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ISOLATION OF HUMAN PLATELET GLYCOPROTEINS

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Summary

Human platelet glycoproteins were isolated from whole platelets by two methods. The first method, that of affinity chromatography on wheat germ agglutinin, is based on the known affinity of lectins for cell surface glycoproteins. When solubilized whole platelets are used as starting material for this procedure, elution with *N*-acetylglucosamine yields primarily a glycoprotein of $M_r \approx 150\,000$ as estimated by sodium dodecyl sulfate-acrylamide gel electrophoresis. The second method is based on the ability of the chaotropic salt lithium diiodosalicylate to extract glycoprotein from particulate cell fractions in water-soluble form. This method yields three major glycopeptides with apparent molecular weights after sulfhydryl reduction of 145 000, 125 000, and 95 000 as estimated on 5.6% sodium dodecyl sulfate-acrylamide gels. Carboxymethylation of these preparations in the presence of sulfhydryl-reducing agent further resolves a glycoprotein of $M_r \approx 165\,000$.

Treatment of whole platelets by periodate oxidation and sodium[^{3}H]-borohydride reduction labels the three major glycoproteins extracted by lithium diiodosalicylate and the glycoprotein of $M_r \approx 150\,000$ isolated on wheat germ agglutinin confirming their surface orientation. However, glycoprotein with $M_r \approx 165\,000$ resolved by carboxymethylation of the lithium diiodosalicylate extracted glycoprotein mixture was not labelled by this method, suggesting that it represents the granule protein with similar electrophoretic characteristics described by others.

Phosphorylation of intact platelets with $^{32}P_i$ also results in labelling of glycoproteins isolated by both methods, suggesting that these molecules traverse the

Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; GP, glycoprotein.

bilipid layer of the platelet membrane, bearing reactive groups on both outer and cytoplasmic surfaces.

Introduction

Platelets circulate through undamaged vessels as discoid structures, adhering neither to intact endothelium nor to each other. Damage to the endothelium, with exposure of subendothelial tissue, induces dramatic changes in the platelet membrane. Pseudopods extend from the platelet surface, coming into contact with and adhering to subendothelial tissues. Platelets approaching the injured area now adhere to one another at the site of injury, and are able to release the contents of their granules to the outside, amplifying the aggregation process and producing a macroscopic platelet-fibrin thrombus. The molecular alterations and/or rearrangements within the membrane responsible for these processes are essentially unknown.

Over the last several years, studies on platelet membrane glycoproteins have been initiated in a number of laboratories. A group of surface-oriented glycoproteins has been identified in membrane preparations which migrate on SDS-acrylamide gels in the 90 000—150 000 dalton range. At least three major glycoprotein species are consistently present, but the total number and electrophoretic behavior of discrete glycopeptides differs among laboratories. George [1] describes five major glycoproteins by electrophoresis of platelet membranes on SDS-acrylamide gels, the relative amounts and behavior of which are dependent on gel pore size and prior sulfhydryl reduction. Phillips and Poh Agin [2] using a two-dimensional electrophoretic system, describe six or seven glycoprotein species in platelet membranes under conditions of sulfhydryl reduction, with molecular weights between 90 000 and 160 000. Okumura and Jamieson [3] observe three major surface glycoproteins and in addition have purified and characterized a loosely bound glycoprotein of molecular weight approx. 150 000, termed 'glycocalicin' which is released in soluble form following platelet homogenization [4].

In contrast with the findings in membrane preparations, solubilize whole platelets appear to contain an additional glycoprotein ($M_r \approx 160\,000$ after sulfhydryl reduction) [5] which is released into the supernatant fraction after treatment of the platelets with thrombin [1,6] and which probably represents the material termed thrombin-sensitive protein by other workers [7].

The purpose of the current study was to develop methods for the purification of platelet glycoprotein components in order to help clarify some of the issues which have arisen concerning the number, behavior and membrane orientation of glycoproteins as studied in crude sonicates or membrane systems. Furthermore, purified glycoprotein preparations will be of great advantage in the study of glycoproteins as receptors for platelet-aggregating agents, their role in inherited abnormalities of platelet function, and as antigens in immune thrombocytopenias.

