by hydrolysis of methylated oligosaccharide alditols with 3 M HCl at 80 °C for 3 h, followed by reduction with NaBH₄, and acetylation as described by Stellner et al. (21), and analyzed by gas chromatography-mass spectrometry (Shimadzu QP-1000, Shimadzu Corp., Kyoto, Japan) with a column (0.26 × 100 cm) of 2% OV-17 or 2% OV-1. Conditions for the analysis were as described previously (22). For the methylation study on the products of glycosidase treatments, the unhydrolyzed sample was analyzed by gas chromatography-mass spectrometry on a column (0.3 × 100 cm) of 2% OV-1. The column temperature was programmed between 180–280 °C at the rate of 5 °C/min.

Glycosidase Treatment—Neuraminidase from Arthrobacter ureafaciens was obtained from Nakarai Chemical Co. (Kyoto, Japan), and α-fucosidase from Chronia fampas from Seikagaku Kogyo Co. (Tokyo, Japan). β-Galactosidase and β-N-acetylgalactosaminidase were purified from jack bean meal (23). β-Galactosidase from Aspergillus niger was purchased from Sigma, and further purified by chromatographies on Sephacryl S-200, DEAE-Sephadex A-50 and hydroxyapatite (24).

Tritiated oligosaccharide alditols (5–20 × 10⁶ cpm, about 0.1–0.4 nmol) were digested at 37 °C with glycosidases (0.5–5 units) in 0.1 ml of 0.05 M sodium acetate buffer, pH 4.0, under a toluene layer for 16–40 h, followed by heating at 100 °C for 3 min to terminate the reaction. The products were analyzed by gel permeation chromatography on a Bio-Gel P-4 column.

RESULTS

Purification of Glycocalicin—When the crude glycocalicin fraction was applied to WGA-Sepharose, most of the sialoglycoproteins were bound to the column and eluted with the buffer containing 0.25 M N-acetylglucosamine (Fig. 1). Analysis by SDS-polyacrylamide gel electrophoresis revealed that glycocalicin was recovered in the bound fraction as reported by Solum et al. (25), and no glycocalicin was observed in the unbound fraction. In order to separate glycocalicin further from some impurities, this fraction was further chromatographed on a Sephacryl S-300 column. When the eluate was monitored by ultraviolet absorption at 280 nm and sialic acid, three major peaks were detected (Fig. 2). The second fraction, which contained large amounts of sialic acid relative to the absorbance at 280 nm, was found to consist of glycocalicin. SDS-polyacrylamide gel electrophoresis of this fraction gave a single band with an apparent Mr of 140,000–150,000 (Fig. 3). About 1.3 mg of glycocalicin was obtained from platelets

Fig. 2. Gel filtration on Sephacryl S-300 of glycocalicin fraction from a WGA-Sepharose column. The glycoprotein fraction bound to WGA-Sepharose was applied to a column (1.2 × 65 cm) of Sephacryl S-300, and the column was eluted with 0.01 M sodium phosphate buffer (pH 7.3) containing 0.5 M sodium chloride. Fractions of 2 ml each were collected, and absorbance at 280 nm (●) and sialic acids (○) were measured.

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified glycocalicin. Purified glycocalicin was analyzed by gel electrophoresis in the presence of SDS with the system of Laemmli (13) using 8% polyacrylamide slab gel. The gel was stained with Coomassie blue (CB, middle) and periodic acid-Schiff (PAS) reagent (left). The right lane contains marker proteins for molecular weight estimation.
prepared from 8 liters of fresh blood. This yield is almost the same as that described by Okumura et al. (9). Glycocalicin could be well stained with periodic acid-Schiff reagent but was only faintly stained with Coomassie blue. This preparation was used for further analysis.

The results of chemical analyses including the amino acid and carbohydrate compositions of purified glycocalicin showed fairly good agreement with those of Okumura et al. (9). It was found to contain approximately 60% (w/w) or 47 mol % carbohydrate consisting of galactose, galactosamine, glucosamine, and N-acetylneuraminic acid as principal sugars in addition to a small amount of mannose and fucose. These results suggest that glycocalicin contains mainly Ser/Thr-linked (O-linked) sugar chains.

