formate and FDP eluted with 0.34 M ammonium formate. Fractions containing D-fructose-6,6-$d_2$ 1,6-diphosphate were combined, acidified with Dowex 50 ($H^+$), and extracted six times with three volume portions of ether to remove formic acid. The sample, which still contained some formic acid, was adjusted to pH 7.0 and lyophilized.

Acknowledgments

The author expresses his gratitude to Dr. Melvin P. Klein, Laboratory of Chemical Biodynamics, University of California, Berkeley, for his interest in this work and for use of the $^{31}P$ nmr spectrometer, and his special gratitude to Dr. Irving Salmeen for demonstration of the use of the spectrometer system, and for helpful discussions on Fourier transform nmr spectroscopy and the optimal conditions for obtaining $^{31}P$ nmr spectra. I also thank Dr. Robert Barker and Dr. C. E. Ballou for supplying some of the samples used in this work.

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# Isolation of Glycopeptides from Low- and High-Density Platelet Plasma Membranes*

A. J. Barber and G. A. Jamieson†

ABSTRACT: Glycopeptides have been isolated from both the low- (*d* 1.090) and high- (*d* 1.120) density membranes isolated by the glycerol-lysis technique. Three size classes of glycopeptide were obtained on treatment with trypsin which were identical with those obtained by proteolytic digestion using intact platelets. However, a chondromucopeptide obtained from intact platelets by trypsin treatment was not obtained using isolated membranes suggesting that it is a product of the platelet "release reaction." Brief digestion of intact platelets with chymotrypsin, which does not induce the release reaction, did not yield the chondromucopeptide and in this case the isolated *macroglycopeptide* was larger than that obtained by tryptic treatment. When intact platelets were subjected to catalytic iodination ($^{125}I$) with lactoperoxidase the incorporation of radioactivity, and its distribution in sodium dodecyl sulfate–polyacrylamide gels, were identical in both the high- and low-density vesicles. The amount of residual sialic acid in both types of vesicle was also equal following limited treatment with neuraminidase. These results show that both types of membrane vesicle are derived from the outer surface of the platelet and may reflect areas of anatomical specialization on the platelet surface, as previously suggested from electron microscopy.

A *macroglycopeptide* is released from intact platelets by brief proteolytic digestion (Pepper and Jamieson, 1970). This macroglycopeptide is unique in having a molecular weight (120,000) considerably larger than that of the glycopeptides isolated from other cell surfaces (Winzler *et al.*, 1967; Winzler, 1969; Buck *et al.*, 1971) and it may be related to the thrombocyte specific antigen (Hanna and Nelken, 1971). It contains both N- and O-glycosidic linkages, has galactose as its major

* From the Blood Research Laboratory, The American National Red Cross, Bethesda, Maryland 20014. *Received June 28, 1971.* This work was supported, in part, by USPHS Grants GM-13057 and AI-09017 and is Contribution No. 230 from the Blood Research Laboratory of the American National Red Cross.

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neutral sugar, and has proline as its principal amino acid. On proteolytic digestion platelets also release glycopeptides which are similar to those which have been obtained from plasma glycoproteins (Pepper and Jamieson, 1969; Mullinger and Manley, 1968) as well as chondromucopeptides derived from the mucopolysaccharides (Jamieson *et al.*, 1971a) present in platelets. All of these may form part of the "fuzzy" coat seen on electron photomicrographs of platelets treated with stains specific for carbohydrate (Hovig, 1965; Behnke, 1968).

A decision as to whether these are truly components of the external surface of platelets is rendered difficult by the fact that specific internal components of platelets are selectively released under the influence of thrombin and other proteolytic enzymes (Holmsen *et al.*, 1969). In view of the importance of separating the membrane-mediated events of platelet:collagen adhesion from the cell-mediated events of collagen-induced platelet aggregation we have attempted to obtain a better definition of the origin of these glycopeptide components.

In the continuation of these studies we have recently isolated two types of membrane vesicles by the glycerol-lysis technique (Barber and Jamieson, 1970). On the basis of ultrastructural and enzymatic data, these vesicles appear to be devoid of intracellular organelles and adsorbed soluble components and are active in mediating collagen adhesion (Barber and Jamieson, 1971; Jamieson *et al.*, 1971a,b). The two types of vesicles had identical carbohydrate content (7%) but differed in their protein and lipid analyses, and in certain of their coagulation properties (Barber *et al.*, 1971).

In order to demonstrate that the macroglycopeptide and its congeners are components of the outer surface of the platelet, itself, and do not arise from cytoplasmic components *via* the release reaction we have now demonstrated: first, that both types of membrane vesicle isolated by the glycerol-lysis technique arise from the outer surface of the platelet; second, that both major types of glycopeptides, including the macroglycopeptide but not the chondromucopeptides, can be isolated from each of them; and, third, that the macroglycopeptide can be isolated from intact platelets following digestion with chymotrypsin, which does not induce the release reaction (Davey and Lüscher, 1965, 1967).

