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Physicochemical Properties of Some Glycopeptides Released from Human Erythrocyte Membranes by Trypsin*

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ABSTRACT: Glycopeptides possessing very weak M and N hemagglutinating inhibitory activity are released both from intact erythrocytes and stroma by the action of trypsin. The variation in chromatographic and electrophoretic behavior between these glycopeptides indicates differences in their molecular weights and net electrical charges. These sialoglycopeptides contain galactosamine and glucosamine and a variety of neutral sugars. The amino acid composition varies significantly from one tryptic glycopeptide to another. One glycopeptide was found to be rich in glutamate and poor in serine, threonine, and aspartate with the NH_2-terminal amino acid being alanine. Two other glycopeptides contained large quantities of serine and threonine, one of these had serine as the NH_2-terminal amino acid while no NH_2-terminal amino acid could be demonstrated in the other glycopeptide.

The M, N blood-group active materials are associated with erythrocyte peripheral zone glycoproteins. All M antigenic determinants contain intrinsic N substance, the specificity of these substances (M, N) apparently being conferred by their terminal neuraminyl groups (Prokop and Uhlenbruck, 1969). No conclusive difference in chemical composition has been found between the M and N blood-group active sialoglycoproteins (Kathan and Adamany, 1967).

Treatment of intact human erythrocytes with trypsin releases glycopeptides which possess low M or N blood-group activity (Seaman and Heard, 1960; Cook *et al.*, 1960; Thomas and Winzler, 1969). It has also been shown that such treatment leads to the loss of M but not N cellular blood-group specificity (Ohkuma *et al.*, 1968). Some of the glycopeptides released into the suspending medium by trypsin treatment have been partially characterized (Winzler *et al.*, 1967).

The present study was undertaken in order to isolate several of the glycopeptides released from either intact human red cells or posthemolytic residues (ghosts) by the action of trypsin and to examine their structure and hemagglutinating inhibitory properties, if any, in relation to the antigenic type of the erythrocytes from which they were derived.

Experimental Sections

Materials

All solutions were made up in water distilled twice in Pyrex glassware. Reagents were of analytical grade unless specified to be otherwise. Standard saline consisted of 0.145 M aqueous sodium chloride solution with the pH adjusted to 7.2 ± 0.2 using 0.5 M aqueous sodium bicarbonate solution. Trypsin (twice crystallized and lyophilized) from bovine pancreas was obtained from the Worthington Biochemical Corp. Rabbit M and N antisera were supplied by Ortho Diagnostics. Outdated O Rhesus-positive bloods were obtained from the Pacific Northwest Red Cross Blood Center, Portland, Ore. Dowex 50W-X2 (200–400 mesh) strongly acidic cation exchanger was obtained from J. T. Baker Chemical Co. Electrophoresis grade acrylamide was purchased from Bio-Rad Laboratories. All grades of Sephadex were supplied by Pharmacia Fine Chemicals, Inc.

Methods

Blood samples were pooled according to their M, N, or MN antigenic specificity after typing with rabbit M and N antisera. Red blood cells from the pooled blood samples were washed four times with standard saline. The washed erythrocytes were then either treated directly with 0.5 mg/ml of trypsin at 38° for 60 min or washed once in 310 mOsmoles/kg of sodium phosphate buffer at pH 5.8 and red cell ghosts prepared as follows. The washed erythrocytes were hemolyzed in 20 mOsmoles/kg of phosphate buffer (Dodge *et al.*, 1963) and the posthemolytic residues dialyzed against doubly distilled water at ≃5°. Cell ghosts were then made up to about 50% v/v suspension in distilled water and trypsin added to give a concentration of 0.5 mg/ml. The system was incubated at 38° for 60 min. The release of the glycopeptides from both intact red blood cells and cell ghosts was followed by assay of the sialic acid liberated into the suspending medium and also by monitoring the gradual decrease, with time, in the electrophoretic mobility of treated erythrocytes and cell ghosts.