In the current study, platelet glycoproteins have been purified (1) by extraction with lithium diiodosalicylate, a chaotropic salt, followed by gel chromatography in SDS, and (2) by affinity chromatography on wheat germ agglutinin

conjugated to Sepharose. Changes in migration of the major glycoproteins in response to sulfhydryl reduction and stabilization by carboxymethylation are also described. Orientation of the glycoproteins within the membrane has been evaluated by employing radiolabelling techniques with specificity for the inner and outer surfaces of the cell. Surface labelling of the glycoproteins has been studied using the technique of mild oxidation and reduction of surface oriented sialic acid by [^{3}H]borohydride described by Liao et al. [8]. Extension of the glycoproteins onto the cytoplasmic surface of the membrane has been studied by phosphorylating intact platelets with ^{32}P-labelled inorganic phosphate, followed by extraction and autoradiography of glycoproteins.

Methods

Phenylmethylsulfonyl fluoride (PMSF) and *N*-acetylglucosamine were obtained from Sigma Chemical Company, St. Louis, MO. 3,5-Diiodosalicyclic acid and β-mercaptoethanol were obtained from Eastman Organic Chemicals. Sodium periodate and phenol were obtained from J.T. Baker, Phillipsburg, NJ. Lithium hydroxide was purchased from Matheson, Coleman, and Bell, East Rutherford, NJ. Biorad (Richmond, CA) provided acrylamide and other reagents for electrophoresis. [^{32}P]Orthophosphoric acid and [^{3}H]borohydride were obtained from New England Nuclear, Boston, MA. Sephadex G-200 and Sepharose 6B were obtained from Pharmacia, Piscataway, NJ.

Platelet preparation. Three types of platelet preparations were used for the experiments to be described: (1) fresh platelets from normal donors, processed immediately after collection, (2) platelet packs obtained from the Red Cross Blood Center in Farmington, CT, less than 24 h after collection or, (3) more than 72 h after collection (i.e. outdated platelets). For experiments using fresh platelets, 2—4 units of fresh blood drawn from normal donors into acid/citrate/dextrose (USP Formula A) were centrifuged twice at 800 rev./min (150 × *g*) for 20 min to remove red blood cells and then at 1800 rev./min (900 × *g*) for 20 min to collect platelets. The platelets were then allowed to sit for 30 min in residual plasma to facilitate resuspension. Platelets were washed twice in Ca^{2+}-free Tyrodes buffer, pH 6.8 [9]. All operations were carried out at room temperature in polypropylene in the presence of 5 ng/ml PMSF (stock solution 150 mM in isopropanol). When platelet packs obtained from the Red Cross were used, 10—50 units were pooled, followed by removal of red cells and collection of platelets as described above. Radiolabelling studies were performed only on the fresh and less than 24-h-old platelets, while extractions of glycoprotein were performed on all three types of platelet preparations. The type of platelet preparation used for the experiments described below will be stated in the text or appropriate figure legend.

Tritium labelling. Platelets from 2—4 units of fresh blood or from ten units of platelets less than 24 h after collection were used for these experiments. The platelet pellet prepared as described above was resuspended in buffer containing 10 mM sodium phosphate and 130 mM NaCl, pH 7.2, at 5 ml/ml packed platelets and tritiated according to Liao et al. [8]. Sodium periodate was added to the platelet suspension at a final concentration of 2 mM, and incubation was continued for 10 min at room temperature. The oxidation step

was terminated by dilution and three phosphate-buffered saline washes, the last wash contained 0.1% glucose. The platelets were then resuspended in 5 ml phosphate-buffered saline/ml packed platelets and incubated with sodium[^{3}H]-borohydride for 20 min at room temperature. Sodium[^{3}H]borohydride was dissolved in 0.01 N NaOH and diluted 1/100 in the incubation mixture to a final concentration of 0.18 mg/ml, equivalent to 0.24 mCi/ml. After incubation, platelets were centrifuged and washed until the counts in the supernatant became constant and then stored at 4° C before being subjected either to the lithium diiodosalicylate-phenol procedure or to solubilization in SDS for affinity chromatography.

