Studies on Glycoproteins. III. Isolation of Sialylglycopeptides from Human Platelet Membranes*

D. S. Pepper and G. A. Jamieson

ABSTRACT: No direct evidence has been obtained for the presence of glycoproteins on platelet membranes despite the importance of membrane phenomena in platelet physiology and blood coagulation. In order to obtain such information a purified membrane fraction has been prepared from homogenized platelets and subjected to proteolytic digestion with trypsin and Pronase. Two major size classes of *N*-acetylneuraminic acid containing glycopeptides of molecular weight approximately 12,000 and 3,500, respectively, have been obtained in equimolar amounts and correspond to a total of about 10^7 such heterosaccharide residues per platelet. Purification by gradient elution from DEAE-Sephadex at pH 2.5 produced three major *N*-acetylneuraminic acid containing peaks in each class. Chemical analysis showed that aspartic acid and glucosamine were the major constituents of all six glycopeptide fractions other than hexose. Gas chromatography of the sugars as their alditol acetates showed that mannose and galactose were found in all six fractions, glucose in four out of six, and that fucose was present in trace amounts. The range of analytical data observed were hexoses, 15–30%; *N*-acetylglucosamine, 15–35%; *N*-acetylneuraminic acid, 7–20%; and amino acids, 28–50%. Isoelectric focussing of partially purified, nondiffusible glycopeptides indicated a range of p*K* from 1.8 to 2.2 for the bound *N*-acetylneuraminic acid which is significantly lower than the accepted value for the free acid; this was confirmed by paper electrophoresis of individual glycopeptides. No evidence was obtained for the presence of alkali-labile *O*-seryl glycosidic linkages in the platelet membrane. These results (i) confirm that glycoproteins are an integral part of the platelet membrane, (ii) demonstrate that they are composed of a variety of complex heterosaccharide units, and (iii) show that the heterosaccharide units of the platelet membrane are significantly different from those isolated from the erythrocyte membrane.

Glycoproteins are known to be important components of the outer membrane of mammalian cells. However, there is no direct evidence for their presence on human platelet membranes although this has been inferred from the existence of myxovirus receptors in a platelet glycoprotein fraction (Pepper and Jamieson, 1968b) and from electrophoretic (Madoff *et al.*, 1964), enzymatic (Hovig, 1965), and ultrastructural (Behnke, 1968) studies. Protein-bound carbohydrate has also been found in extracts of whole platelets (Bezkorovainy and Doherty, 1962; Bezkorovainy and Rafelson, 1964; Mullinger and Manley, 1968).

Despite these implied relationships virtually nothing is known about the biochemical structure of the platelet membrane apart from some recent studies on the nature of the phospholipid component (Marcus *et al.*, 1966). This dearth of information is due, in part, to the inherent difficulties in working with platelets because of their short lifespan outside the body (24 hr) and their tendency to aggregation, to viscous metamorphosis, shape changes, etc.; in part, to the difficulty in obtaining sufficient material for adequate biochemical studies and, in part, to the absence of any method for preparing well-characterized platelet membranes.

However, many reactions of the platelet seem to be mediated by membrane phenomena. These include aggregation by thrombin and by ADP, adhesion to collagen, adhesion to glass, and adhesion to other surfaces, and the retraction of fibrin clots (for reviews, see Schulz, 1968, Johnson *et al.*, 1961, Kowalski and Niewiarowski, 1967, Johnson and Seegers, 1967, Brinkhous, 1967, and Marcus and Zucker, 1965). In addition, the outer, or surface, membrane of the platelet is unusual in that it is derived from the endoplasmic reticulum of its precursor cell, the megakaryocyte. Thus, the platelet outer membrane corresponds to the inner, or smooth, membranes of most other cells and these appear to differ significantly from plasma membranes in composition and density. Approximately nine different antigenic groupings have been detected on the platelet surface, several of which are immunologically identical with groupings found on granulocytes, lymphocytes, and other cell types (Shulman *et al.*, 1964). In the case of lymphocytes, several of these membrane antigens have been found to be glycoproteins (Shimada and Nathenson, 1967).

As part of a general investigation on the component glycoproteins of the membranes of human blood cells we have prepared a membrane fraction from homogenized human platelets and have isolated and characterized a series of glycopeptides obtained by proteolytic digestion with trypsin followed by Pronase. A preliminary account of this work has already appeared (Pepper and Jamieson, 1968a).

Materials and Methods

Platelet homogenates were isolated from 4-hr-old human acid–citrate–dextrose blood by two cycles of differential centrifugation (Pert *et al.*, 1967) and were finally freed of all traces

* From the Blood Research Laboratory, The American National Red Cross, Washington, D. C., and from the Department of Biochemistry, Georgetown University School of Medicine, Washington, D. C. *Received March 25, 1969.* This work was supported, in part, by U. S. Public Health Service Grant 13057. Contribution No. 145 from the Blood Research Laboratory, The American National Red Cross.

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of red blood cells by low-speed centrifugation and lysis in ammonium oxalate (Pepper and Jamieson, 1968b). The platelets isolated from 36 donor units (18 l. of blood) were subsequently pooled and dialyzed against distilled water at 4° for 18 hr. The swollen platelets were homogenized for 5 min in a close tolerance Teflon Potter-Elvehjem apparatus (Marcus *et al.*, 1966) cooled in ice.

