Isolation of a Macroglycopeptide from Human Platelets*

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ABSTRACT: Proteolysis of intact human platelets with trypsin, pronase, or papain for 10–30 min at 37° released 30–60% of total cell sialic acid in the form of three distinct size classes of glycopeptides. Analytical values for molecular weights and carbohydrate composition were similar for each class of glycopeptide irrespective of the particular enzyme used in its preparation. Relatively more of the high molecular weight glycopeptide, GP-I (mol wt 120,000), was released by trypsin while the lowest molecular weight glycopeptide, GP-III (mol wt 5000), was preferentially released by either pronase or papain. The intermediate glycopeptide, GP-II (mol wt 22,500), was obtained in small amounts by either proteolytic procedure. GP-I was resistant to further proteolysis and GP-I and GP-II gave single peaks on chromatography on DEAE-cellulose while GP-III could be subfractionated into several components identical with those obtained by prolonged proteolysis of isolated platelet membranes (Pepper and Jamieson (1969), *Biochemistry 8*, 3362). GP-I isolated from combined tryptic and pronase digests contained *ca.* 22% sialic acid, 16% galactose, and equal amounts of glucosamine and galactosamine (15% of each). Proline (6%) was the principal amino acid with smaller amounts of threonine (5.5%) and serine (3.5%). About half the hexosamine was in alkali-labile linkages. GP-III contained *ca.* 18% sialic acid, 15% galactose, 10% mannose, and 33% glucosamine; it was devoid of galactosamine and of alkali-labile carbohydrate. GP-II was intermediate in properties between these two glycopeptides but was not related to GP-I in a degradative sequence.

The small amounts of GP-II available precluded extensive structural characterization. Thus, GP-I has many of the properties of a mucin and may extend as a semiflexible rod from the surface of the platelet. It may be related to the fuzzy coat seen in electron photomicrographs of platelet thin sections and may be involved in platelet adhesion during hemostasis and in platelet immune lysis.

The presence of glycoprotein components on the outer membrane of human blood platelets has been inferred from electrophoretic (Madoff *et al.*, 1964), enzymatic (Hovig, 1965), and ultrastructural (Behnke, 1968; Rambourg and Leblond, 1967, 1969) studies. Previous work from this laboratory has shown that a glycoprotein-rich fraction can be solubilized from the platelet membrane (Pepper and Jamieson, 1968) and that a complex family of glycopeptides can be isolated following prolonged proteolytic digestion of these membranes (Pepper and Jamieson, 1969).

This investigation was designed to study the effect of *brief* proteolytic digestion of the intact platelet with different proteases in order to isolate a simpler mixture of the precursor glycopeptides relatively free of contaminating noncarbohydrate peptides.

Experimental Section

Preparation of Platelet Digests. Fresh human platelet concentrates prepared for clinical use (Pert *et al.*, 1967) were further freed of erythrocytes by differential centrifugation and lysis (Pepper and Jamieson, 1968). After washing twice in ammonium oxalate (1% w/v) containing EDTA (0.1% w/v), pH 7.5, each platelet unit was resuspended in 10 ml of the same buffer or in saline solution (0.9%) buffered with 0.001 M phosphate buffer to pH 7.5. One milligram of pronase, trypsin, or papain was added to the washed platelet concentrate and digestion was allowed to proceed at 37° for 30 min. In the case of trypsin the reaction was stopped by the addition of 2 mg of soybean trypsin inhibitor. All platelet digestion mixtures were centrifuged at 10,000 rpm (18,000*g*) at 0° for 5 min to remove the treated platelets and any cellular debris, and the supernatant was frozen at −20° until required.

The pooled supernatants (110 mg dry weight) from 30–40 platelet units were concentrated by rotary evaporation and desalted on a column of Sephadex G-25 (1.2 × 55 cm) by elution with distilled water adjusted to pH 8.5 with ammonium hydroxide (solutions of lower pH were found to decrease the yield of high molecular weight components by decreasing their solubility). The digests were desalted by gel filtration on Sephadex G-25 to prevent precipitation during concentration under reduced pressure (final volume 1–3 ml) and were then subjected to gel filtration on columns of Sephadex G-75 or G-200 together with Blue Dextran and glucose markers. After separation the individual glycopeptides were desalted on Sephadex G-25, lyophilized, and redissolved in distilled water to give analytical stock solutions of *ca.* 10 mg/ml.

Chemical Analysis. Sialic acid, hexose, hexosamines, and amino acids were determined as before (Pepper and Jamieson, 1969) with the following modifications: hexosamines were determined after hydrolysis at 100° with 2 N

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HCl for 16 hr or 4 N HCl for 2 hr and hydrolyses were carried out in 10 ml of tissue culture tubes with Teflon-lined screw caps (Gatt and Berman, 1966); hexoses and methylpentoses were identified as their alditol acetates following hydrolysis in 0.1 N H_2SO_4 plus Dowex 50-X8 (200–400 mesh, H^+ form, 50% v/v) at 100° for 16 hr. Following hydrolysis, the supernatant solution was removed and combined with twice-concentrated 0.5-ml water washes of the resin. Hydrochloric acid was then removed from the combined solutions with Dowex 1-X8 (200–400 mesh, HCO_3^- form) to a final pH of 5 and the supernatant solution and the resin washings were recombined. After reduction with $NaBH_4$ to the corresponding alditols, Na^+ ions were removed by treatment with Dowex 50-X8 (200–400 mesh, H^+ form) which also served to destroy any unreacted BH_4^- ion. Borate was removed by three successive additions and evaporations of methanol. The alditol acetates were then prepared and analyzed by gas–liquid chromatography as previously described (Pepper and Jamieson, 1969); 50 μg of *myo*-inositol was added prior to hydrolysis to serve as an internal standard. Published data for the relative mass yields of each alditol acetate were assumed (Lehnhardt and Winzler, 1968).