Total neutral hexose in the sialoglycopeptides was determined by the phenol–sulfuric acid colorimetric method (Dubois *et al.*, 1956) and the individual sugars by the gas–liquid chromatographic procedure of Lehnhardt and Winzler (1968). Total hexosamines and the individual amounts of glucosamine

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and galactosamine were obtained after hydrolysis with 3 N HCl for 4 hr using a Technicon AutoAnalyzer (Kominz, 1962). Sialic acid was determined by the method of Svennerholm (1956). Amino acids were determined after hydrolysis for 22 hr in constant-boiling HCl (5.7 N) at 105–110° in sealed evacuated tubes using a Technicon AutoAnalyzer. Tryptophan was determined by the method of Bencze and Schmid (1957). NH_2-terminal amino acids were identified by the Sanger fluorodinitrobenzene method as modified by Fraenkel-Conrat *et al.* (1955), and the dansyl chloride[1] procedure of Gray (1967). Partial specific volumes were calculated from the amino acid and carbohydrate composition by the method of Schachman (1957). The partial specific volumes used for the sugars were 0.62 for hexose and hexosamine and 0.59 for sialic acid (Bezkorovainy and Doherty, 1962). Conventional equilibrium sedimentation studies for molecular weight estimation were performed with a Spinco Model E analytical ultracentrifuge (Chervenka, 1970). A 12-mm double-sector cell was used at speeds of 20,410–29,500 rpm. All photographic plates were read on a Gaertener microcomparator. Apparent molecular

$$\text{apparent molecular weight, } M_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{2.303(\text{d} \log c)}{\text{d}(r^2)}$$

$$\text{average molecular weight, } M_z = \frac{M_{wb}c_b - M_{wm}c_m}{c_b - c_m}$$

weights were calculated from plots of log c (where c is concentration in fringes at various radial positions in the cell) *vs.* r^2 (where r is the radial distance (centimeters) from the center of the rotor) and R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume of solute, ρ density of the solution, and ω is the angular velocity of the rotor in radians per second. In calculating M_z, M_{wb} is the molecular weight at the cell bottom, M_{wm} the molecular weight at the meniscus, c_b concentration of the solute at the cell bottom, and c_m concentration of the solute at the cell meniscus.

Durrum-type paper electrophoresis was performed using 0.025 M potassium biphthalate buffer at pH 5.9 at a current of 1.25 mA/30-cm strip (Durrum, 1949). Disc polyacrylamide gel electrophoresis was carried out in 20% gel using the method of Davis (1964). Electrophoretic mobility measurements on cells and posthemolytic residues were carried out in a cylindrical chamber apparatus as described by Seaman and Heard (1961). The calcium, potassium, and sodium ion contents of the sialoglycopeptides were estimated by flame photometry (Kingsley and Shaffert, 1953).

Results

About 0.75 μmole of sialic acid was released per ml of packed ghosts or about 3 ml of packed intact red blood cells by incubation with the trypsin for 60 min. During the same time interval the electrophoretic mobility of the cells or ghosts in standard saline decreased from the control value of -1.08 ± 0.03 to -0.73 ± 0.04 μ per sec per V per cm. Eylar *et al.* (1962) have reported that the total sialic acid content of human erythrocytes is in the range of 0.45–0.56 μmole/ml of packed red blood cells.

[1] Abbreviations used were: dansyl chloride, 5-dimethylamino-1-naphthalenesulfonyl chloride; AP-I, acidic sialo substance one; AP-II, acidic sialo substance two; LAP-I, LAP-II and LAP-III, less acidic substances one, two, and three, respectively.

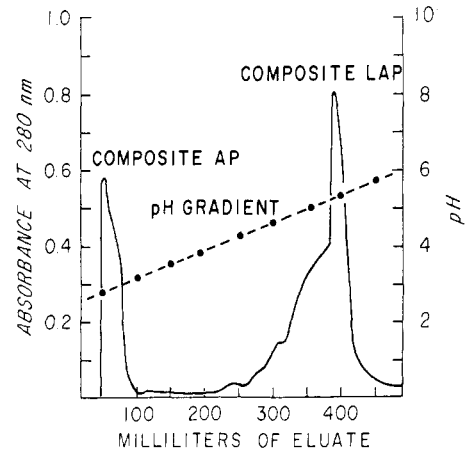

FIGURE 1: Separation of the crude glycopeptide fraction released from stroma by trypsin into the most acidic tryptic glycopeptide fraction and the less acidic glycopeptide fraction on a sulfonated polystyrene cation-exchange resin (Dowex 50W-X2). Column (2.3 × 40 cm) was loaded with 60 mg of the trichloroacetic acid soluble desalted crude glycopeptide and equilibrated with 0.1 M aqueous sodium citrate buffer and eluted with a pH gradient of 2.65–5.50 and ionic strength gradient of 0.1–0.5 M. (--●--) pH gradient; (—) variation in absorbance at 280 nm, a value of 0.1 of a unit corresponding to 0.70 mg of glycopeptide/ml.