Gas Chromatography of Alditol Acetates. Glycopeptides (60–200 μg plus 100 μg of *myo*-inositol as internal standard) were hydrolyzed in 2 N HCl for 3 hr at 100°. The monosaccharides were then derivatized as their alditol acetates (Sawardeker *et al.*, 1965; Lehnhardt and Winzler, 1968). The hydrolysis samples were neutralized with NH_4OH and reduced with 1 mg of $NaBH_4$ in 1 N NH_4OH for 3 hr at 20°. Excess $NaBH_4$ was destroyed with acetic acid, the solution was evaporated to dryness, and boric acid was removed by four successive additions and evaporations of methanol. The alditols were then acetylated at 100° for 3 hr with equal volumes of pyridine and acetic anhydride. After evaporation to dryness the alditol acetates were desalted by solvent extraction and recovered in the chloroform layer of a chloroform–water mixture (1:1, v/v). The organic extract was evaporated to dryness and redissolved in 10–50 μl of chloroform immediately prior to injection onto the gas chromatography column. All operations were carried out without transfer in Teflon-lined screw-capped conical-bottom centrifuge tubes and all evaporations were performed in a Biodryer (Virtis Co., Gardner, N. Y.) with a special rotor built to hold 48 of the conical centrifuge tubes. Standard sugars were derivatized by identical procedures.

Amino Acid Analysis. Individual amino acids were determined on the Beckman Model 120 short and long columns following the hydrolysis of samples (50–200 μg) in 6 N HCl at 107° for 20 hr under N_2. Glucosamine and galactosamine were quantitated on the long column of this instrument, following hydrolysis with 3 N HCl for 5 hr at 107°.

Sialic acid was determined by the thiobarbituric acid method of Aminoff (1961) after hydrolysis with 0.1 N H_2SO_4 at 85° for 30 min.

Total hexoses were determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956). Molar extinction coefficients at 485 mμ relative to mannose (1.00) were found to be galactose, 0.83; glucose, 0.79; and fucose, 0.51.

Isoelectric Focussing. An LKB Model 8101 electrofocussing column (110 ml) was used with a 1% (w/v) Ampholine carrier of pH range 3–10. Samples (10–100 mg) in distilled water or 1% (w/v) glycine were inserted in the middle sucrose gradient steps in lieu of water. After running for 36 hr at 20° (400 V, 10 mA decreasing to 1 mA) (Svennson, 1967) the sucrose gradient was pumped out and 5-ml fractions were collected, dialyzed exhaustively for 2 days against distilled water containing 0.02% (w/v) NaN_3, and assayed for 278-mμ absorption and sialic acid content.

High-Voltage Paper Electrophoresis. Duplicate samples of glycopeptide (50–100 μg) in water were spotted onto Whatman No. 3MM paper premoistened with formic acid buffer (pH 2.0, 0.9 M) or pyridine–acetic acid buffer (pH 3.5, 0.7 M) and run at 1000 V (30 V/cm) for 30 min in a water-cooled flat-plate apparatus. Glucose was added to correct for endosmosis. Free amino groups were identified with a cadmium–ninhydrin spray (Heilmann *et al.*, 1957). Bound sialic acid was hydrolyzed with 0.1 M trifluoroacetic acid by wrapping the chromatogram in a thin sheet of plastic, sandwiching between two clamped glass plates, and heating at 100° for 20 min. Free sialic acid was then identified with the thiobarbituric acid spray reagent of Warren (1960).

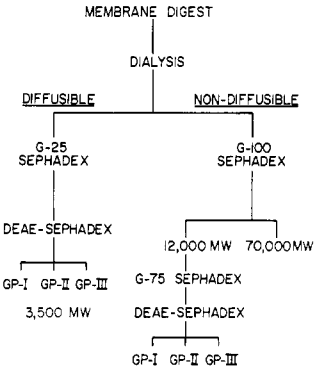

FIGURE 1: Scheme for the isolation of glycopeptides from platelet membranes.

Results

Isolation of Glycopeptides. The scheme for the isolation of the glycopeptide fractions is summarized in Figure 1. The homogenized platelets from 18 l. of blood (about 2.5 g on a dry weight basis) were spun at 6000 rpm (4300*g*) for 15 min to pellet intact platelets. The supernatant solution was spun at 20,000 rpm (48,000*g*) for 60 min and a yellow pellet was obtained which was clearly divided into a lower granular phase and an upper gel phase. These were easily separated with a spatula and were examined by electron microscopy. The upper phase consisted entirely of empty membrane vesicles (Figure 2a) while the lower (granular) precipitate corresponded to granules, intact platelets, and other dense platelet bodies (Figure 2b). The membrane fraction was suspended in 20 ml of 0.1 M phosphate buffer (pH 7.1), containing a few drops of chloroform to retard bacterial growth, and incubated with 20 mg of trypsin at 45°, with stirring, for 24 hr. After trypsinization two 20-mg lots of Pronase were added at 24-hr intervals. No further degradation was noted after 48 hr. However digestion was routinely allowed to proceed for 72 hr for the isolation of glycopeptides. The yellow-brown, insoluble lipid material (650 mg) was removed by centrifugation at 34,000 rpm (126,000*g*) for 2 hr and the clear yellow supernatant solution was dialyzed for 48 hr against 1 l. of distilled water. The dialyzable and nondialyzable peptides were then concentrated under reduced pressure and desalted on G-25 Sephadex. In a typical experiment the yields of diffusible and nondiffusible solutes, recovered by lyophilization, were 1.2 g and 138 mg, respectively. A total of four separate platelet batches was processed in this way.