Proteolysis. Experiments were first undertaken to determine the extent to which total platelet sialic acid could be released in a soluble form by various combinations of enzyme time, temperature, and buffer. Individual platelet units were usually suspended in 10 ml of saline solution giving a final pH of 6.9 or, infrequently, in ammonium oxalate (0.1 M) containing EDTA (0.002 M), for digestion with trypsin or pronase, and in 0.2 M phosphate buffer (pH 6.2) containing cysteine (0.01 M) and EDTA (0.01 M), for the papain digestion. One milligram of enzyme was added and samples of 1.0 ml were withdrawn at intervals of 5 min, cooled in ice, and immediately centrifuged at 6000 rpm (5000*g*) for 4 min; 0.5 ml of clear supernatant was removed for total sialic acid assay. In a series of ten experiments, the range of release of total sialic acid with trypsin incubation was 24–26% at 20° and 34–44% at 37°. The use of a chelating buffer (EDTA) effected a slight reduction in the percentage released with trypsin but the addition of Ca^{2+} to a concentration of 0.01 M in saline solution did not increase the rate. The available Ca^{2+} is presumably derived from the platelets themselves. Similar, but faster, release kinetics were observed when using pronase (1 mg/platelet unit) but papain released only about 30% NANA[1] in the same time. The overall range of observed release of total sialic acid was 30–60% (Figure 1). This value is similar to the carbohydrate released by tryptic digestion of HeLa cells (Shen and Ginsburg, 1968) or human erythrocytes (Winzler, 1969). The release of glycopeptides from platelets, determined as soluble NANA, was dependent on the prior treatment of the platelet concentrate (storage temperature, time after collection, etc.) and on whether the concentrates were digested singly or combined; combining platelet units prior to digestion led to a higher release. On average, each platelet unit (72 mg of protein) released into the supernatant 200 μg of bound sialic acid and approximately 5 mg of proteins, glycopeptides, and larger peptides. This dry weight was increased by the presence of trypsin and inhibitor (maximum 3 mg) but was decreased by the process of desalting on Sephadex G-25 where a considerable amount of peptide and amino acid material was discarded.

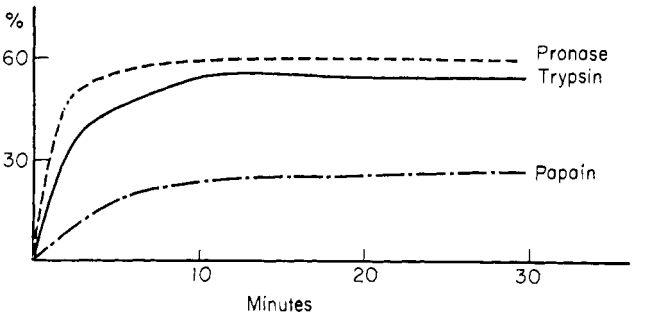

FIGURE 1: Rate of release of glycopeptides from human platelets determined as total soluble NANA. Platelet protein, 7 mg/ml; trypsin, pronase, or papain, 0.1 mg/ml.

In another type of experiment the platelet concentrates were treated first with trypsin (30 min/37°) and the reaction was stopped with soybean trypsin inhibitor. The treated platelets were then washed twice with normal saline and retreated with pronase (30 min/37°). Under these conditions there was an additional slight release (*ca.* 15%) of soluble sialylglycopeptides corresponding to GP-III but with no observable additional release of GP-I or GP-II. In the reverse experiment, pronase followed by trypsin, the extra release was *ca.* 3%, mainly of GP-III but with a number of small, ill-defined additional peaks.

Gel Filtration. When the supernatants from pooled platelet digests, generally from 30–40 units, were subjected to gel filtration two distinct types of molecular weight profile were observed depending on the proteolytic enzyme used. Tryptic digestion produced one major sialylglycopeptide peak which was eluted slightly later than the void volume, V_0, on Sephadex G-200 together with lesser amounts of two lower molecular weight components (Figure 2a). In contrast, pronase digests yielded the lowest molecular weight component as the major fraction with lesser amounts of the two higher molecular weight fractions (Figure 2b). The elution pattern of the papain digests on Sephadex G-200 was similar to that obtained with pronase (Figure 2c). In view of the similar elution volumes for the components of each of the proteolytic digests the highest molecular weight peak was designated GP-I, the intermediate peak GP-II, and the lowest molecular weight peak GP-III, in each case. The sialylglycopeptides GP-I, GP-II, and GP-III from each of the proteolytic digestions were eluted as single peaks when resubjected to gel filtration on Sephadex G-200, G-150, and G-75, respectively.