Phosphorylation. Platelets from 2—4 units of fresh blood were collected and washed once in Ca^{2+}-free Tyrodes buffer, and once in 125 mM Tris, 20 mM citric acid, 45 mM NaCl, 5 mM KCl, at pH 6.8. The platelets were then resuspended at a concentration of 1 ml packed platelets/7—10 ml Tris buffer as above except that the citrate concentration was reduced to 2 mM, and 5 mM glucose was added. Neutralized $H_3{}^{32}PO_4$ (10 mCi) was added to 20—40 ml platelet suspension with carrier phosphate at concentration of 0—20 mM, and the mixture was incubated at 37° C for 90 min with shaking. Incubation was terminated by dilution, centrifugation and washes with the Tris wash buffer (above) until the cpm of ^{32}P in the supernate became stable. The platelets were then subjected either to the lithium diiodosalicylate-phenol procedure or solubilized in SDS for affinity chromatography.

Wheat germ-Sepharose affinity chromatography. Wheat germ agglutinin, bought at a local natural food store, was purified by ovomucoid-Sepharose 4B affinity chromatography as previously described [10]. 40 mg of purified wheat germ were coupled to 20 ml Sepharose 4B after activation with cyanogen bromide as described by Cuatrecasas and Anfinsen [11]. The wheat germ-Sepharose beads were washed with 2% SDS in 15 mM sodium phosphate, 250 mM NaCl, 0.025% NaN_3, pH 7.2, and then equilibrated with 0.05% SDS in the same buffer. Washed, packed platelets from 2—4 units of fresh blood were solubilized by the addition of an equal volume of 6% SDS, 4 M urea, 20 mM Tris and 3% β-mercaptoethanol, pH 8.3. The SDS concentration was decreased to 0.05% by addition of the phosphate buffer (no added mercaptoethanol) and the solubilized platelets were passed through a column containing 10 ml of wheat germ-conjugated Sepharose beads. After several column volume washes with the 0.05% SDS/phosphate buffer, 1/2 column volume of 0.1 M *N*-acetylglucosamine in the 0.05% SDS/phosphate buffer was applied to the column, and the column was turned off and allowed to stand for several hours. After elution of the glycoprotein with additional *N*-acetylglucosamine, the column was regenerated in 2% SDS/phosphate buffer. The *N*-acetylglucosamine eluate was dialyzed against distilled water for several days and lyophilized.

Lithium diiodosalicylate-phenol extraction procedure. Lithium diiodosalicylate was prepared from diiodosalicyclic acid and lithium hydroxide as previously described [12,13]. Platelets were suspended in Ca^{2+}-free Tyrodes solution (pH 6.8) with added PMSF (6 ng/ml) at a concentration of 1 ml of packed platelets/10 ml buffer. Sonication was performed on a Branson Sonifier at 75% maximum voltage in three 30-s bursts with continuous icing of the sample. Disruption of the platelets was checked for completeness in a phase

contrast microscope. Sonicates were collected at 20 000 rev./min (45 000 × *g*) for 60 min at 4° C, washed once in Ca^{2+}-free Tyrodes and once in 20 mM Tris, pH 7.2, and lyophilized. Lyophilized platelet sonicates were solubilized in 0.3 M lithium diiodosalicylate in 20 mM Tris, pH 7.2, at 30 mg sonicate/ml. The solution was stirred at room temperature for 5 min, diluted with 2 vols of cold distilled water and stirred at 4° C for 10 min. The solution was then filtered through cheesecloth and centrifuged at 20 000 rev./min (45 000 × *g*) for 60 min. The supernatant was removed, mixed with an equal volume of 50% phenol for 15 min at 4° C and the mixture centrifuged in a swinging bucket rotor (Sorvall Hg4L) at 3000 rev./min for 60 min. After warming to room temperature, the aqueous layer containing the glycoprotein was removed, dialyzed exhaustively against water, and lyophilized.