Gel Filtration. The diffusible and nondiffusible samples were resuspended separately in a minimum volume of water (1–3 ml) and applied to columns of Sephadex G-25 or G-100, respectively. In the case of the nondiffusible material a small but reproducible peak of sialic acid containing material (apparent mol wt 70,000) (Determan, 1966) was eluted just after void volume (Figure 3). The main glycopeptide peak from this fractionation was further chromatographed on G-75 or G-50 Sephadex to remove the peptide material (Figure 4). A single

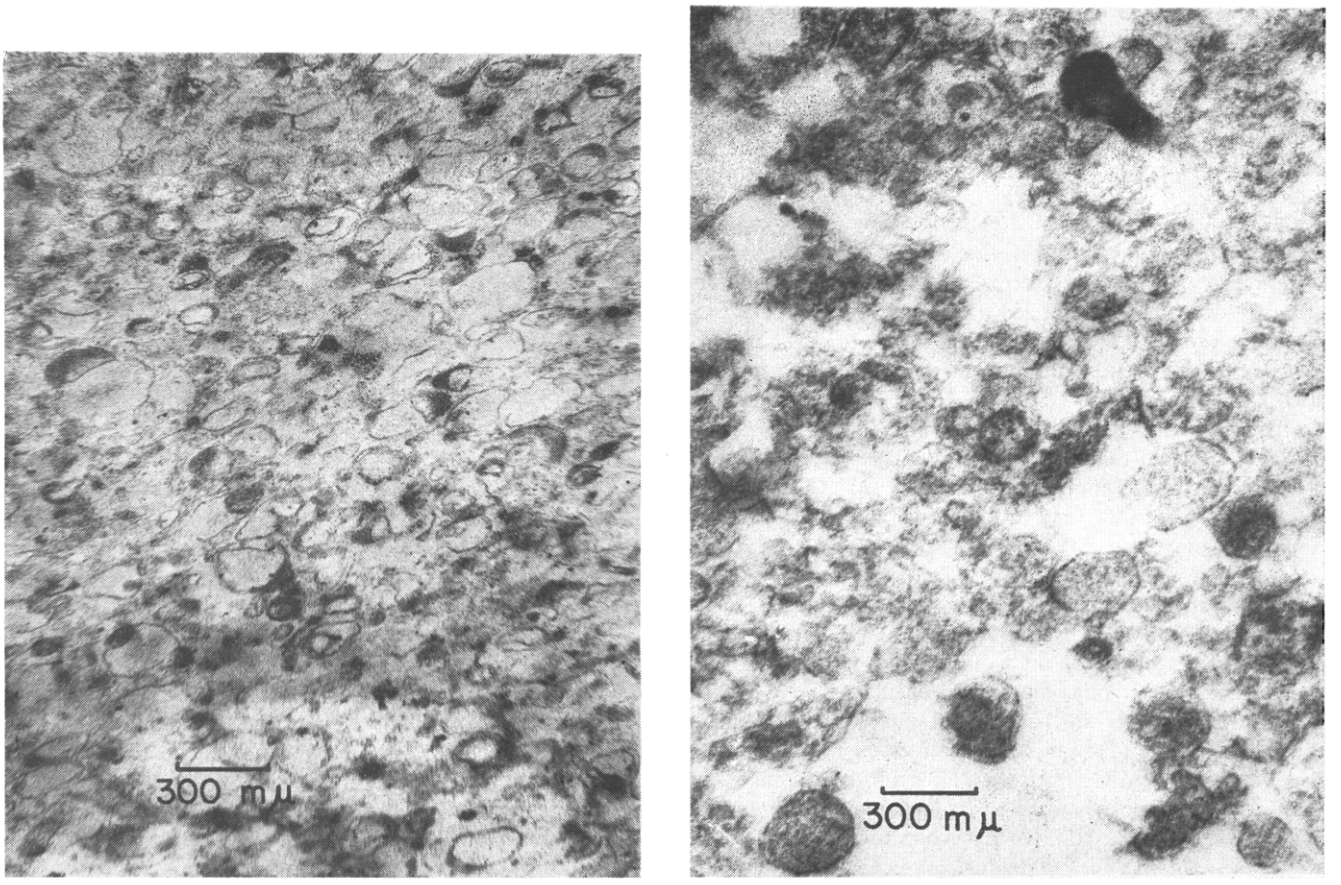

FIGURE 2: Electron micrograph of membrane (a, left) and granule (b, right) phases from the 48,000*g* pellet. Samples were suspended in 4% (w/v) glutaraldehyde, repelleted at 126,000*g*, and embedded in epoxide resin after staining with osmium tetroxide. Thin sections were examined at 76,000X final magnification.

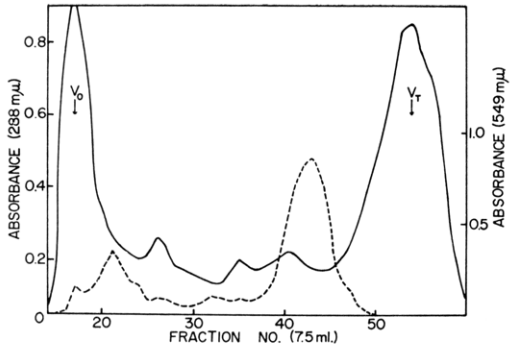

FIGURE 3: Chromatography of 179 mg of crude digest (nondiffusible) on G-100 Sephadex (2.5 × 85 cm) in 0.15 M NaCl plus 0.02% (w/v) NaN_3. Flow rate 12 ml/hr; 7.5-ml fractions were collected. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ----.

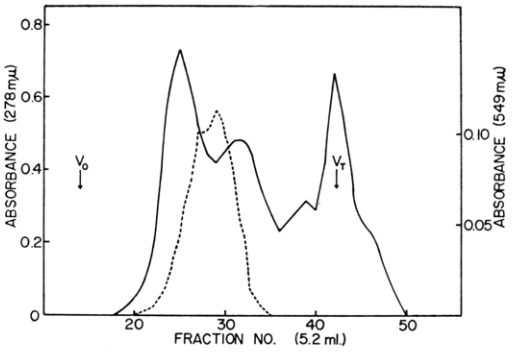

FIGURE 4: Chromatography of 38 mg of partially purified digest (nondiffusible) on G-75 Sephadex (1.2 × 120 cm) in 0.15 M NaCl plus 0.02% (w/v) NaN_3. Flow rate 12 ml/hr; 5-ml fractions were collected. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ----.