When tryptic GP-I (*ca.* 6 mg, 1200 μg of NANA) was subjected first to gel filtration on Sephadex G-200 (Figure 2a) and then treated with 1 mg of pronase in normal saline (0.01 M in Ca^{2+}) at 37° for 24 hr followed by a second, identical treatment a relatively small amount of lower molecular weight material, corresponding in elution volume to GP-III, was obtained with a corresponding change in the elution volume of GP-I to the value obtained by direct pronase digestion of intact platelets (Figure 3).

Individual pronase and trypsin glycopeptide peaks from

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

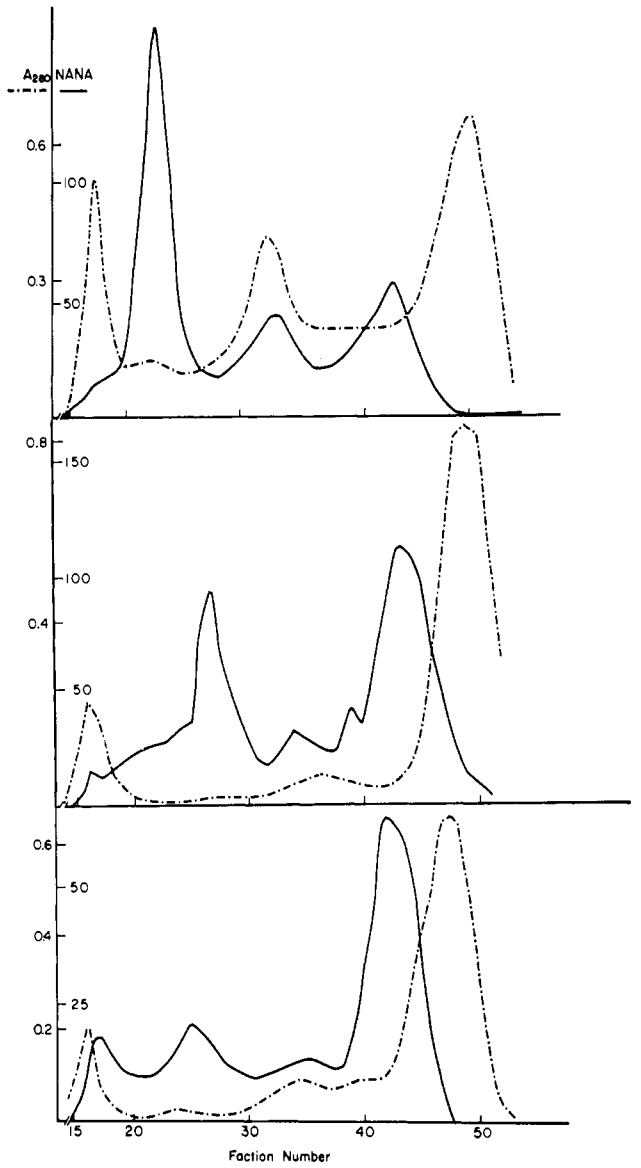

FIGURE 2: Gel filtration of crude soluble digests of human platelets on a column (2.5 × 95 cm) of Sephadex G-200 eluted with Aronsonn–Gronwall (1957) buffer diluted 1:20 in saline. Flow rate, 12 ml/hr; fraction volume 10 ml. Ultraviolet absorption (280 nm), ----; NANA (μg/fraction), ——. Fractions were pooled as indicated. (a, top) Tryptic digest (170 mg dry wt). GP-I, 20–25; GP-II, 29–35; GP-III, 38–48. (b, middle) Pronase digest (1.15 mg of NANA). GP-I, 20–25; GP-II, 32–37; GP-III, 40–48. (c, bottom) Papain digest (0.68 mg of NANA). GP-I, 15–18; GP-II, 22–28; GP-III, 33–48.

a number of runs were pooled, concentrated by rotary evaporation, and desalted on a column of Sephadex G-25. The data for the individual peaks from the pooled products of several chromatographic runs are presented in Table I. Thus, unless otherwise stated, the terms GP-I, GP-II, and GP-III refer to glycopeptides isolated from combined pools of tryptic and pronase digests. In earlier experiments (Pepper and Jamieson, 1969) there was considerable loss of the high molecular weight glycopeptides due to precipitation at the pH of distilled water (pH 5–7). In subsequent work, care was taken to maintain the pH of the eluting solution at 8.5 to effect complete recovery from the columns.

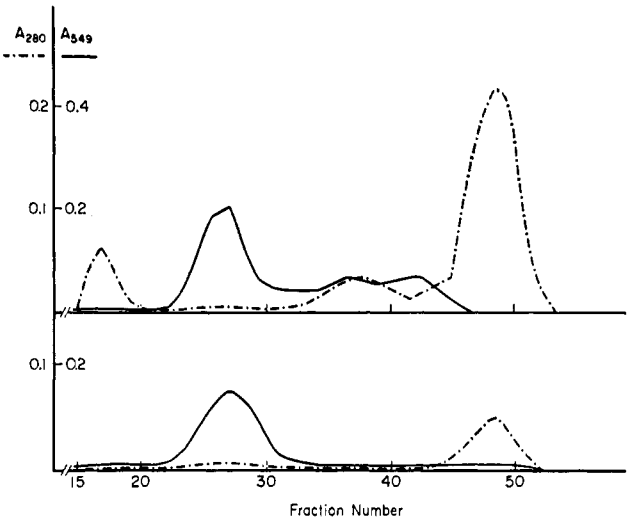

FIGURE 3: Upper: gel filtration of 6 mg of tryptic GP-I treated at 37° for 24 hr with 1 mg of pronase. Conditions as in caption to Figure 2. Lower: gel filtration of the main glycopeptide peak in the upper elution diagram (fractions 25–30) redigested (24 hr/37°) with a further 1 mg of pronase. Conditions as above.