## Methods

*Preparation of High- and Low-Density Platelet Membranes.* Membranes were isolated from homogeneous platelet preparations using a combination of stepwise and continuous sucrose gradient centrifugation following hypotonic glycerol lysis, as previously described (Barber and Jamieson, 1970; Barber *et al.*, 1971).

*Chemical Analysis.* Protein, sialic acid, hexosamine, and phosphorus were determined by standard methods as previously described (Barber and Jamieson, 1970).

*Polyacrylamide Gel Electrophoresis.* Lipid was removed from the high- and low-density membrane fractions and from the lysate by extraction with a 20-fold volume of chloroform–methanol (2:1, v/v) (Folch *et al.*, 1957). The lipid-extracted material was dried and dissolved in 0.01 M sodium phosphate buffer (pH 7.0), 1% sodium dodecyl sulfate, and 1% $\beta$-mercaptoethanol by incubation at 37° for 2 hr. The solubilized platelet fractions were applied to gels containing 10% polyacrylamide with 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969). The gels (5 × 10 mm) were run at 8 mA/gel for 4 hr and stained for either protein or carbohydrate. Coomassie brilliant blue (0.05%) in a mixture of methanol and glacial acetic acid was used to stain for protein. Staining was performed at room temperature overnight and the gels were destained electrophoretically. Glycoproteins were detected after fixation in a solution of 40% aqueous ethanol, 15% acetic acid using the periodic acid–Schiff (PAS) stain. Approximately 120 $\mu$g of protein was applied to each gel for detection when using the coomassie blue stain and 240 $\mu$g when using the PAS stain. Molecular weights were estimated from the gels by calibrating with standard proteins (Weber and Osborn, 1969). Tracing of the gels was performed using a densitometer (Photovolt Corp., New York, N. Y.) equipped with a 545-nm filter.

*Limited Sialidase Treatment of Low- and High-Density Membranes.* This was carried out essentially under the conditions described by Steck *et al.* (1970) for erythrocyte ghosts. High- and low-density membrane fractions were dialyzed overnight at 4° against 0.1 M Tris-acetate (pH 5.7) and adjusted to a final protein concentration of 1 mg/ml. *Clostridium perfringens* sialidase (Sigma) (EC 3.2.1.18) (40 $\mu$g/mg of membrane protein) was added to each and the mixture incubated at 37° for 1 hr. The membranes were removed by centrifugation and the total sialic acid content of the supernatant solution determined.

*Limited Trypsin Treatment of Low- and High-Density Membranes.* This was carried out essentially under the conditions described by Steck *et al.* (1970) for erythrocyte ghosts. High- and low-density membrane fractions were dialyzed overnight at 4° against 5 mM phosphate buffer (pH 8.0) and adjusted to a final protein concentration of 1 mg/ml. Crystalline pancreatic trypsin (Calbiochem) (EC 3.4.4.4) (125 $\mu$g/mg of membrane protein) and $CaCl_2$ (final concentration, 0.01 M) were added and the mixture was incubated at 37° for 1 hr. Soy bean trypsin inhibitor (Worthington Biochemical Corp.; 1 mg inhibits 1.5 mg of trypsin) was added to stop the reaction and the released sialoglycopeptides were separated from the membranes by centrifugation (105,000*g*, 1 hr, 4°). Sialic acid was determined as previously described on the soluble supernatant after hydrolysis with $H_2SO_4$. A similar experiment was performed in which the incubation mixture was made isotonic by the addition of 0.25 M sucrose. After the membranes were collected by centrifugation the sucrose was separated from the glycopeptides by chromatography on Sephadex G-25.

*Iodination of Intact Platelets with Lactoperoxidase.* Less than 2 hr after phlebotomy, human platelets (from approximately 1 l. of blood) were freed of residual erythrocytes, washed (twice), and suspended in 10 ml of a pH 7.5 isotonic buffer (Baenziger *et al.*, 1971) at a final concentration of $2 \times 10^{10}$ cells/ml. Lactoperoxidase, a gift from Dr. Phillip Majerus, Washington University, St. Louis, Mo., had been isolated by the method of Morrison and Hultquist (1963). We are indebted to Dr. Majerus for details of the lactoperoxidase technique in its application to platelets (Baenziger, 1971). Lactoperoxidase (final concentration $3.3 \times 10^{-7}$ M; Phillips and Morrison, 1970) and 0.2 ml of [$^{125}$I]KI (60 $\mu$Ci, 0.1 $\mu$mole/ml) were added to the platelet suspension. The iodination catalyzed by lactoperoxidase was initiated and maintained by the addition of a freshly prepared solution of hydrogen peroxide (8 $\mu$M) at the rate of 0.001 ml/sec. The reaction was carried out at 30° with gentle mixing for 45 min and was stopped by the cessation of peroxide addition. The platelets were then washed three times in cold buffer (0.001 M EDTA–0.01 M Tris-HCl (pH 7.5)–0.15 M NaCl) and the membrane and other fractions isolated by the glycerol-lysis technique. Protein was determined by the procedure of Lowry *et al.* (1951) and radioactivity with a $\gamma$ spectrometer.