The sialoglycopeptides released from the posthemolytic residues by treatment with trypsin were isolated by spinning down the treated posthemolytic residues at 27,300*g* for 30 min at 0° in a Sorval RC2-B preparative centrifuge, followed by addition of trichloroacetic acid up to a concentration of 6% w/v to the clear supernatant fluid in order to precipitate any proteins present. The supernatant fluid recovered by centrifugation which contained the soluble heterogeneous sialomucoids was freed of trichloroacetic acid by extraction with diethyl ether and the aqueous phase then lyophilized. An aqueous solution of the sialomucoid fraction was desalted by passage through a Sephadex G-10 or G-25 column.

Separation of the more acidic sialoglycopeptides from the less acidic ones was achieved on a cation-exchange resin (Dowex 50W-X2, sulfonated polystyrene) employing sodium citrate buffer and the developer with a pH and ionic strength gradient of 2.65–5.50 and 0.1–0.5 M, respectively (as shown in Figure 1). The term acidic is used in relation to behavior of the sialoglycopeptide on the Dowex cation exchanger rather than to its amino acid composition or sialic acid content. The more acidic sialosubstance on this criterion appeared to be mainly a single component by standard physicochemical procedures and is designated, AP-I.[1] The less acidic sialosubstance fraction was separated into two components (LAP-I and LAP-II) using a Sephadex G-50 superfine gel with distilled water as the developer (see Figure 2).

LAP-I was subjected to recycling chromatography (Porath and Bennich, 1962) (1.25 × 95 cm, Sephadex G-50 superfine column; four cycles; flow rate 12 ml/hr, Milton Roy minipump) using water as the developer. Fractions 30–40 were pooled and lyophilized to yield a purified LAP-I. Figure 3 shows the separation of the OD_{280}-absorbing components from the major sialic acid containing substance. However when sialo substance LAP-II was subjected to recycling chromatography, two components were obtained, the sialo substance remaining with the more OD_{280}-absorbing portion being termed LAP-III, and the other component representing purified LAP-II. The LAP-III was found to be quite hetero-

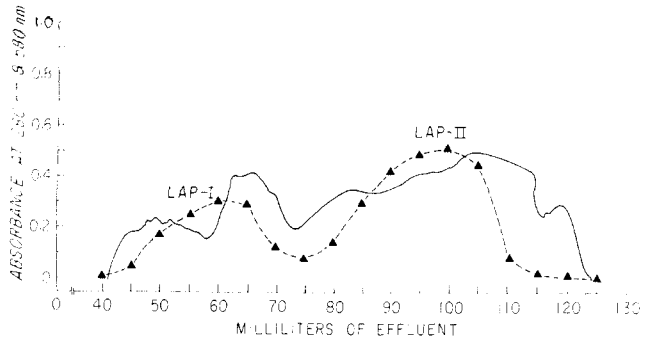

FIGURE 2: Purification of the less acidic glycoprotein fraction (initial separation shown in Figure 1) by gel filtration on a G-50 Sephadex superfine gel column (1.5 × 84 cm) with distilled water as the developer. (—) Absorbance at 280 nm, a value of 0.1 of a unit corresponding to 0.70 mg of glycopeptide/ml. Sialic acid is represented by --▲-- which corresponds to the absorbance at 580 nm, using the method of Svennerholm where an absorbance of 0.1 of a unit corresponds to 80 μg/ml of sialic acid.

geneous and not amenable to further separation by the procedures outlined.