Immunochemistry. The crude soluble glycopeptide fractions from each enzyme digest, partially purified by gel filtration, were examined in micro-Ouchterlony diffusion plates against a variety of antisera. No precipitin lines were observed with antisera against IgA, IgM, β-lipoprotein, haptoglobin, α_2-macroglobulin, α_1-lipoprotein, thrombosthenin, or platelet membranes but a major precipitin band was observed with antialbumin in GP-II and GP-III; no other plasma protein or glycoprotein was detected which would have produced glycopeptides in any significant amount. However, no precipitin lines were observed in any of the most purified fractions following chromatography on DEAE-Sephadex.

Molecular Weight Determination. Molecular weights of the purified glycopeptides, isolated from combined pronase and tryptic digests by gel filtration and chromatography, were calculated by an indirect method based on the determination of sedimentation coefficient and Stokes radius (Pepper, 1967).

For the determination of sedimentation coefficients the individual glycopeptides were made up to 10 mg/ml in saline solution buffered to pH 7.5 with 0.001 M phosphate buffer and 0.4-ml volumes were placed in a synthetic boundary cell and run at 56,100 rpm in the Spinco Model E ultracentrifuge. A single peak was observed for GP-I although both GP-II and GP-III showed some evidence of heterogeneity (Figure 4). The calculated sedimentation coefficients are given in Table III.

Stokes radii were determined from the plot of the partition coefficient, K_D, against the Stokes radius, r_s (Siegel and Monty, 1966), on columns of Sephadex G-75 or G-200 using, as standards, human fibrinogen (r_s, 107 Å), human serum albumin (r_s, 35.2 Å), soybean trypsin inhibitor (r_s, 22.6 Å), and equine cytochrome *c* (r_s, 18.9 Å). Blue Dextran and glucose were used as markers of the void volume, V_0, and total volume, V_t, respectively. The Stokes radius of each of the purified glycopeptides (Table III) was then determined by interpolation for the appropriate Sephadex column.

TABLE I: Yield and NANA Content of Crude Glycopeptide Fractions.

	Pronase Digest (70 platelet units, 350 mg of digest)			Trypsin Digest (43 platelet units, 170 mg of digest)		
	GP-1	GP-II	GP-III	GP-I	GP-II	GP-III
Dry weight (mg)	16.4	19.6	24.4	11.8	31.1	45.6
NANA (μg)	210	112	354	295	137	36
NANA % (w/w)	1.28	0.57	1.45	2.5	0.44	0.08

TABLE II: Composition of Purified Glycopeptides Eluted from DEAE-Sephadex A-50.

	GP-I		GP-II		GP-III-0		GP-III-I		GP-III-II		GP-III-III		GP-III-IV	
	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole	g/100 g	Moles/ Mole
Fuc	0.65	5	2.1	3	1.0	0.3	2.95	1	3.0	1	2.03	0.7	1.57	0.5
Man	0.83	6	2.7	4	8.6	2.6	11.5	3.4	9.9	3	6.9	2.0	6.5	2
Gal	16.5	118	16.5	23	7.7	2.3	13.0	4.0	13.8	4	16.6	5	13.9	4.1
Glc	0.92	7	3.8	5	6.5	2.0	6.0	2.0	1.8	0.6	1.5	0.4	3.5	1
NANA	22.4	89	31.8	25	6.8	1.0	15.5	2.6	18.6	3	18.0	3	28.3	5
Glcn[a]	15.1	86	13.5	15	17.1	4.0	32.8	8	37.0	9	33.8	8.1	27.5	6.6
Galn[a]	14.2	84	10.6	13										
Asx	0.51	11	2.23	4	9.9	4.0	4.28	1.8	3.4	1.5	3.09	1.2	3.89	1.6
Glx	2.92	26	3.33	6	8.8	3.0	1.75	0.7	1.55	0.6	2.3	1	1.66	0.6
Gly	0.51	11	0.99	4	3.4	3	0.81	0.7	0.59	0.6	0.77	0.7	0.85	0.7
Pro	5.95	71	1.75	4	4.9	2	1.04	0.5	1.40	0.7	2.08	1	1.0	0.5
Ser	3.53	47	2.73	6.5	4.2	2	2.34	1.3	1.66	0.9	2.13	1.2	1.6	0.9
Thr	5.48	63	2.93	7	2.4	2	2.40	1	1.83	0.8	2.76	1.3	2.43	1.2
Ala	1.15	19	0.95	3	3.1	2	0.79	0.5	0.94	0.4	0.97	0.7	0.71	0.5
Leu	1.31	14	1.53	3	2.3	1	0.95	0.4	0.82	0.4	1.2	0.5	1.1	0.5
Val	0.62	8	0.74	2	2.3	1	0.62	0.3	0.41	0.2	0.66	0.4	0.37	0.2
Ile	1.00	10	0.74	1	2.6	1	0.47	0.2	0.47	0.2	0.60	0.3	0.36	0.2
Lys	0.76	7	0.54	1	2.6	1	0.29	0.1	0.55	0.2	0.96	0.4	0.55	0.2
Arg					2.9	1	0.41	0.1	0.55	0.2	0.65	0.2	0.29	0.1
His	1.78	15	0.19	0.3	1.1	0.3	0.39	0.1	0.34	0.1	0.59	0.2	0.36	0.1
Phe	1.59	13	0.02	0	1.1	0.3	0.85	0.3	0.52	0.1	1.01	0.3	1.74	0.6
Tyr	1.76	13	0.02	0	0.8	0.3	0.77	0.3	0.57	0.1	1.12	0.3	1.93	0.6

[a] Assumed to be N acetylated.