*Polyacrylamide Gel Electrophoresis of Iodinated Membrane Fractions.* Removal of lipid, the preparation of the sample,

the gels, and fixation were performed as described above. The distribution of radioactivity in the polyacrylamide gels was determined by slicing the gels serially in 1.5-mm sections and counting the $\gamma$ emission from each slice on a $\gamma$ spectrometer.

*Isolation of Low- and High-Density Membranes from Sialidase-Treated Platelets.* Eight units (approximately $8 \times 10^{11}$ platelets) of erythrocyte-free, washed human platelets were suspended in 20 ml of buffered saline solution. *Cl. perfringens* sialidase (Sigma) (EC 3.2.1.18) (0.4 mg/ml, approximately 12 $\mu$g/mg of protein) was added and the mixture incubated at 37° for 30 min. The reaction mixture was centrifuged and, after removal of the supernatant, the platelets were suspended in buffer (0.001 M EDTA–0.01 M Tris-HCl (pH 7.5)–0.15 M NaCl) and the membrane fractions isolated by the glycerol-lysis technique as previously described (Barber and Jamieson, 1970; Barber *et al.*, 1971).

*Isolation of Glycopeptides from Platelet Membranes.* High- and low-density platelet membranes were dialyzed exhaustively at 4° against Tris-HCl buffer (0.05 M, pH 7.8) containing 0.01 M $CaCl_2$ at a final protein concentration of 0.8 mg/ml. After the removal of samples for determination of total sialic acid, crystalline trypsin (Calbiochem) was added to a final concentration of 0.6 mg/ml and the membrane fractions were incubated at 37°. Aliquots (1 ml) were removed at various time intervals, added to a solution of soy bean trypsin inhibitor (1 mg/ml), and centrifuged at 105,000*g* for 1 hr. The supernatant solutions were assayed for sialic acid both before and after hydrolysis with 0.1 N $H_2SO_4$ at 85° for 30 min.

For preparative experiments, high- and low-density platelet membrane fractions were suspended in Tris-HCl buffer (0.05 M, pH 7.8) containing 0.01 M $CaCl_2$ to a final protein concentration of 4.6 and 7.0 mg per ml, respectively. Crystalline trypsin (5 mg/ml) was added and the membrane fractions were incubated for 65 min at 37°. The reaction was terminated by the addition of soy bean trypsin inhibitor (6 mg/ml), followed by centrifugation at 105,000*g* for 1 hr at 4°. The supernatant solutions were then subjected separately to gel filtration on columns (2.5 × 95 cm) of Sephadex G-200, equilibrated, and eluted with Aronsonn–Gronwall buffer (Aronsonn and Gronwall, 1957) diluted 1:20 in saline. The ratio of trypsin to membrane protein in these experiments was between 0.75 and 1.0.

*Chymotryptic Digestion of Intact Platelets.* Washed human platelets (Pepper and Jamieson, 1970) were suspended in physiological saline solution at 37° and treated with chymotrypsin (in a ratio of 1 mg of enzyme/unit platelets, Calbiochem, A grade; the ratio of chymotrypsin to trypsin activity in these preparations was 0.005). Aliquots were removed at appropriate times, the treated platelets were removed by centrifugation (10,000 rpm, 18,000*g* for 5 min), and total sialic acid was determined in the soluble supernatant solution by the thiobarbituric acid assay following acid hydrolysis.

In preparative experiments 8 platelet units (*ca.* $8 \times 10^{11}$ platelets) were treated with 8 mg of chymotrypsin for 90 min and the soluble supernatant was subjected to gel filtration on a column (2.5 × 92 cm) of Sephadex G-200 in Aronsonn–Gronwall (1957) buffer diluted with normal saline solution.