**Isolation and Fractionation of Sugar Chains of Glycocalicin**—In order to release Ser/Thr-linked (O-linked) sugar chains, purified glycocalicin was subjected to alkaline borohydride treatment under the conditions described under “Experimental Procedures.” More than 80% of carbohydrates of glycocalicin were recovered after chromatography on a Sephadex G-50 column, as estimated with the periodate-resorcinol reaction. This oligosaccharide alditol fraction was fractionated by HPLC with anion exchange resin, Hitachi Custom Resin #2630 (16). As shown in Fig. 4, most oligosaccharides were found to be acidic; they were bound to the column and eluted with a gradient of aqueous sodium chloride solution. One major oligosaccharide alditol fraction designated as I1 and six minor fractions designated as I-1-1-6 were obtained. The proportion of each oligosaccharide alditol fraction estimated from radioactivity in Fig. 4 are as follows: I-1, 4%; I-2, 6%; I-3, 8%; I-4, 5%; I-5, 4%; I-6, 5%; II, 67%. Oligosaccharide I1 in the major fraction and oligosaccharides I-1-1-6 in the minor fractions were found to be disialylated and monosialylated oligosaccharides, respectively, based on results of the sialic acid assay and their elution positions. The structure of oligosaccharide II was elucidated in the present study because of its preponderance and the relative ease of its preparation.

**Structure of the Major Sugar Chain of Glycocalicin**—The results of compositional and methylation analyses of oligosaccharide I1 are shown in Table I. Oligosaccharide I1 was found to be composed of galactose, N-acetylgalactosamine, N-acetylgalactosamine, and N-acetylneuraminic acid in a molar ratio of 2:1:1:2. Methylation analysis of oligosaccharide I1 revealed that all galactose and N-acetylglucosamine residues were substituted at C-3 and C-4, respectively. An N-acetylgalactosamine residue was found as a reducing terminal group, forming branches at C-3 and C-6. After neuraminidase treatment followed by methylation analysis, 2 mol of galactose residues originally substituted at C-3, were converted to nonreducing terminal groups. This indicated that 2 mol of N-acetylneuraminic acid residues were linked to C-3 of galactose residues in the intact oligosaccharide II.

Neuraminidase-treated oligosaccharide II was subjected to gel permeation chromatography on Bio-Gel P-4 of tritiated oligosaccharide alditols. Experimental details are given in the text. a, sequential glycosidase treatment of oligosaccharide II was performed and the product at each step was analyzed by gel permeation chromatography on a Bio-Gel P-4 column. Desialylated oligosaccharide II (— — —) was successively treated with β-galactosidase (jack bean) (— —), β-N-acetyhexosaminidase (— — —), and β-galactosidase (A. niger) (— — —). Desialylated oligosaccharides I-1-1-6 were analyzed for molecular size. Closed circles (1-10), open circles (1-5) and the closed square indicate the elution positions of standard sugars. •, glucose oligomers; ○, N-acetylgalactosamine oligomers; □, Gal-GalNAcAl prepared from human erythrocyte glycoprotein A.

![Fig. 4. HPLC on Hitachi Resin #2630 of oligosaccharide alditols from glycocalicin.](http://www.jbc.org/)

![Fig. 5. Gel permeation chromatography on Bio-Gel P-4 of tritiated oligosaccharide alditols.](http://www.jbc.org/)
mval of 1 residue of N-acetylhexosamine, and gave a radio-
active peak at the position corresponding to that of Gal-
GalNAcol. Although the presence of 2 mol of nonreducing
terminal galactose residues was found on methylation analysis
of asialo-oligosaccharide II, a decrease of only 1 residue was
observed after the β-galactosidase (jack bean) digestion. It has
been reported that the β-galactosidic linkage in Galβ1-
3GalNAcol is resistant to β-galactosidasen from several
sources including jack bean (26, 27). As the possibility of
the existence of this type of β-galactosyl linkage in oligosaccha-
ride II was indicated, we examined the susceptibility of the
disaccharide alditol obtained after β-galactosidase (jack bean)
and β-N-acetylhexosaminidase digestions to the β-galacto-
sidase purified from A. niger which is known to cleave this type
of β-galactosyl linkage (28). This β-galactosidase treatment
of the disaccharide alditol yielded a radioactive product that
eluted at the same position as N-acetylgalactosaminitol from
a Bio-Gel P-4 column (Fig. 5A), indicating that the disaccha-
ride alditol could be hydrolyzed by this enzyme as expected.
These results indicate that the sugar sequence of asialo-
The anomeric configuration of each monosaccharide was con-
firmed also by a chromium trioxide oxidation experiment (29).

The core structure of oligosaccharide II was elucidated by
gas chromatography-mass spectrometric analysis of the per-
methylated disaccharide alditol prepared from cold oligosac-
charide II by successive treatments with neuraminidase,
β-galactosidase, and P-N-acetylhexosaminidase. From the mass
spectrum shown in Fig. 6, the structure of the core disaccha-
ride alditol was identified as hexosyl (1 → 3) N-acetylhexo-
saminitol, i.e. Galβ1 → 3GalNAcol.

On the basis of the results of compositional analyses, meth-
ylation and glycosidase treatment, the structure of oligosac-
charide II, the major O-linked sugar chain of glycocalicin, was
proposed to be a hexasaccharide alditol as shown in Fig. 7.