Criteria for Homogeneity of the Isolated Sialo Substances. The purity of the sialo substances was evaluated on the basis of the following procedures. (1) Paper electrophoresis (Durrum type) using 0.025 M aqueous potassium biphthalate buffer at pH 5.9 with a current of 1.25 mA/30-cm strip. Both LAP-I and LAP-II components appeared to be homogeneous and yielded single bands on staining with ninhydrin or resorcinol–HCl. The anodic mobility of LAP-I (4.1 cm) was less than that of LAP-II (6.1 cm) indicating that LAP-I possesses a lower net negative charge. (2) Disc electrophoresis in 20% polyacrylamide gel (stacks at pH 8.9, runs at pH 9.5) using the method of Davis (1964). The samples were fixed for 1 hr in 20% w/v aqueous sulfosalicyclic acid and after periodate oxidation the gels were stained with periodic acid–Schiff reagent. Both LAP-I and LAP-II appeared to be homogeneous and each yielded only single bands on staining with periodic acid–Schiff reagent. LAP-I had an R_F value of 0.38 while LAP-II gave an R_F of 0.72. The high anodic mobility of LAP-II in 20% polyacrylamide gel suggests that this sialo substance may, in addition to possessing a greater net negative charge than LAP-I, also be smaller in size. The elution volume of LAP-II from Sephadex G-50 superfine, evolving as it does after the LAP-I substance, is also consistent with the notion of smaller size (see Figure 2).

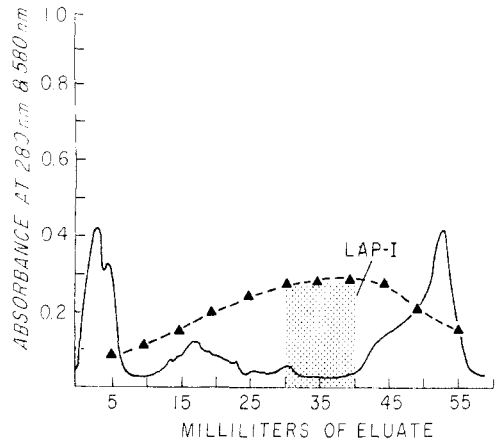

FIGURE 3: Graphic profile of recycling LAP-I glycopeptide by gel filtration on a G-50 Sephadex superfine gel column (1.25 × 95 cm) with distilled water as the developer. (—) Absorbance at 280 nm, a value of 0.1 of a unit corresponding to 0.70 mg of glycopeptide/ml. The sialic acid content is represented by --▲-- for the absorbance at 580 nm, using the method of Svennerholm where an absorbance of 0.1 of a unit corresponds to 64 μg/ml of sialic acid. Shaded portion depicts cuts made to obtain the LAP-I glycopeptide essentially free of material which absorbs at 280 nm.

TABLE I: Amino Acid Composition of Glycopeptides Released from Human Red Blood Cell Ghosts by Trypsin. Values Expressed as g/100 g of Glycopeptide.

Amino Acids	Acidic Glycopeptide AP-I	Less Acidic Glycopeptides LAP-I	LAP-II
Lysine	1.51	1.41	0.13
Histidine	1.61	1.22	1.01
Arginine	1.25	1.71	2.10
Aspartic acid	1.83	1.16	0.34
Threonine	4.73	4.22	1.80
Serine	5.00	4.45	1.93
Glutamic acid	1.05	0.81	6.54
Proline	0.85	0.51	1.78
Glycine	0.82	0.41	0.85
Alanine	1.19	0.56	2.26
Valine	1.28	0.87	2.96
Methionine	1.15	0.53	
Isoleucine	0.72	0.47	0.80
Leucine	0.38	0.15	
Tyrosine	1.27	0.04	0.28
Phenylalanine	0.64		
Tryptophan	1.09	ND[a]	ND[a]

[a] Not determined.

Physicochemical characteristics of the more acidic sialo substance AP-I were found to correspond closely to the glycopeptide described by Winzler *et al.* (1967) with essentially the same amino acid composition except for the presence of methionine and a trace of phenylalanine. On further purification of sialo substance AP-I on Sephadex G-100 superfine employing 0.1 M NaCl at pH 6.8 as the developer the content of methionine was increased to 1.15 g/100 g and that of phenylalanine to 0.64 g/100 g (see Table I).

In addition another component (acidic P-II [AP-II]) with a lower sialic acid and neutral carbohydrate content with virtually no glucosamine or galactosamine and no methionine or phenylalanine was obtained. The yield and purity of AP-II has so far precluded an extensive quantitative investigation of its composition.

The quantities of the products recovered from Sephadex G-100 superfine, based upon optical density at OD_{280}, indicated AP-I as representing 88.4% and AP-II as 11.6% of the total amount of composite sample applied to the column (see Figure 4). The two components (AP-I and AP-II) were rechromatographed on Sephadex G-100 superfine until free of cross contamination and then desalted through Sephadex G-25 fine prior to chemical analysis. The AP-I glycopeptide was essentially salt free on the basis of ashing at 800° in a platinum boat. However examination of the salt content, Ca^{2+}, K^+, and Na^+, of the sialoglycopeptides by flame photometry

TABLE II: Molecular Weight of the Glycopeptides.