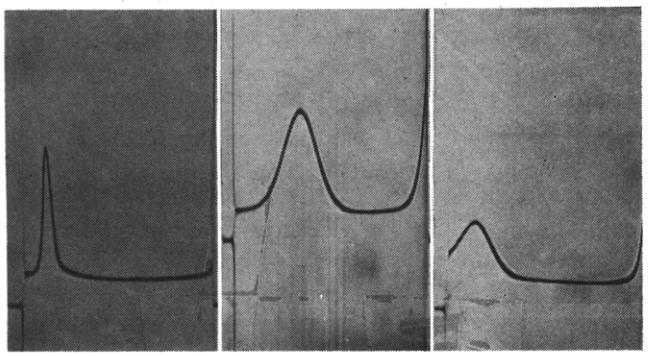

FIGURE 4: Ultracentrifugation of platelet glycopeptide preparations in cup valve synthetic boundary cell at 56,100 rpm. Solvent, 0.85% NaCl, pH 7.5. Temperature 20°. The pictures were taken at the times indicated. Left: GP-I (1.0%); 20 min. Middle: GP-II (1.8%); 40 min. Right: GP-III (2.5%); 55 min.

The partial specific volume, $\bar{v}$, for each glycopeptide was then calculated from the analytical data (Gibbons, 1966) and the values for $\bar{v}$ (Table III) and s were inserted in the Svedberg equation

$$M = \frac{sRT}{D(1 - \bar{v}\rho)}$$

which, from the Stokes–Einstein equation

$$r_s = \frac{KT}{6\pi\eta D}$$

can be expressed as

$$M = \frac{sr_s 6\pi\eta N}{(1 - \bar{v}\rho)}$$

where s is the sedimentation constant at zero concentration, R, the gas constant (8.31×10^7 ergs), T, the absolute temperature (20° or 293° A), N is Avogadro's number (6.023×10^{23}), η is the solvent viscosity (0.010 for water), and ρ, the solvent density (1.00 for water). Molecular weight values for the glycopeptides calculated by this method are given in Table III.

From these values the frictional coefficient, f/f_0, was calculated from the following equation (Tanford, 1961)

$$f/f_0 = \frac{r_s}{\sqrt[3]{3\bar{v}M/4\pi N}}$$

These calculated values are given in Table III.

Ion-Exchange Chromatography. Pooled pronase and trypsin samples of each molecular weight class (GP-I, GP-II, and GP-III) were washed separately into columns of DEAE-Sephadex A-50 equilibrated with water at pH 2.5 and eluted with concave gradients of NaCl as described in the caption to Figure 5. Both GP-I (26 mg) and GP-II (46 mg) yielded protein fractions which were not absorbed to the column and single peaks of sialylglycopeptide which eluted at 0.25 M NaCl, pH 2.4 (Figure 5a,b); in each case the yield of purified glycopeptide from 110 platelet units was 6.2 mg. However pooled GP-III (57 mg) produced a nonadsorbed peak (GP-III-0) plus a series of closely spaced peaks (GP-III-I to GP-III-IV) which eluted higher molarities (Figure 5c). The dry weight of each major low molecular weight peak was GP-III-0, 31 mg; GP-III-I, 3.4 mg; GP-III-II, 3.5 mg; GP-III-III, 2.0 mg; and GP-III-IV, 3.8 mg.

TABLE III: Physical Properties of Glycopeptides.

	GP-I	GI-II	GP-III
Diffusion coefficient ($\times 10^7$)	2.15	7.96	11.74
Stokes radius (Å)	100	27	18.3
Sedimentation coefficients ($s^{1\%}_{20,w}$), S	3.2	2.2	0.76
Partial specific volume ($\bar{V}$)	0.688	0.700	0.677
Molecular weight	120,000	22,500	5000
Frictional ratio (f/f_0)	3.16	1.47	1.67

Chemical Analysis. The analytical data for the individual purified glycopeptides from the pooled digests are shown in Table II. Similar data were obtained for the partially purified glycopeptides obtained after gel filtration alone, although in these cases the proportion of carbohydrate was lower and the proportion of amino acids higher. Unlike GP-III, which had only glucosamine, the higher molecular weight glycopeptides GP-I and GP-II contained equimolar amounts of glucosamine and galactosamine. The predominant amino acids in GP-I were proline and threonine, in GP-II glutamic and aspartic acids and threonine and serine, while in GP-III it was aspartic acid.