## Results

*Release of Glycopeptides from Membranes under Saturating Enzyme Concentrations.* At saturating trypsin concentrations both the total amount, and the rate of release, of soluble glycopeptides were slightly lower in the case of the low-density membrane fraction (Figure 1). However, the differences were small

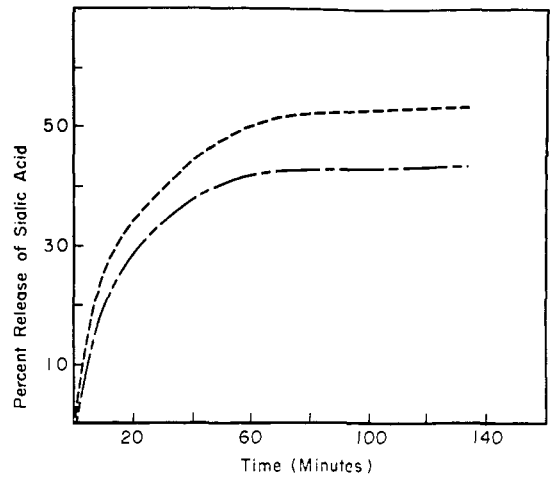


FIGURE 1: Rate of release of glycopeptides from human platelet membranes treated with trypsin, determined as total soluble NANA. High-density membrane fraction, ———; low-density membrane fraction, – – – – – –. Membrane protein, 0.8 mg/ml; trypsin, 0.6 mg/ml.

and the total release of 40–50% of the total bound sialic acid was similar to that found with intact platelets using trypsin and other proteolytic enzymes (Pepper and Jamieson, 1970) and also in HeLa cells (Shen and Ginsburg, 1969) and human erythrocytes (Winzler, 1969).

*Release of Glycopeptides from Membranes under Limiting Enzyme Concentrations.* In order to accentuate any possible differences in the accessibility of trypsin and neuraminidase to the glycoproteins of the high- and low-density platelet membranes, these were treated with low concentrations of the two enzymes. The results were the same in each case: 68 and 70%, respectively, of the total sialic acid present was released from the high- and low-density membrane fractions by neuraminidase while 28% of the total, in the form of soluble glycopeptides, was released following treatment with limiting amounts of trypsin (Table I). Similar results were obtained when the membrane vesicles were maintained in an isotonic environment during enzymatic treatment.

*Isolation of Membranes from Neuraminidase-Treated Platelets.* Low- and high-density membranes which had been isolated from intact platelets following treatment with the neuraminidase had identical concentrations of bound sialic acid on a protein basis (Table II). The sialic acid remaining in the high- and low-density membranes comprised 28 and 23%, respectively, of that in the two membrane fractions isolated

TABLE I: Accessibility of Neuraminic Acid and Sialoglycopeptides of High- and Low-Density Platelet Membranes to Low Concentrations of Neuraminidase and Trypsin.

| | Sialic Acid | | |
|---|---|---|---|
| | Content (nmoles/mg of Protein) | Percentage Released by | |
| Material | | Neuraminidase | Trypsin |
| Upper band (*d* 1.090) | 56 | 68 | 28 |
| Lower band (*d* 1.120) | 42 | 70 | 28 |

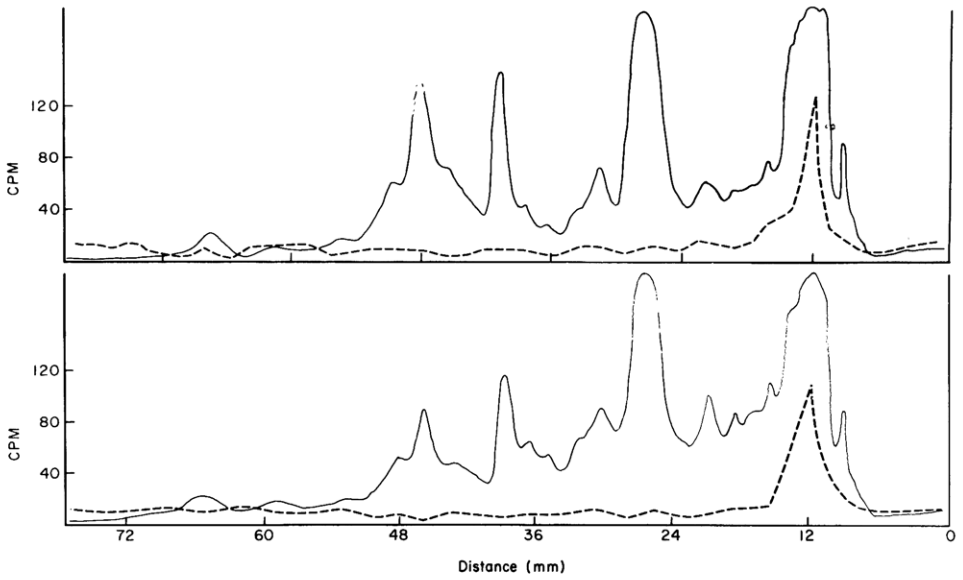


FIGURE 2: Distribution of membrane protein on polyacrylamide gel electrophoresis of iodinated low- (upper) and high- (lower) density membrane fractions. After the removal of lipid membrane protein was dissolved in 1% sodium dodecyl sulfate. Anode is at left. Sample (*ca.* 120 μg of protein). Densitometer tracing (——) after staining with coomassie blue; (— — — —), radioactivity determined on 1.5-mm sections.

from untreated platelets. The released sialic acid could be quantitatively determined in the supernatant solution following neuraminidase treatment. The sialic acid in the membrane fractions which was not cleaved by neuraminidase probably represents sialic acid in ganglioside linkage which is resistant to enzymatic attack.