Characterization of Minor Sugar Chains—Structural anal-
yses of sugar chains in minor fractions, which were deter-
mined to be monosialylated oligosaccharides according to the
HPLC method reported previously (16), 1-1-1-6, were partially
performed, because of the limited availability of the samples.

Oligosaccharides 1-1-1-6 were analyzed for molecular size
by gel permeation chromatography on a Bio-Gel P-4 column
after neuraminidase treatment (Fig. 5b). Asialo-oligosaccha-
rides 1-2 and 1-3 were eluted from the column at a position
identical with that of asialo-oligosaccharide II. Furthermore,
sequential glycosidase digestions of asialo-oligosaccharides 1-
2 and 1-3 gave the same results as for asialo-oligosaccharides
II (data not shown). This suggested that oligosaccharides 1-2
and 1-3 had an identical structure to that of oligosaccharide
II except that only 1 N-acetylneuraminyl residue was present
in oligosaccharides 1-2 and 1-3. The difference between 1-2
and 1-3 seems to be the heterogeneity in the location of the
N-acetylneuraminyl residue.

Desialylated oligosaccharide 1-1 appeared to have a higher
molecular weight than desialylated oligosaccharide II. When
this oligosaccharide was treated with α-fucosidase (C. lampas),
a decrease in size corresponding to 1 monosaccharide unit was
observed in a Bio-Gel P-4 column, and the resulting radioac-
tive product co-migrated with asialo-oligosaccharide II. The
subsequent digestions with β-galactosidase and β-N-acetyl-
hexosaminidase gave identical results with those in the case
of asialo-oligosaccharide II. These results suggest that oligo-
saccharide I-1 has a hexasaccharide structure in which an α-
fucosyl residue is attached to a pentasaccharide, 1-2 or 1-3.
The complete structure of this minor oligosaccharide remains
to be elucidated. On the other hand, desialylated oligosaccha-
ride I-5 was found to be eluted at the same position as standard
Gal-GalNAcol (Fig. 5b). β-Galactosidase (A. niger) treatment of asialo-I-5 revealed that this oligosaccharide was
β-galactosyl-N-acetylgalactosaminol.

Asialo-1-4 and asialo-1-6 were identified as β-Gal—β-
GalNAc-X (X may be a degraded GalNAc residue) and
galactitol, respectively, from the results of sequential glycosidase
treatment and gel permeation chromatography (data not shown).
Intact I-4 and I-6 may be the respective monosialo-
ylated forms of those neutral oligosaccharide alditols, and are
presumably by-products derived from oligosaccharide II during
alkaline borohydride treatment, because a glycosidic link-
age at the C-3 position of an N-acetylgalactosaminide residue
is known to be very labile on even mild alkaline treatment (30).

DISCUSSION

The structure of the major O-linked sugar chain of human
platelet glycocalicin has been elucidated as shown in Fig. 7.
This hexasaccharide structure is identical with that of an
oligosaccharide isolated from plasma membranes of an ascites
hepatoma (31), and some sugar chains having a similar struc-
ture to this hexasaccharide have been found in several tumor
cells (26, 32). Thus, the present study indicates that the
distribution of this type of sugar chain is not restricted to
glycoproteins of tumor cell membranes, but that it can also
be found on a normal cell surface such as that of normal human
platelets.

In addition to the major hexasaccharide we obtained minor
sugar chains, some of which lacked 1 N-acetylneuraminyl
residue as can be seen in I-2 and I-3, and 1 residue each of
N-acetylnuneuraminic acid, galactose, and N-acetylglucosamine as
in the case of I-5 compared with the major sugar chain shown in
Fig. 7. The possibility is that they are intermediates during
the biosynthetic processes to form the major hexasaccharide.
Among these minor oligosaccharides, oligosaccharide I-1 has
a somewhat unique monosialyl and monofucosyl structure
instead of a disialyl form as in oligosaccharide II. The number
of oligosaccharides chains/mol of glycocalicin can be estimated
to be approximately 50–60 based on its apparent
M r, 140,000, its carbohydrate content, and the proportion of each
oligosaccharide chain.

In the case of human erythrocyte membranes, structural
analyses of sugar chains of glycoproteins present on the cell
surface have been intensively carried out. Human glubophorin
A, known as a majorialoglycoprotein of erythrocyte mem-
branes (33), has high contents of carbohydrates as in the case
of glycocalicin in human platelets. The structure of the major

![Fig. 6. Mass spectrum of the methylated derivative of the core disaccharide alditol derived from oligosaccharide II.](image)

![Fig. 7. Proposed structure for oligosaccharide II, the major sugar chain of human platelet glycocalicin.](image)
sugar chain of glycoporphin A has been elucidated to be NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc (34), and this is different from that of glycolipid. However, both major sugar chains share a common Galβ1-3GalNAc sequence as the core structure. A mucin type sugar chain containing a GlcNAc residue such as the one found in glycolipid may be distributed among various membrane glycoproteins.

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