A variety of approaches was made to determine whether alkali-labile linkages were present between carbohydrate side chains and hydroxyamino acids although the inadequate amounts of GP-II precluded structural studies for this class. Solutions of GP-I and GP-III-I were made up to 1300 μg/ml in 0.5 N NaOH and hyperchromicity at 241 nm (Carubelli *et al.*, 1965) of sixfold and twofold, respectively, was observed over a period of 3 days at 25° (Figure 6). At the end of this period, no further change in optical density was observed, and it was calculated that the hyperchromic shifts corresponded to the production of 40 and 14%, respectively, of the maximum yield of α-aminoacrylic and crotonic acids had all serine and threonine residues been susceptible to β-elimination. Attempts to measure the quantitative disappearance of serine and threonine, and the production of alanine and α-aminobutyric acid, following treatment with alkaline borohydride (0.1 N NaOH–0.4 M $NaBH_4$, 25°/24 hr), were unsuccessful as were attempts to equate the amount of undegraded, nondialyzable hexosamine and diffusible Elson–Morgan chromogen. However, hexosamine analysis of the nondialyzable fraction of the high molecular weight glycopeptide GP-I showed a decrease in the galactosamine to glucosamine ratio from 1:1 to 0.1:1 following alkaline reductive cleavage. The detection of glucose as a minor component of each glycopeptide is of dubious significance since these glycopeptides were chromatographed in the presence of Blue Dextran and Sephadex, which may yield homopolymers of glucose by bacterial action.

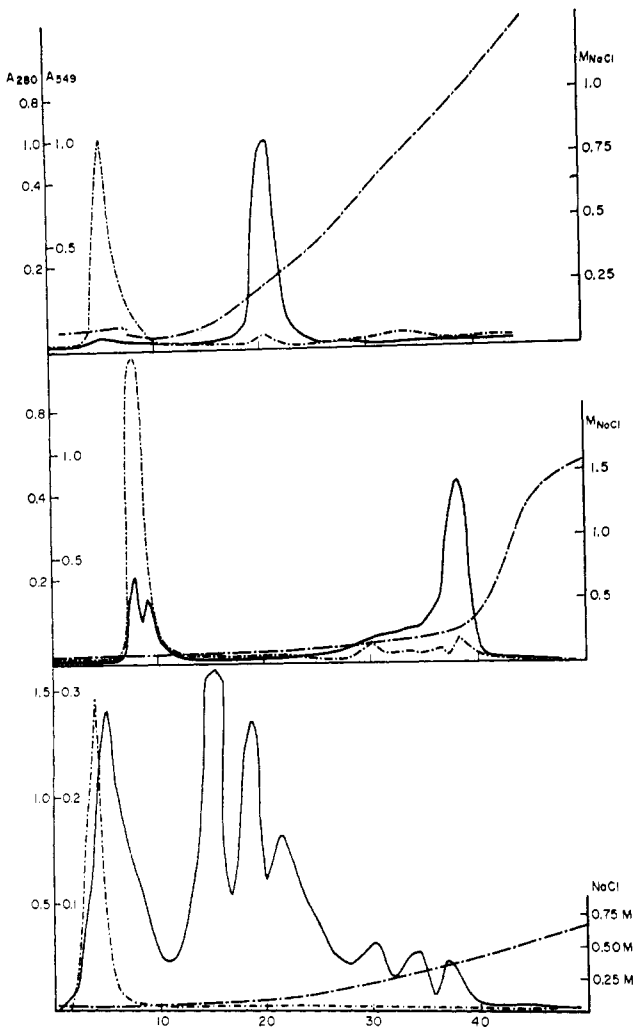

FIGURE 5: Chromatography of individual glycopeptides of combined tryptic and pronase digests on DEAE-Sephadex A-50 in water–HCl (pH 2.5) with increasing NaCl concentration. Column size, 1.5 × 30 cm. Flow rate, 18 ml/hr. Ultraviolet absorbance (280 nm), –·–·–·–; NANA, ——; molarity NaCl, —·—·—. (a, upper) GP-I. Fraction size, 8.4 ml. Pooled glycopeptide fractions, 18–23. (b, middle) GP-II. Fraction size, 3.8 ml. Pooled glycopeptide fractions, 63–70. (c, lower) GP-III. Fraction size, 8.1 ml. Pooled subfractions were GP-III-0, 1–10; GP-III-I, 13–17; GP-III-II, 17–20; GP-III-II, 20–28; GP-III-IV, 28–40.

Gel Electrophoresis. Approximately 500 μg of each glycopeptide purified by chromatography on DEAE-Sephadex was dissolved in 0.05 ml of water, run in duplicate in polyacrylamide disc electrophoresis, pH 8.3 Tris–glycine buffer (Ornstein, 1964; Davis, 1964), and fixed in 7% acetic acid. One set was stained with Amido Black or coomassie blue to locate proteins and glycoproteins, the other set with periodic acid–Schiff (PAS) reagent to detect carbohydrates (Van Neerbos and De Vries-Lequin, 1969; V. T. Marchesi, personal communication). Only in the case of GP-I was any fixed band observed; a strong band of mobility 0.30, relative to albumin, was stained with Schiff reagents but did not give any reaction with Amido Black or coomassie blue, presumably due to the high carbohydrate content. No other PAS-positive or protein bands were detected in the purified GP-I, although several slow moving protein bands were detectable in the crude glycopeptide fraction prior to DEAE chromatography.