*Distribution of Radioactivity in Isolated Fractions Following Iodination of Intact Platelets with Lactoperoxidase.* Lactoperoxidase catalyzes iodide incorporation into the exposed tyrosine and histidine groups on proteins and the reaction has been shown to occur *via* an enzyme–substrate complex between the protein substrate and lactoperoxidase (Morrison *et al.*, 1970).

When intact platelets were incubated with the enzyme lactoperoxidase, $^{125}I$, and hydrogen peroxide, the highest specific activity was found in the isolated membrane fraction (Table III). The specific activity of the soluble and intracellular debris fractions equalled about 3 and 18%, respectively, of that found in the combined membrane fractions.

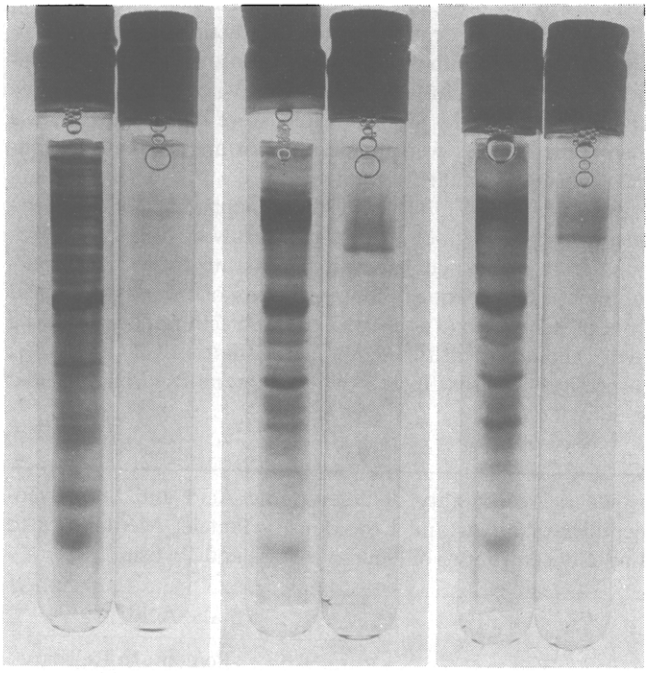


FIGURE 3: Polyacrylamide gel electrophoresis of protein and glycoprotein fractions of platelets. (a) Lysate, (b) low-density membrane, and (c) high-density membrane. In each case the left-hand gel (120 μg of protein) was stained with coomassie blue while the right-hand gel (240 μg of protein) was stained with the periodic acid–Schiff reagent. Anode was at the bottom and the sample was applied at the cathodal end.

TABLE II: Sialic Acid Content of High- and Low-Density Platelet Membranes Isolated after Neuraminidase Treatment of Intact Platelets.

| Material | Sialic Acid Content (nmoles/mg of Protein) | |
|---|---|---|
| | Control | Sialidase-Treated Platelets |
| Upper band (*d* 1.090) | 56 | 13 |
| Lower band (*d* 1.120) | 42 | 13 |

TABLE III: Distribution of Radioactivity in Isolated Fractions Following Iodination of Intact Platelets with Lactoperoxidase.

| Fraction | Sp Act. (cpm/mg of Protein) |
|---|---|
| Soluble | 361 |
| Debris | 2416 |
| Upper band (*d* 1.090) | 7302 |
| Lower band (*d* 1.120) | 6500 |