Centrifugal Parameters	Glycopeptides AP-I	LAP-II
Partial specific volume, $\bar{v}$ (cm^3/g)	0.634	0.642
Molecular weight (M_{app})	12,011	6345
At meniscus	10,634	3164
At bottom	14,068	9525
Average molecular weight (M_z)	14,767	4494

yielded the following results. AP-I contained 5.4% w/w Na^+, LAP-I contained 10.3% w/w Na^+, and LAP-II contained 20.6% w/w Na^+, 7.6% w/w K^+, and 0.6% w/w Ca^{2+}. The amino acid content of AP-I, LAP-I, and LAP-II is presented in Table I. Note that the values expressed as g/100 g of glycopeptide in Tables I and III include the salt content of glycopeptides. If correction is made for the various salt contents, then the amino acid and carbohydrate content accounts for 96.5% of AP-I, 84.0% of LAP-I, and 89.0% of LAP-II.

For the estimation of molecular weights by equilibrium sedimentation, samples of the AP-I and the LAP-II were each dissolved at a concentration of 0.2% w/v in 0.1 M NaCl at pH 6.8–7.0. Apparent molecular weights of 12,000 and 6300, respectively, were found for the AP-I and LAP-II trypsin fragments (Chervenka, 1970) (see Table II). The plots (log concentration of sialo substance *vs.* [radial distance]^2) tended to slope upward very slightly, implying some heterogeneity of both sialo substances. The upward slope for the LAP-II substance was more marked than that for the AP-I substance. Not enough AP-II and LAP-I substances were obtained to permit molecular weight estimations.

Molecular weights were estimated also employing a K15/75 Sephadex G-100 superfine column fitted with flow adapters (total bed volume (V_t) = 1.5 × 69 cm = 122 ml) and equilibrated with 0.1 M NaCl. Ribonuclease A, mol wt ≃13,700, chymotrypsinogen A, mol wt ≃25,000, and ovalbumin, mol wt 45,000, standards were used to prepare a linear molecular weight selectivity curve. AP-I and AP-II components were chromatographed together through the Sephadex G-100 column under standard calibration conditions. Calculation of the results gave apparent molecular weights of ≃51,000 and ≃3500 for each, respectively, based upon the selectivity curve (Granath and Kvist, 1967). The difference from the apparent molecular weight obtained by the gel filtration method *vs.* sedimentation equilibrium probably reflects the effects of the branched and extended glycopeptide chains on the exclusion volume of the glycopeptides.

Using the dansyl chloride method (Gray, 1967) the NH_2-terminal groups in LAP-I and LAP-II were shown to be, respectively, serine and alanine (see Table III). No NH_2-terminal amino acid residue could be demonstrated in the AP-I sialo substance by the dansyl chloride method nor by the fluorodinitrobenzene method (Fraenkel-Conrat *et al.*, 1955). The carbohydrate composition of the various sialo substances are also presented in Table III.

Hemagglutination inhibitory assays were performed by serial dilution on both the AP and LAP composite glycopeptides against rabbit M and N antisera on four-times standard saline washed M and N red blood cells. It was found that these glycopeptides only weakly inhibited (<1000th the effect of the intact cells from which the glycopeptides were derived) the M and N antisera hemagglutinating activity to about the same degree with initial glycopeptide concentrations in standard saline of 1 mg/ml.