NANA[1]-containing peak was obtained in each case (yield 20 mg) which had an apparent molecular weight of 12,000 (Determan, 1966). The diffusible material gave a single peak of NANA-containing material on Sephadex G-25 (Figure 5) with an apparent molecular weight of 3500 (Ackers, 1967). Digestion with trypsin alone produced glycopeptides of the same size range as trypsin followed by Pronase but the noncarbohydrate-containing peptides were smaller in the trypsin-plus-Pronase digests thus facilitating purification. The stability of the tryptic glycopeptides to prolonged Pronase digestion demonstrates that the various size classes of glycopeptide revealed by the dialysis and chromatographic experiments are not the products of incomplete digestion.

[1] Abbreviation used is: NANA, *N*-acetylneuraminic acid.

TABLE I: Composition of Partially Purified (Nondiffusible) Glycopeptides (GP) Eluted from DEAE-Sephadex A-50 at pH 2.85.

	GP I		GP II		
	g/100 g	Moles/12,000 g	g/100 g	Moles/12,000 g	GP III[b]
NANA	2.4	0.99	13.0	5.05	
Glucosamine[a]	14.3	8.44	33.6	19.84	
Mannose	5.3	3.92	4.5	3.33	
Galactose	4.9	3.63	9.6	7.10	
Fucose	1.3	1.07	1.9	1.56	
Asx	15.0	15.55	9.9	10.37	
Thr	6.0	7.12	4.3	5.07	
Ser	3.4	4.70	2.5	9.48	
Glx	11.9	11.08	6.0	5.58	
Pro	11.3	14.46	5.3	6.48	
Gly	5.6	11.90	3.7	7.87	
Ala	2.4	4.00	2.0	3.48	
Val	4.6	5.54			
Ile	5.4	5.68	1.6	1.69	
Leu	3.4	3.74	1.3	1.39	
Weight of sample (mg)	5.9		4.7		2.4
Peak eluted at ionic strength of	0.035		0.110		0.330

[a] As *N*-acetyl. [b] Complete analysis of GP III was not possible because of lack of material.

Ion-Exchange Chromatography. The diffusible and the nondiffusible glycopeptides were separately applied to DEAE-Sephadex A-50 column at pH 2.5 and eluted with a linear gradient of NaCl (0.01–0.25 M in distilled water adjusted to pH 2.5 with HCl) (Figures 6 and 7). Preliminary experiments at pH values of 7.2, 6.2, 5.2, and 2.9 indicated that only partial resolution of glycopeptides from each other and from peptide material was obtained. However, the low p*K* of the bound sialic acid enables satisfactory adsorption to be effected at pH 2.5, or lower. Three major peaks containing sialic acid and hexose were eluted at ionic strengths from 0.01 to 0.10 for both the diffusible and nondiffusible glycopeptides (Tables I and II; Figures 6 and 7).

This procedure has the disadvantage that any fucosylglycopeptides without sialic acid will not be retained on the column and that prolonged contact with solutions of low pH may hydrolyze some NANA.

Isoelectric Focussing. As an alternative procedure for the isolation of sialic acid containing glycopeptides the crude digest after dialysis (25 mg of nondiffusible solute) was run in the electrofocussing column (Figure 8). The general application of this method for the isolation of sialylglycopeptides is currently under investigation. Although it has the advantage of preparing substantially peptide-free glycopeptide material directly from crude digests in a single step, it is inferior to

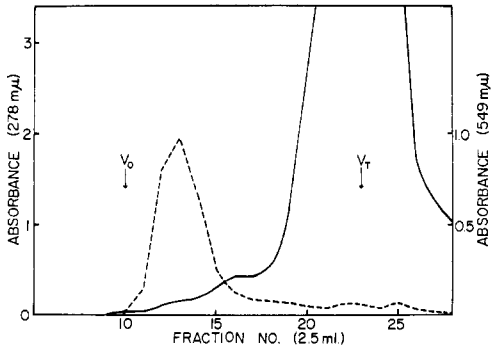

FIGURE 5: Chromatography of 12 mg of crude digest (diffusible) on G-25 Sephadex (1.1 × 55 cm) in water. Flow rate 8 ml/hr; 2.5-ml fractions were collected. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ---.

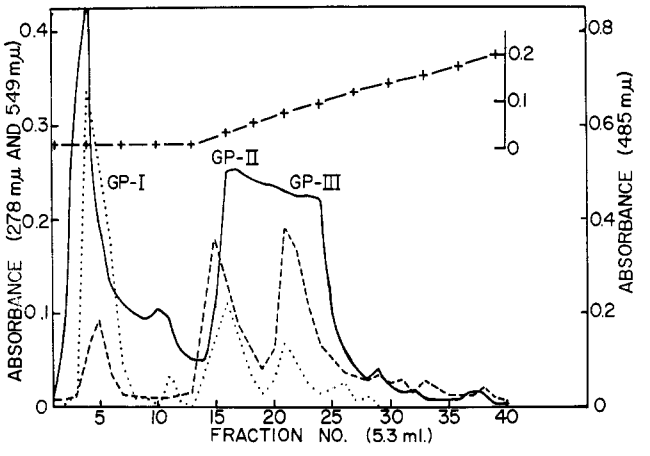

FIGURE 6: Chromatography of 12 mg of partially purified (diffusible) glycopeptides on DEAE-Sephadex A-50 (1.2 × 30 cm) at pH 2.5 Flow rate 6 ml/hr; 5-ml fractions were collected. Linear gradient from 0.01 to 0.25 M NaCl. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ---; hexose (absorbance at 485 mμ), · · ·; NaCl, +—+—.