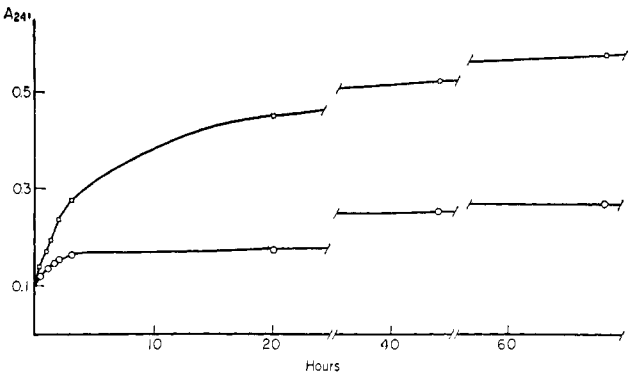

FIGURE 6: Hyperchromic shifts at 241 nm observed with solutions (1300 μg/ml) of GP-I and GP-III-I in 0.5 N NaOH at 25°. GP-I, ——□——; GP-III-I, ——⊙——.

Discussion

These results indicate that three major classes of glycopeptides may be isolated by brief proteolytic digestion of intact human platelets. Digestion with trypsin yields mainly a glycopeptide fragment of mol wt 120,000 containing approximately 70% carbohydrate with galactose as the principal neutral sugar but with equimolar amounts of glucosamine and galactosamine. Although this glycopeptide is, presumably, the proteolytic product of a larger membrane-bound precursor its molecular weight (120,000) is considerably higher than that of several intact glycoproteins. We propose the loosely defined term *macroglycopeptide* for proteolytically derived products of this type. In this case, proline is the principal amino acid but the macroglycopeptide also contains a high proportion of serine and threonine. While extensive structural studies were precluded by the small amounts of material available the hyperchromicity at 241 nm following alkaline treatment, the relative loss of galactosamine from the nondialyzable residue would indicate that GP-I contains approximately half its hexosamine in *O*-glycosidic linkages. This macroglycopeptide GP-I is essentially resistant to further proteolysis and yields only a small amount of lower molecular weight glycopeptides when subjected to prolonged digestion with pronase. However it should be recognized that the compositional data of Table II refer to a mixture of tryptic and pronase digestion products and are, therefore, probably heterogeneous even though they form a distinct class on the basis of molecular size and chemical analysis. This resistance to proteolytic digestion is probably not due to the compactness of the molecule, since the calculated frictional ratio (3.2) indicates an extended molecular form, but may arise from the high carbohydrate content and the unusual amino acid composition.

However, digestion of the intact platelets with either pronase or papain leads, in addition, to a lower molecular weight fragment which has aspartic and glutamic as its principal amino acids, is devoid of *N*-acetylgalactosamine, and contains significant amounts of mannose but with galactose as the principal neutral sugar. The molecular

weight of this fraction has been determined as 5000 although it may be subfractionated on the basis of charge by chromatography on DEAE-cellulose at low pH. The order of elution from DEAE-cellulose parallels the increasing content of sialic acid (15.5–28.2%).

We have previously described (Pepper and Jamieson, 1969) the isolation of a glycopeptide fraction from isolated platelet membranes after prolonged digestion with trypsin and pronase. The material then designated "nondiffusible glycopeptides" GP-I, GP-II, and GP-III corresponds exactly to those designated GP-III-I, GP-III-II, and GP-III-III in the present work. This may be seen from the similarity of elution patterns from DEAE-cellulose, the similar chemical analyses of each fraction, and the absence of alkali-labile linkages in both cases although the present material is more homogeneous as evidenced by its lower ultraviolet absorption (280 nm) and its higher carbohydrate content.

An important difference, however, is the molecular weight of these "nondiffusible glycopeptide" which, in the earlier work, was estimated to be 12,000 from elution data alone but in the present work has been determined as 5000 (GP-III). The "diffusible glycopeptide" was not detected in the present work and probably represents a low molecular weight cut of the diffusible glycopeptide which was effected by the previous dialysis procedure.

The present indirect method for the determination of molecular weights (Pepper, 1967) has the advantage that calibration in terms of Stokes radius is valid whether the standard marker molecules are proteins or polysaccharides (Laurent and Granath, 1967) and is independent of the size, shape, composition (and, therefore, of $\bar{v}$), or molecular weight of the marker molecules. This is particularly important for glycoproteins and glycopeptides since adequate standards for calibration in terms of molecular weight do not exist. The validity of this indirect method is shown in the case of fibrinogen (mol wt 340,000; r_s, 107 Å) which does not fall on a straight line of log M_w *vs.* V_e (Andrews, 1965) but fits nicely to a line of r_s *vs.* V_e; this is directly related to our present observations in the case of GP-I in which we are dealing with an asymmetric molecule of high molecular weight.

The glycopeptide now designated GP-II probably corresponds to the material of estimated molecular weight 70,000 described in the earlier work (Pepper and Jamieson, 1969) which was not investigated due to insufficient quantities. However, this molecule, too, is asymmetric and a better estimate of its molecular weight, utilizing the indirect method, is 22,500. Its chemical analysis is intermediate between that of GP-I and GP-III. Thus, it contains glucosamine and galactosamine with galactose as the major hexose, but with a larger weight content of mannose than GP-I. The failure to detect the high molecular weight GP-I in the earlier studies probably resulted from its precipitation at pH values close to, or below, neutrality.