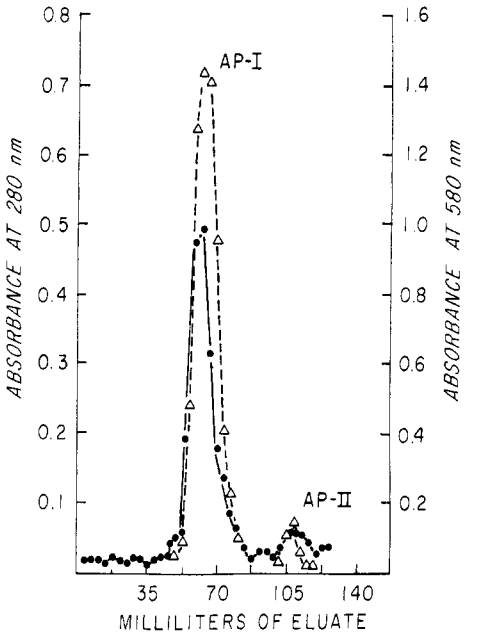

FIGURE 4: Separation of AP-I glycopeptide from AP-II glycopeptide on a G-100 Sephadex superfine column (1.5 × 70 cm) with 0.1 M aqueous NaCl as the developer. (—●—) Absorbance at 280 nm, a value of 0.1 of a unit corresponding to 0.35 mg of glycopeptide/ml. Sialic acid is represented by --△-- which corresponds to the absorbance at 580 nm using Svennerholm's method where an absorbance of 0.1 of a unit represents 64 μg/ml of sialic acid.

Discussion

The glycoproteins isolated from human erythrocyte membranes have been shown to contain hexose, hexosamines, and sialic acid and to possess both viral hemagglutinating inhibitory properties and M–N blood-group activity. No significant differences in carbohydrate composition have been found for glycoproteins derived from MM as opposed to NN or MN cells (Kathan and Adamany, 1967). Studies by Thomas and

TABLE III: Some Characteristics of Glycopeptides Released from Human Red Cell Ghosts by Trypsin. Values Expressed in g/100 g of Glycopeptide.

	Glycopeptides AP-I	LAP-I	LAP-II
Acetylglucosamine	5.39	4.77	2.04
Acetylgalactosamine	11.08	10.15	1.80
Total neutral hexoses	18.20	14.25	6.62
Fucose	2.60	0.97	
Mannose		1.91	
Galactose	13.60	9.02	6.58
Glucose	1.76	0.49	1.13
N-Acetylneuraminic acid	29.89	25.76	26.71
NH_2-terminal amino acid		Serine	Alanine

Winzler (1969) suggest that the major oligosaccharide in the surface glycoprotein is a tetrasaccharide, which is the same in preparations from both M and N erythrocytes thereby suggesting that serological specificity is not a function of the tetrasaccharide.

Trypsin treatment of the human red blood cell produces about a 30% decrease in the electrophoretic mobility (Seaman and Heard, 1960) with the concomitant release of sialoglycopeptides of molecular weight 6000–12,000. The neuraminyl-containing moieties probably have to be arranged perpendicularly in the peripheral zone of the cell rather than tangentially so as to afford access to enzymes, antibodies and other components. The fragments released probably represent portions of the viral and M–N blood-group active surface glycoprotein. Initial amino acid analyses, paper electrophoresis, and disc gel electrophoresis indicated that the initial crude product was quite heterogeneous.

The glycopeptides AP-I, AP-II, LAP-I, and LAP-II have been isolated from all preparations of both intact red blood cells and cell ghosts. The relative quantities of the sialoglycopeptides obtained from intact red cells was approximately the same as those obtained from ghosts, however the large quantities of sodium chloride necessary to maintain isotonicity during the intact cell treatment made the resulting work-up and isolation of the components much more difficult, thus the majority of the study was confined to red blood cell ghosts. Although the inner surface of the cell membrane of ghosts is probably accessible to trypsin, the possible increase in sites of tryptic attack in ghosts does not lead to the appearance of any additional sialoglycopeptides. Studies by Winzler *et al.* (1967) and Winzler (1969) on the glycopeptides released by trypsin from posthemolytic residues as opposed to the intact erythrocyte also support these findings.

All the sialoglycopeptides were comparable in their sialic acid content and thus apparently of comparable acidity, however they differed in their behavior on Dowex 50 cation exchangers. In order to differentiate between the sialoglycopeptides the somewhat arbitrary designation of more or less acidic based solely on ionic exchange behavior was used. About 30% of the sialo substances bind strongly to the Dowex 50 cation-exchange resin and are only eluted by appreciable increases in the pH and ionic strength of the developer whereas the other apparently more acidic sialo substances are eluted in the column void volume. Electrophoretic data suggest that the sialoglycopeptides which are strongly bound by the Dowex 50 resin carry a net negative charge which is difficult to reconcile with their apparent affinity for a strong cation-exchange resin. On a weak anion exchanger such as DEAE-cellulose all of the sialoglycopeptides are eluted from the column together with little or no resolution as was also found by Winzler *et al.* (1967). Complete separation of the individual glycopeptides in water on gels of cross-linked dextrans or polyacrylamide could not be achieved. For example, AP-I when chromatographed in water always elutes as an apparently single homogeneous peak, however when 0.1 M sodium chloride is used as the developer (Figure 4) a small sialoglycopeptide (AP-II) separates from the main component. AP-I still appeared to be heterogeneous on the basis of the amino acid composition and the equilibrium sedimentation studies (upward curvature of the log c *vs.* r^2 plot) although the periodic acid–Schiff reagent stained bands obtained for AP-I on disc electrophoresis indicated that the sample was homogeneous. Insufficient quantities of LAP-I were obtained to permit an estimate of its molecular weight by equilibrium sedimentation studies. The electrophoretic mobilities of the AP-I and LAP-I on polyacrylamide (R_F values of, respectively, 0.36 and 0.38) are very close and imply comparable molecular weights.