TABLE II: Composition of Purified Glycopeptides (Diffusible and Nondiffusible) Eluted from DEAE-Sephadex A-50 at pH 2.50.

	Diffusible						Nondiffusible					
	GP I		GP II		GP III		GP I		GP II		GP III	
	g/100	Moles/ 3,500 g	g/100	Moles/ 3,500 g	g/100	Moles/ 3,500 g	g/100	Moles/ 12,000 g	g/100	Moles/ 12,000 g	g/100	Moles/ 12,000 g
NANA	2.7	0.3	6.9	0.8	6.2	0.8	9.5	3.9	18.9	7.8	16.0	6.6
Acetylglucosamine	35.0	6.0	ND		31.0	5.3	30.0	17.7	34.0	20.1	35.0	20.7
Mannose	20.5	4.4	7.5	1.6	7.1	1.5	6.8	5.0	7.8	5.8	7.3	5.4
Galactose	5.7	1.2	4.1	0.9	6.5	1.4	9.7	7.2	14.8	11.0	11.1	8.2
Glucose	7.1	1.5	15.0	3.2	0	0	0	0	1.7	1.3	2.9	2.2
Asx	10.2	2.9	ND[a]		12.3	3.7	11.8	12.7	5.5	5.7	6.1	6.3
Thr	4.3	1.3	ND		3.1	1.1	4.1	4.7	2.2	2.6	3.0	3.5
Ser	2.3	0.7	ND		3.9	1.6	3.9	5.4	1.4	1.9	2.3	3.2
Glx	4.3	1.0	ND		8.2	2.2	6.2	5.7	4.4	4.1	3.7	3.4
Pro	2.3	0.7	ND		3.0	1.0	2.5	3.1	1.3	1.6	2.8	3.5
Gly	1.5	0.8	ND		3.1	1.9	3.5	7.3	1.4	3.0	2.0	4.1
Ala	0.7	0.3	ND		3.8	1.8	3.0	5.1	1.8	3.0	1.6	2.8
Val	1.8	0.6	ND		1.4	0.5	2.7	3.2	1.0	1.3	1.0	1.3
Ile	0.8	0.3	ND		1.4	0.4	1.0	1.2	0.7	0.8	1.1	1.2
Leu	0.9	0.3	ND		2.4	0.7	1.5	1.5	1.1	1.3	1.6	1.7
Weight of sample (mg)	1.0		0.6		1.4		2.2		1 2		1.1	
Peak eluted at ionic strength	0.010		0.030		0.080		0.054		0.084		0.108	

[a] ND, not determined due to insufficient sample.

chromatography on DEAE-Sephadex at the present stage of development since individual sialylglycopeptide peaks were not obtained. It does, however, have the advantage of indicating an unequivocal pI range for the glycopeptide material (Svennson, 1967) of 1.8–2.0 which is significantly lower than the value of 2.6 generally claimed for the carboxyl group of free NANA (Scheinthal and Bettelheim, 1968). A possible explanation is that the p*K* of bound NANA is significantly lower than that of the free acid but the presence of sulfate or phosphate ester linkages in the glycopeptides cannot be excluded on the basis of the present evidence; further investigation of this point was precluded by the small amounts of material available.

Paper Electrophoresis. When each glycopeptide was run in high-voltage paper electrophoresis a single spot was observed for each glycopeptide peak apart from small traces of free amino acids and sialic acid arising from the acid conditions employed in ion-exchange chromatography and in paper electrophoresis (Figure 9). These spots had the characteristic brown color of glycopeptides compared with the pink color of free amino acids (Marshall and Neuberger, 1964). Bound NANA was detected only in the same spot as was identified by the ninhydrin spray. The mobility on paper at pH 2.2 and 3.5 paralleled the order of elution from DEAE-Sephadex for both sets of glycopeptides. The nondegradable glycopeptide of mol wt 70,000 present in the nondiffusible fraction was also run in high-voltage paper electrophoresis at pH 3.5 in pyridine–acetate buffer and gave a ninhydrin- and sialic acid positive streak with anodal mobility; this material was not investigated further.

Chemical Analysis. The glycopeptide fraction (mol wt 12,000) isolated from Sephadex G-75 or G-50 (Figure 4) had a hexose content of 12.3% and a NANA content of 5.4%. Material which had been further purified on an electrofocussing column but not resolved into separate glycopeptide peaks gave a hexose content of 28.6%. The neutral sugar components were found to be fucose, 2.5%; mannose, 7.0%; galactose, 10.6%; and glucose, 8.5%.

Analytical data for material eluted from DEAE-Sephadex at pH 2.85, but only partially resolved into individual glycopeptide peaks is shown in Table I. Analytical data for separate glycopeptide peaks from both the diffusible and nondiffusible fractions on DEAE-Sephadex at pH 2.50 is presented in Table II together with the weight yield of the purified fractions.