Thus, brief proteolytic digestion of intact human platelets leads to three types of glycopeptide distinguishable on the basis of their molecular weights, amino acid and carbohydrate analyses, and homogeneity on ion-exchange chromatography. GP-I and GP-III can also be obtained in good yield from highly purified platelet membranes isolated by a glycerol–lysis technique (A. J. Barber, D. S. Pepper, and G. A. Jamieson, unpublished).

The question of the nature of the cytochemical substrate of the proteases used in this work is not easily answered. It is presumed that the glycopeptides arise from the outer surface of the platelet but trypsin and other proteases are known to induce the platelet release reaction (Holmsen *et al.*, 1969) and glycosaminoglycans, characteristic of thrombin-induced release (Riddell and Bier, 1965), have been found in the tryptic and pronase digests. It should also be noted that the relative amounts of the various glycopeptides obtained depend on the proteolytic enzyme used.

When studied in the electron microscope (Rambourg and Leblond, 1967, 1969; Hovig, 1968; Behnke, 1968) the platelet, and its precursor the megakaryocyte, are seen to have a layer of carbohydrate-rich material varying in thickness from 200 to 500 Å. Hovig (1965) showed that the ultrastructure of thin sectioned platelets was almost unchanged after trypsin treatment but that a second membrane structure appears within the cytoplasm. The qualitative similarity of the digestion products of trypsin and pronase suggests that the effect of the latter on the platelet would be essentially similar. It is unlikely that either enzyme penetrates the cell membrane since this results in complete solubilization of the cell (Stewart and Ingram, 1967; Ward and Ambrose, 1969) and a much higher proportion of its dry weight would be recovered.

Compared to the digestion of soluble glycoproteins, the digestion of the platelet outer surface to produce glycopeptides proceeds with considerable speed. This may be due to the fact that the enzyme:substrate ratio was 1:1 in terms of membrane (A. J. Barber, D. S. Pepper, and G. A. Jamieson, unpublished data) although only 1:75 in terms of total platelet protein. With trypsin, pronase, or papain the release is essentially complete in 10 min. The nature of the products of digestion with either enzyme are similar in several respects, although pronase releases a higher proportion of the platelet-bound sialic acid and gives larger quantities of GP-II and GP-III than does trypsin. The overall release of NANA is relatively low with papain and it is found predominantly in GP-II and GP-III. The observed data are most simply explained by postulating three different types of glycoprotein molecules extending out from the platelet membrane. The subfractionation of GP-III would indicate that several different types of low molecular weight glycoproteins are present on the platelet membrane although this may be a reflection of the large pools from which they are derived and the fact that they are a combination of both tryptic and pronase digests. Moreover, the fact that neither GP-I nor GP-II has so far been resolved by chromatography on DEAE-cellulose does not preclude the possibility that they may also represent families of closely related glycopeptides. In view of the high molecular weight of GP-I, and the fact that it constitutes about one-half of the total glycopeptide, it is unlikely, but not impossible, that the three glycopeptides arise from the scission of a single glycoprotein. More probably, the glycoprotein precursor of GP-I is equally susceptible to trypsin and pronase while the precursors of GP-II and GP-III are considerably more susceptible to pronase and papain than to trypsin. Confirming this view, we have been unable to detect the formation of GP-II or GP-III from GP-I by prolonged digestion with pronase, and, indeed, the chemical analysis of each fraction is sufficiently different (Table III) to make such a possibility unlikely.

Insufficient material was available to determine whether GP-I could give rise to GP-III on prolonged digestion. The number of individual glycopeptides corresponds to approximately 10^5 chains of the macroglycopeptide GP-I, 10^5 chains of the high molecular weight glycopeptide GP-II, and 10^6 chains of the low molecular weight glycopeptide GP-III per platelet. This last value is in reasonable agreement with the value of 10^7 determined in the earlier work for the number of "nondiffusible" glycopeptides (Pepper and Jamieson, 1969).

The largest glycopeptide previously examined has been isolated by limited tryptic digestion of the human red cell (Winzler *et al.*, 1967; Thomas and Winzler, 1969) and has a molecular weight of 10,000. The platelet glycopeptide GP-I is over ten times larger and probably extends out from the platelet surface as a semiflexible rod forming the outer "fluffy" coat of the platelet which may be involved in platelet adhesion during hemostasis (Hovig, 1968). This coat may be defective in such clotting abnormalities as Glanzmann's thrombosthenia in which a specific membrane antigen is lacking (Nachman and Marcus, 1968).

Platelets which have been treated briefly with papain mimic the platelets from patients with paroxysmal nocturnal hemoglobinuria in their susceptibility to lysis by quinidine-sensitive antibodies (Aster and Enright, 1969). This could be interpreted as indicating a membrane glycoprotein defect in paroxysmal nocturnal hemoglobinuria platelets. The question of whether platelet-bound HL-A (lymphocyte) antigens are integral parts of these surface glycoproteins remains to be answered although it has been suggested (Shulman *et al.*, 1964) that they do contain carbohydrate. Evidence is also accumulating (Michal, 1969; Marcus, 1969) that the serotonin receptors on platelets may be sialic acid dependent as they are in other cells (Woolley and Gommi, 1964; Carrol and Sereda, 1968; Wesemann and Zilliken, 1968; Wesemann, 1969).

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