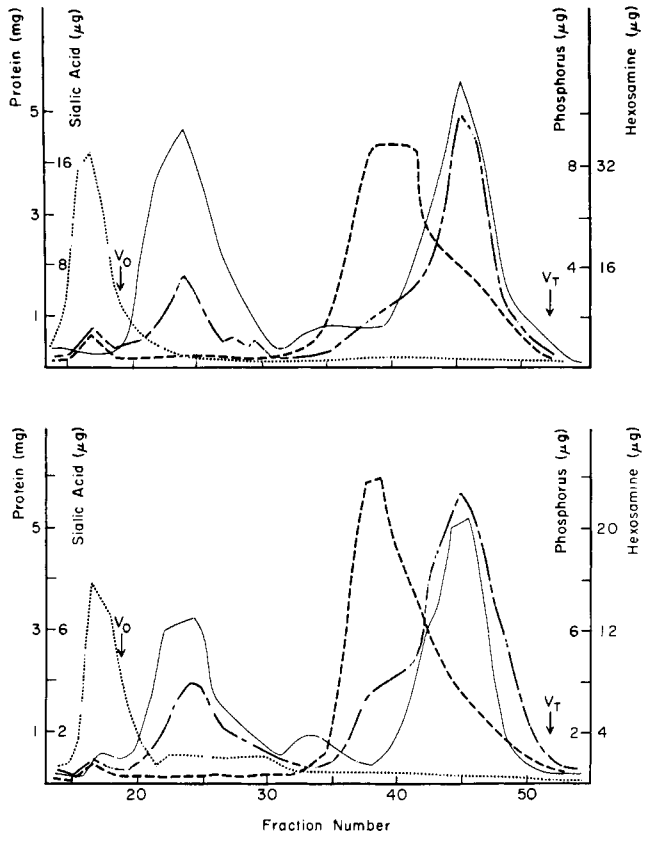


FIGURE 4: Gel filtration of trypsin digests of human platelet membranes on Sephadex G-200 (2.5 × 95 cm) eluted with Aronsonn-Grönwall buffer diluted 1:20 in saline. Flow rate, 12 ml/hr; fraction volume, 9 ml. Upper, low-density membrane fraction; lower, high-density membrane fraction. Sialic acid (——), hexosamine (— — — —), phosphorus (--------), and protein (— — — — —). The ordinates are given in micrograms or milligrams per fraction.

*Polyacrylamide Gel Electrophoresis of Iodinated Membrane Fractions.* Membrane proteins obtained from iodinated, intact platelets were separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The distribution of radioactivity among the membrane proteins is identical in both the high- and low-density membranes (Figure 2) and indicates that only a closely related group of high molecular weight components, present in each fraction, contains radioactive iodide.

*Polyacrylamide Gel Electrophoresis of Lysate and Membrane Fractions.* Polyacrylamide gel electrophoresis of the lysate preparation, following lipid extraction and treatment with sodium dodecyl sulfate, revealed approximately 30 protein bands (Figure 3a) and two very faint, slow-migrating glycoprotein bands. Both the protein and glycoprotein patterns were identical in the case of the isolated low- and high-density membrane fractions (Figure 3b,c). Approximately 20 protein bands which had comparable mobilities in each fraction, together with the two slow-moving glycoproteins, were observed. The greater intensity of the carbohydrate-containing bands in the case of the latter fractions is in accord with the higher carbohydrate content of the membrane fractions compared with the lysate (Barber and Jamieson, 1970). Similarly, very weakly staining glycoprotein bands were observed in the soluble and debris fractions. When membranes from which the lipid had not been removed were run under similar conditions, a very fast-moving, PAS-staining band was observed which was not visualized with the corresponding coomassie blue stain, and probably represented glycolipid. The molecular weight of the membrane glycoproteins was estimated as being approximately 175,000 using standard protein markers and not corrected for the anomalous effects of the carbohydrate content.

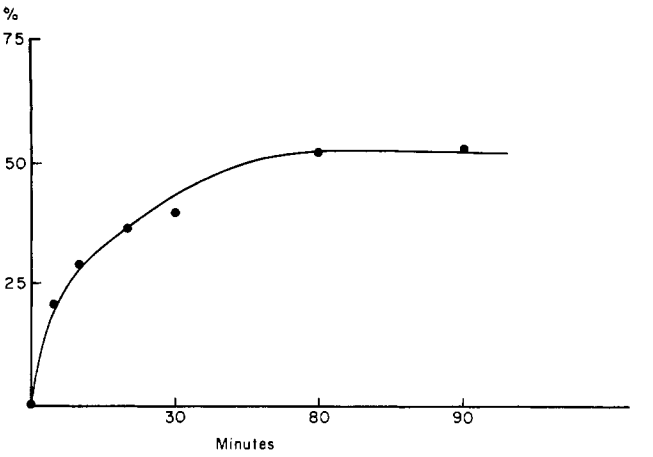


FIGURE 5: Rate of release of chymotryptic glycopeptides from human platelets determined as total soluble NANA. Platelet protein, 8 mg/ml; chymotrypsin, 0.1 mg/ml.