The individual hexoses identified were mannose, glucose, and galactose. Small amounts of fucose (2.0%) were identified in several preparations. In addition, a small but reproducible peak appeared prior to mannose on gas chromatography but was not identifiable with known standards (Figure 10). This component appears to be a hydrolysis product of glycopeptide material generally and not an artifact of derivatization. In most cases, galactose was the major hexose while glucosamine was the only hexosamine present. The sialic acid values showed large variation but correlated generally with the order of elution from DEAE-Sephadex. A decrease in the hexose: NANA ratio is evident with increasing the elution volume of the

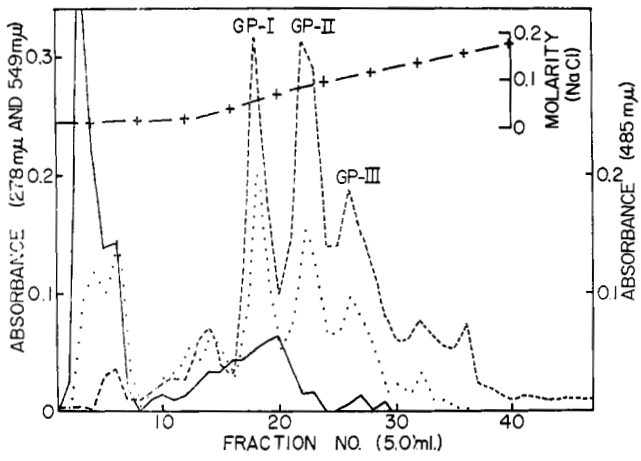

FIGURE 7: Chromatography of 20 mg of partially purified (nondiffusible) glycopeptides from DEAE-Sephadex A-50 (1.1 × 30 cm) at pH 2.5. Flow rate 12 ml/hr; 5-ml fractions were collected. Linear gradient from 0.01 to 0.25 M NaCl. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ---; hexose (absorbance at 485 mμ), · · ·; NaCl, +—+—+.

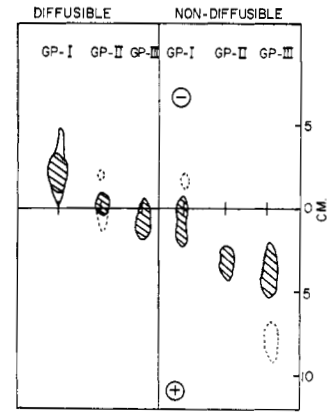

FIGURE 9: High-voltage paper electrophoresis of diffusible and nondiffusible glycopeptides (100 μg) at pH 3.5 in 0.9 M pyridine–acetic acid buffer; 1000 V for 30 min at 20°. Open circles indicate ninhydrin-positive spots, hatched circles indicate thiobarbiturate-positive spots, and broken lines indicate faint reactions.

glycopeptides (Figures 6 and 7; Table II). Amino acid analysis (Table II) indicated that aspartic acid was the major amino acid in every case. Small amounts of aromatic and basic, but no sulfur-containing amino acids were detected. Alkaline reduction (0.1 N NaOH and 0.3 M $NaBH_4$, 24 hr at 20°) produced no glucosaminitol or loss of serine and threonine.

Discussion

These results confirm the presence of glycoproteins in the outer membrane of the human platelet and indicate that a variety of complex heterosaccharide units must be present. We have previously shown that these glycoproteins can be solubilized and possess influenza virus receptor activity (Pepper and Jamieson, 1968b) and a complement-fixing antigen (unpublished data). In addition recent work has shown that NANA is an important receptor for the uptake of serotonin in the contraction of smooth muscle (Wesemann and Zilliken, 1968). Platelets, which also have a contractile function, are known to take up large quantities of serotonin and these platelet membrane glycoproteins may be related to the receptor sites.

Using a number of enzyme markers, Marcus *et al.* (1966) have shown that platelet outer membranes can be isolated relatively free of other intracellular organelles. Although it cannot yet be determined to what extent our preparations are contaminated with the membranes of platelet granules and mitochondria their homogeneity demonstrated in the electron photomicrographs, taken together with the presence of hemagglutination inhibition activity in the soluble glycoprotein fraction strongly indicates platelet outer membranes as the major component of this fraction.

The analytical data of the diffusible glycopeptides differ from the nondiffusible glycopeptides in several respects. The diffusible glycopeptides contain 1 mole, or less, of NANA per mole of glycopeptide and have galactose:mannose ratios of 1:4, 1:2, and 1:1 for diffusible glycopeptides I, II, and III, respectively. The nondiffusible glycopeptides I, II, and III have molar contents of NANA of 4, 8, and 7, respectively, and ga-

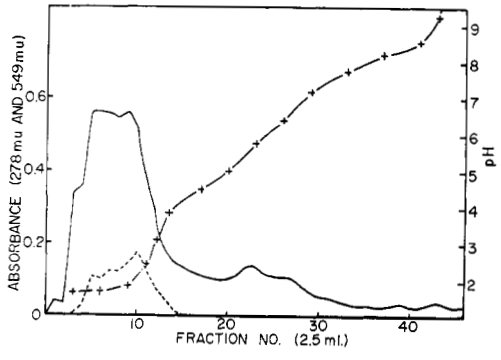

FIGURE 8: Isoelectric focussing of crude nondiffusible glycopeptides (25 mg) in a pH gradient from 1.5 to 10.5 (2.5-ml fractions were collected). Final voltage/current 400 V/1 mA at 20°. Absorbance at 278 mμ, —; NANA (absorbance at 549 mμ), ----. pH, —×—.

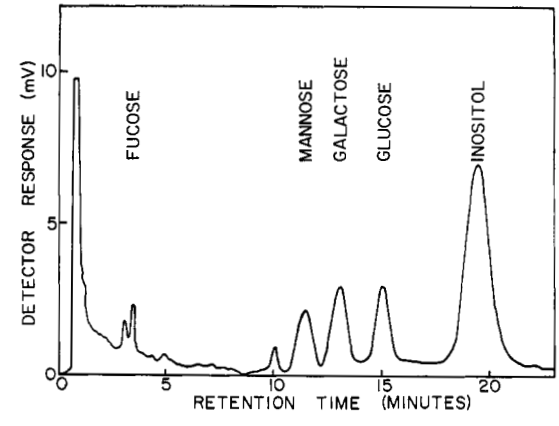

FIGURE 10: Gas chromatography of alditol acetates of partially purified (nondiffusible) glycopeptides on a 4-ft column of 3% ECNSS-M on Gas Chrom Q (100–200 mesh), 195°, N_2 flow rate 80 ml/min. Flame ionization detector. The internal standard of *myo*-inositol represents 8 μg.