Gas chromatography of the neutral sugars in the glycopeptides indicated the presence of glucose in all the preparations (Table III). Glucose has not been reported previously to be present in the glycopeptides liberated from erythrocytes by proteolytic enzymes, although Barber and Jamieson (1970) have reported the presence of nondialyzable glucose in platelet lysate preparations. The glucose may have arisen from the breakdown of cross-linked dextrans used as support media in the columns for the separation and purification of the glycopeptides. The values for the amino acid composition of the glycopeptides are expressed in g/100 g since the molecular weight of only two of the components has been determined and a comparison on a mole basis is therefore not feasible (Table I). The acidic sialoglycopeptide fraction prior to separation into AP-I and AP-II had an amino acid composition close to that of the trypsin fragments described by Winzler *et al.* (1967) with the exception of the presence of significant amounts of methionine and phenylalanine.

Only two types of protein–carbohydrate linkages have been confirmed for glycoproteins, namely, the *N*-glycosidic linkage of *N*-acetylglucosamine to asparagine as exemplified by the II D-3 glycopeptide obtained from the membrane of erythrocytes by tryptic hydrolysis (Kornfeld and Kornfeld, 1970) and the *O*-glycosidic linkage of carbohydrate to the hydroxyl group of hydroxyamino acids as for example in M and N blood-group glycoproteins where Lisowska (1969) has reported the presence of *O*-(*N*-acetylgalactosaminyl)-L-serine and -threonine. The presence of appreciable quantities of serine and threonine in the AP-I and LAP-I glycopeptides, like the material described by Winzler *et al.* (1967) and Thomas and Winzler (1969), suggests that the predominant carbohydrate–protein linkage is probably *via* these amino acids. On the other hand, the high content of glutamate and low complements of serine, threonine, and aspartate in LAP-II are indicative of oligosaccharides linked *N*-glycosidically through glutaminyl residues. The content of serine and threonine however does leave open the possibility of some *O*-glycosidic linkages to the serine and threonine residues in the LAP-II glycopeptide.

It seems probable that the glycopeptides released from the surface of the human red blood cell by trypsin are the free terminal portions of peripheral zone glycoproteins split off at the arginine or lysine residues which are accessible to the enzyme. It is also conceivable that the glycopeptides could have arisen from the same parent glycoprotein split at several points by the action of the trypsin. Winzler *et al.* (1967) have suggested that most likely these tryptic glycopeptides represent the terminal portion of the viral hemagglutination inhibitor described by Kathan and Winzler (1963). The weak M and N inhibitory hemagglutinating activity found by us for the AP and LAP composite glycopeptides is consistent with this view.

Rosenberg and Guidotti (1969) using a sodium dodecyl sulfate solubilized red blood cell membrane preparation have by the use of the cyanate method demonstrated the presence of no fewer than seven NH_2-terminal amino acids, two of which, alanine and serine have been found as the NH_2-terminal amino acids of the glycopeptides LAP-II and LAP-I, respectively. No NH_2-terminal amino acid could be demonstrated for the AP-I glycopeptide, suggesting that the NH_2-terminal position is blocked. The presence of single NH_2-terminal amino acids for each glycopeptide denotes homogeneous preparations, although the results of the sedimenta-

tion equilibrium studies performed on the LAP-II glycopeptide indicate some heterogeneity.

The results presented here support the concept of the presence of glycoprotein subunits in the peripheral zone of the erythrocyte membrane, which are relatively superficial, accessible to trypsin and the glycopeptide moieties of which are somewhat heterogeneous both as regards amino acid composition and content of individual carbohydrates.

Acknowledgments

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