*Isolation of Tryptic Glycopeptides from High- and Low-Density Membranes.* When the soluble glycopeptides resulting from the tryptic digestion of the high- and low-density platelet membranes were subjected to gel filtration on Sephadex G-200 an elution pattern was obtained which was essentially identical with that obtained with intact platelets (Figure 4). This consisted of a high molecular weight glycopeptide with $V_r$[1] = 1.26 and a low molecular weight glycopeptide with $V_r$ = 1.84. In addition, a small amount of a third glycopeptide was obtained with a $V_r$ of 2.37. These values are in good agreement with the values found for the glycopeptides GP-I, GP-II, and GP-III isolated from intact platelets (Pepper and Jamieson, 1970). In addition, the sialic acid to hexosamine ratios were identical for the high molecular weight glycopeptide (1:1) and the low molecular weight glycopeptide (1:0.5) isolated from both sources.

The peak of phosphorus-containing material is probably due to the presence of phospholipid micellar material which is released from the membranes during enzymatic treatment and is eluted slightly ahead of the void volume due to its low water solubility, high degree of aggregation, and high negative charge. The only hexosamine peaks detectable were coincident with the sialic acid peaks of the major glycopeptide.

*Release of Glycopeptides by Chymotrypsin Digestion of Intact Platelets.* Chymotrypsin releases about 50% of total platelet sialic acid in the form of soluble glycopeptides (Figure 5) similar to the results obtained with trypsin and other proteases (Pepper and Jamieson, 1970). On gel filtration two peaks corresponding to GP-I and GP-III were obtained although GP-II was, apparently, not released under these circumstances (Figure 6). It may be noted that the chymotryptic macroglycopeptide isolated under these conditions was eluted at the void volume ($V_0$) on gel filtration while the tryptic macroglycopeptide had $V_r$ = 1.29.

[1] Abbreviations used are: $V_r$, relative elution volume, equals $V_e/V_0$, where $V_e$ equals the elution volume and $V_0$ the void volume of the column; NANA, *N*-acetylneuraminic acid.

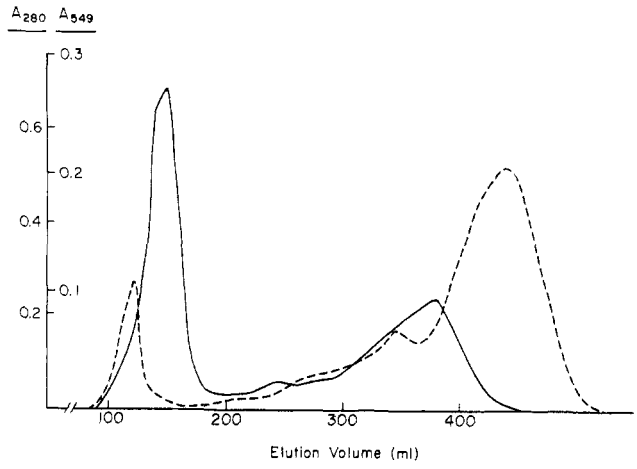


FIGURE 6: Gel filtration of crude soluble chymotryptic digest of human platelets on a column (2.5 × 92 cm) of Sephadex G-200 eluted with Aronsonn–Gronwall (1957) buffer diluted 1:20 in saline. Flow rate, 10 ml/hr; fraction volume, 9.5 ml. Ultraviolet absorption (280 nm), ------; NANA (absorbance 549 nm),——.

## Discussion

Determination of the surface structure of the blood platelet presents particular problems because of the possible effects of the selective release of cytoplasmic components (Holmsen *et al.*, 1968) and the possible compartmentalization of surface components in the invaginations of the surface connected system (Hovig, 1968). We have, in particular, endeavored to separate these cellular reactions from the purely membrane aspects of platelet biochemistry by isolating purified preparations of platelet membranes and by the use of enzymes which reportedly do not induce the release reaction.

Several possibilities could be envisaged for the origin of the two types of platelet membrane isolated by the glycerol-lysis technique. Firstly, these may have arisen by the separation of the inner and outer surface of the membrane in a manner analogous to the splitting which occurs mechanically during the application of the freeze-cleave technique (Weinstein, 1969); secondly, they could have arisen by formation of the "inside-out" and "outside-outside" type of vesicles which have been shown to be formed from erythrocyte membranes under carefully defined ionic conditions (Steck *et al.*, 1970); thirdly, they could represent areas of anatomical specialization in the membrane as has been suggested on the basis of differential staining prior to electron microscopy (Nakao and Angrist, 1968).

The similarity in sialic content of the two membrane bands when isolated from neuraminidase-treated platelets, their equal accessibility to neuraminidase and trypsin, the identical labeling with $^{125}I$ following catalytic iodination with lactoperoxidase, and the identity of the glycopeptides released from each of the membranes with those isolated from intact platelets all strongly suggest that the third possibility is correct; namely, that the two types of vesicle represent areas of platelet membrane specialization. This interpretation receives some support from the differences which have been found in the procoagulant activities of the phospholipids extracted from both types of membrane (A. J. Barber, G. A. Jamieson, and D. C. Triantaphyllopoulos, submitted for publication).