lactose:mannose ratios of 1:0.66, 1:0.5, and 1:0.66. These differences may reflect microheterogeneity in the diffusible and nondiffusible glycopeptides or different antigen types in the donor pool. *N*-Acetylglucosamine is found in particularly high concentrations in the nondiffusible glycopeptides and both diffusible and nondiffusible glycopeptides were devoid of galactosamine although this comprises one-quarter of the total hexosamine of intact platelets (A. J. Barber, D. S. Pepper, and G. A. Jamieson, 1969; unpublished data). This supports the observation of Olsson and Gardell (1967) that 94% of the total galactosamine of human platelets occurs in the glycosaminoglycan fraction. Fibrinogen is a minor but tenacious component (2%) of platelet membranes even after extensive washing (Nachman *et al.*, 1967). However NANA comprises 0.8–1% of platelet membranes while fibrinogen contains only 0.37% NANA. Hence fibrinogen glycopeptides could make only a negligible contribution to the analytical data obtained.

There are several possibilities regarding the relation between the high molecular weight (12,000) nondiffusible glycopeptides and the low molecular weight (3500) diffusible glycopeptides. The nondiffusible glycopeptide cannot be composed of a series of identical heterosaccharide chains corresponding to the diffusible glycopeptide in view of their differing over-all composition. However the question of whether the two heterosaccharides were derived from a single glycoprotein chain or from two different ones cannot be resolved.

On the basis of the recoveries of diffusible and nondiffusible glycopeptides it can be calculated that the high (12,000) and low (3500) molecular weight glycopeptides are present in approximately equal numbers on the platelet membranes and correspond to a total of about 10^7 glycopeptides per individual blood platelet although each glycopeptide may contain several heterosaccharide chains.

Of particular interest is the high content of aspartic acid which ranges from a minimum of 3 moles/mole for the diffusible glycopeptides to a maximum of 12 moles/mole for the nondiffusible glycopeptides. Although these fractions were homogeneous on paper electrophoresis the elution patterns obtained on ion-exchange chromatography (Figures 6 and 7) indicate that they are not pure and it is possible that contamination with heterogeneous acidic polypeptides is responsible for the high aspartic acid content. Another possibility is that there are a number of aspartyl residues in the immediate environs of the point of attachment of the single glycopeptide chain and that these polyaspartylpeptides are resistant to proteolysis. A third possibility is that the isolated glycopeptides are, in fact, units containing several carbohydrate chains joined to multiple aspartyl residues occurring in close proximity on the membrane and that the resistance to proteolysis is a reflection of the high carbohydrate density near the points of attachment. Answers to these questions must await the large-scale purification of the intact glycoproteins of the platelet membrane. *O*-Glycosidic linkages between *N*-acetylgalactosamine and serine or threonine, of the type found in erythrocyte membranes, appear to be absent from platelet membranes.

The nature of the high molecular weight components (mol wt 70,000) separated by gel filtration of the nondiffusible glycopeptides is unknown. The amount of this fraction was insufficient for further investigation. It may be resistant to proteolysis because of its amino acid composition or because the NANA is part of a glycolipoprotein unit.

It is of interest to compare these data on platelet membrane glycoproteins with the information available on erythrocyte membrane glycoproteins. Winzler *et al.* (1967) have shown that tryptic digestion of whole red cells yields a single sialylglycopeptide of mol wt 10,000 which contains *N*-acetylgalactosamine in an alkali-labile *O*-glycosidic linkage to the hydroxyl groups of serine and threonine. In contrast the sialylglycopeptides of the human platelet membrane lack *N*-acetylgalactosamine, contain relatively less serine and threonine and more aspartic and glutamic acids, and are resistant to alkaline hydrolysis. These facts, together with the isolation of two size ranges of sialylglycopeptides in the present case, indicate fundamental differences in the membrane glycoproteins of human platelets and red cells.

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References

Ackers, G. K. (1967), *J. Biol. Chem. 242*, 3237.
Aminoff, D. (1961), *Biochem. J. 81*, 384.
Behnke, O. (1968), *J. Ultrastruct. Res. 24*, 51.
Bezkorovainy, A., and Doherty, D. (1962), *Arch. Biochem. Biophys. 86*, 412.
Bezkorovainy, A., and Rafelson, M. E. (1964), *J. Lab. Clin. Med. 64*, 212.
Brinkhous, K. M. (1967), Platelets: Their Role in Hemostasis and Thrombosis, Stuttgart, F. K. Schattauer-Verlag.
Determan, H. (1966), *Protides Biol. Fluids 14*, 563.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.
Heilmann, J., Barrollier, J., and Watzke, E. (1957), *Z. Physiol. Chem. 309*, 219.
Hovig, T. (1965), *Thromb. Diath. Haemorrhag. 13*, 84.
Johnson, S. A., Monto, R. W., Rebuck, J. W., and Horn, R. C. (1961), Blood Platelets, Boston, Mass., Little Brown.
Johnson, S. A., and Seegers, W. H. (1967), Physiology of Hemostasis and Thrombosis, Springfield, Ill., C. C Thomas.
Kowalski, E., and Niewiarowski, S. (1967), Biochemistry of Blood Platelets, New York, N. Y., Academic.
Lehnhardt, W. F., and Winzler, R. J. (1968), *J. Chromatog. 34*, 471.
Madoff, M. A., Ebbe, S., and Baldini, M. (1964), *J. Clin. Invest. 43*, 870.
Marcus, A. J., and Zucker, M. B. (1965), The Physiology of Blood Platelets, New York, N. Y., Grune and Stratton.
Marcus, A. J., Zucker-Franklin, D., Saifer, L. B., and Ullman, H. L. (1966), *J. Clin. Invest. 45*, 14.
Marshall, R. D., and Neuberger, A. (1964), *Biochemistry 3*, 1596.
Mullinger, R. N., and Manley, G. (1968), *Biochim. Biophys. Acta 170*, 282.
Nachman, R. L., Marcus, A. J., and Zucker-Franklin, D. (1967), *J. Lab. Clin. Med. 69*, 651.
Olsson, I., and Gardell, S. (1967), *Biochim. Biophys. Acta 141*, 438.
Pepper, D. S., and Jamieson, G. A. (1968a), *Fed. Proc. 27 (2)*, 3364.
Pepper, D. S., and Jamieson, G. A. (1968b), *Nature 219*, 1252.

Pert, J. H., Zucker, M. B., Lundberg, A., Yankee, R., and Henderson, E. (1967), *Vox Sanguinis 13*, 119.
Sawardeker, J. S., Sloneker, J. H., and Jeans, A. (1965), *Anal. Chem. 37*, 1602.
Scheinthal, B. M., and Bettelheim, F. A. (1968), *Carbohydrate Res. 6*, 257.
Schulz, H. (1968), Electron Microscopy of Blood Platelets and Thrombosis, New York, N. Y., Springer-Verlag.
Shimada, A., and Nathenson, S. G. (1967), *Biochem. Biophys. Res. Commun. 29*, 828.
Shulman, N. R., Marder, V. J., Hiller, M. C., and Collier, E. M. (1964), *Progr. Hematol. 4*, 222.
Svennson, H. (1967), *Protides Biol. Fluids 15*, 515.
Warren, L. (1960), *Nature 186*, 237.
Wesemann, W., and Zilliken, F. (1968), *Z. Physiol. Chem. 349*, 823.
Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P. (1967), *Biochemistry 6*, 2195.

The *O*-Acetyl Derivatives of *N*-Acetylmuramic Acid*

Toshiaki Osawa,† Pierre Sinaÿ,‡ Margaret Halford, and R. W. Jeanloz§

ABSTRACT: The 4-*O*-acetyl, 6-*O*-acetyl, and 4,6-di-*O*-acetyl derivatives of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] were synthesized starting from benzyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside. The 4-*O*-acetyl derivative was obtained by preparation of the 4,6-*O*-benzylidene derivative of the benzyl ester, followed by removal of the benzylidene group, tritylation at O-6, acetylation at O-4, detritylation, and hydrogenolysis of both benzyl groups. The 6-*O*-acetyl derivative was obtained by formation of the internal ester at O-4 and acetylation at O-6, followed by hydrogenolysis of the benzyl aglycon group. The 4,6-di-O-acetyl derivative was obtained by acetylation at O-4 and O-6 of the benzyl ester of the starting material, followed by removal of both benzyl groups.

The various derivatives were characterized by infrared spectroscopy and by paper and gas–liquid partition chromatography. The 6-*O*-acetyl derivative was identical with a compound isolated from *Staphylococcus aureus* cell-wall.

The presence of *O*-acetyl groups has been reported in numerous polysaccharides of bacterial origin, and it has been suggested that the resistance of some bacterial cell walls toward egg-white lysozyme (Brumfitt *et al.*, 1958; Brumfitt, 1959) or to attack by bacteriophages (Brumfitt, 1960) was caused by *O*-acetyl groups linked to the cell wall structure.

After enzymic degradation of *Staphylococcus aureus* cell wall, Ghuysen and Strominger (1963) isolated a mono-*O*-acetyl derivative of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose]. On the basis of the results of the periodate oxidation of this derivative and of the parent disaccharide, these authors proposed the structure of a 4-*O*-acetyl derivative. After it was shown that the *N*-acetylmuramic acid residue is linked in the bacterial cell wall at C-4 (Jeanloz *et al.*, 1963; Sharon *et al.*, 1966), the periodate oxidation of the monoacetyl derivative and of the parent disaccharide was reinvestigated, and the new results were interpreted to suggest a 6-*O*-acetyl derivative of muramic acid (Tipper *et al.*, 1965). Since the results of the periodate oxidation have in the past led to erroneous interpretations of structures containing muramic acid (Salton and Ghuysen, 1959, 1960; Ghuysen and Strominger, 1963), the synthesis of the various *O*-acetyl derivatives of *N*-acetylmuramic acid was accomplished in order to obtain standards for comparison with natural products.

Results and Discussion

Benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (II) (Scheme I) (Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965; Jeanloz *et al.*, 1968) was selected as starting material for the synthesis of the three possible *O*-acetyl derivatives of *N*-acetylmuramic acid, 4-*O*-acetyl (X), 6-*O*-acetyl (XV), and 4,6-di-*O*-acetyl (XVI), because the mild conditions used for removal of the benzyl aglycon group by catalytic hydrogenation would not affect the *O*-acetyl groups.

For the preparation of the 4-*O*-acetyl derivative X, the carboxyl group of the lactyl side chain was protected by esterification with benzyl alcohol, in order to avoid the formation

* From the Laboratory for Carbohydrate Research, Department of Biological Chemistry and Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts 02114. *Received March 25, 1969.* This is Publication LIX of the series Amino Sugars and No. 479 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital. This investigation has been supported by research grants from the National Institute of Allergy and Immunology, National Institutes of Health, U. S. Public Health Service (Grant AI-06692), and from the National Science Foundation (Grant GA-5031). A preliminary note has been published (Jeanloz and Osawa, 1964).

† Present address: Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

‡ Fellow from the Centre National de la Recherche Scientifique (France).

§ To whom inquiries should be sent.