In addition to the three size classes of sialylglycopeptide tryptic treatment of intact platelets gives a high molecular weight peak (GP-O) which is devoid of sialic acid but rich in hexosamines and neutral sugars, and corresponds to the mucopeptides of chrondroitin 4- (or 6-) sulfate arising from the chondromucoproteins present in the platelet (Jamieson *et al.*, 1971a). This chondromucopeptide is not found when isolated platelet membranes are treated with trypsin suggesting that the chondromucoprotein is not a component of the membrane itself but is probably released from the platelet interior during tryptic digestion. This view is supported by the fact that chymotrypsin, which does not effect the platelet release reaction (Davey and Lüscher, 1965, 1967), also fails to yield the chondromucopeptide from intact platelets.

These results confirm the origin of the unique macroglycopeptide of platelets, and the lower molecular weight glycopeptides, as being the outer surface of the platelet. They suggest that the platelet may show areas of anatomical specialization which do not occur in erythrocyte membranes but which have been suggested in liver cells (Evans, 1970) and in nuclear membranes (Kashing and Kasper, 1968).

## Acknowledgments

We are grateful to Mrs. Carol Wu for her skillful experimental assistance and to Dr. Evan C. Stone, Jr., of the Washington Regional Red Cross Blood Center for providing the platelets used in this study under NIH Contract 70-2005.

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# Immunoglobulin Synthesis in a Cell-Free System*

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ABSTRACT: A cell-free system prepared from rabbit lymph nodes synthesizes predominately immunoglobulin heavy and light chains. The enzymatic and ionic requirements for optimal protein synthesis are shown. The synthesis of complete heavy and light chains is directed by two different size classes of polysomes. The number of ribosomes in the polysomes is that expected for mRNA molecules coding for individual complete polypeptide chains. Synthesis of the heavy and light chains also occurs if lymph node polysomes are incubated with supernatant enzymes from rabbit reticulocytes, rabbit liver, or rat liver. Analysis of the cyanogen bromide cleavage peptides of the heavy chain suggests that the bulk of the amino acid incorpration occurs by a COOH-terminal completion of polypeptides already initiated on polysomes *in vivo*.

Antibodies are synthesized in the plasma cells of lymph nodes. These proteins are characteristically composed of two heavy chains, each containing about 440 amino acids, and two light chains with about 215 amino acids each (Edelman and Gall, 1969). In the completed IgG molecule, these are bound together by disulfide linkages. The $NH_2$-terminal regions of these two types of chains vary greatly in their amino acid sequence, whereas the rest of the molecule is relatively invariant within a species. It has been suggested that these two regions may be coded for by different genes (Dreyer and Bennett, 1965; Wang *et al.*, 1970). Kinetic labeling experiments with whole cells suggest that the heavy and light chains are made as single intact units (Fleischman, 1967; Lennox *et al.*, 1967). However, it is of interest to study this question by *in vitro* experiments with the protein synthetic apparatus of the cell.

In a hyperimmunized rabbit, more than 70% of the protein synthesized by the lymph nodes *in vivo* is immunoglobulin (Becker *et al.*, 1970). The maximum synthesis of immunoglobulin occurs 3–5 days after a booster injection of antigen when cell proliferation has begun to subside (Mach and Vassali, 1965). This can be correlated with the appearance of two classes of polysomes in the lymph nodes (Becker and Rich, 1966), the development of two rapidly labeled RNA species (Kuechler and Rich, 1969a,b), and a concomitant rapid increase in serum antibody titer. The small polysomes contain 6–8 ribosomes and the larger ones contain slightly over twice this number of ribosomes (Becker *et al.*, 1970). By comparing the size of the polysome with the size of the proteins being synthesized in other cell types (Warner *et al.*, 1963; Heywood and Rich, 1968), we can make an estimate of the molecular weight of protein made by these two classes of polysomes. The biphasic lymph node polysome pattern is consistent for a tissue synthesizing predominantly polypeptide components containing 210–220 residues (light chains) and 440–480 residues (heavy chains), respectively. Experiments have also been carried out on closely related myeloma tumors which support this interpretation (Shapiro *et al.*, 1966; Williamson and Askonas, 1967; Schubert, 1968).

In the present paper the cell-free system prepared from rabbit lymph nodes is described; a brief report has been made earlier (Ralph *et al.*, 1967). Here we demonstrate that this system synthesizes the intact immunoglobulin molecule. Incubation of separate heavy and light polysome fractions yields labeled heavy and light chains, respectively. Furthermore, substitution of soluble enzymes from other tissues yields the same products, showing that the specificity for synthesis resides in the polysomes, presumably through its mRNA,

* From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received January 12, 1971*. This research was supported by grants from the National Institutes of Health and the National Science Foundation.

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