Gel chromatography. Lyophilized lithium diiodosalicylate extracts were solubilized in a final concentration of 3% SDS, 2 M urea, 10 mM Tris, 1 mM EDTA, and 3% β-mercaptoethanol, pH 8.3, heated to 100° C for 3 min, and applied to columns of Sephadex G-200 (90 × 2.5 cm) or Sepharose 6B (90 × 1.5 cm) equilibrated with 1% SDS in 10 mM Tris, 1 mM EDTA, and 0.5% β-mercaptoethanol, pH 8.0. 3—5-ml fractions were collected and A_{280} determined. Pooled fractions were dialyzed against water at room temperature for one day, at 4° C for three days, and lyophilized.

Lipid extraction. Glycoprotein prepared by the lithium diiodosalicylate procedure was extracted at 4° C twice with absolute ethanol, once with chloroform/methanol, once with chloroform/methanol/conc. HCl, and again with 100% ethanol.

Carboxymethylation. Lithium diiodosalicylate glycoprotein preparations were dissolved in 8 M urea in 20 mM Tris-HCl (pH 8.0) in the presence of 100 mM β-mercaptoethanol at a final concentration of 1 mg protein/ml estimated by the Lowry method [14] and incubated at 37° C for 3 h. Recrystallized iodoacetic acid neutralized with NaOH was added slowly to a final concentration of 150 mM with titration to keep the pH between 8.3 and 8.5. After 1 h, the reaction was stopped by the addition of excess β-mercaptoethanol; samples were dialyzed against 50 mM NH_4HCO_3 and lyophilized. Where indicated in the text, carboxymethylation was carried out in the absence of mercaptoethanol.

SDS-polyacrylamide gel electrophoresis. Samples to be electrophoresed were heated to 100° C for 3 min in 3% SDS, 2 M urea, 1 mM EDTA and 3% β-mercaptoethanol. Mercaptoethanol was omitted only for samples shown in Fig. 5 as described in the text and legend for that figure. Samples were electrophoresed on 5.6% acrylamide tube or slab gels in a modified system of Fairbanks et al. [15]: the gels contained 0.5 M urea and 1% SDS and the buffer contained 1% SDS in 40 mM Tris, 2 mM EDTA, and 20 mM sodium acetate brought to pH 7.4 with acetic acid. The tube gels were run at 8 mA/tube for 2.5—3 h, until the tracking dye had reached 9 cm. Slab gels were run 3—4 h at 60—70 V until the tracking dye had reached 9 cm. Gels were fixed and stained with Coomassie blue or with Schiff stain for carbohydrate [15]. Gels to be stained with Coomassie blue and Schiff were loaded with 75 and 150 μg protein, respectively, as measured by the Lowry method [14]. Tube gels on which labelled samples (^3H or ^{32}P) had been electrophoresed were cut into 1 mm slices; the slices were incubated overnight in 0.4 ml 30% H_2O_2 + 0.2 ml 60%

$HClO_4$. Scintillation fluid was then added and samples were counted in a Beckman LS230 scintillation counter. Slab gels on which ^{32}P-labelled samples had been electrophoresed were also fixed, dried, and autoradiographed on Kodak X-OMAT R film. Slab gels on which ^{3}H-labelled samples had been electrophoresed were stained with Coomassie blue and photographed. The gels were then soaked in dimethylsulfoxide (Me_2SO), followed by 22% diphenyloxazole (PPO) in Me_2SO, dried and autoradiographed on Kodak X-OMAT R film as described by Bonner and Laskey [16].

Molecular weight estimates were made by the method of Weber and Osborn [17] using as standards bovine serum albumin (67 000), transferrin (85 000), β-galactosidase (135 000) and fibrinogen (340 000).

Results

A. Isolation of platelet glycoprotein by wheat germ-Sepharose affinity chromatography

Lectins are known to have a strong affinity for cell surface glycoproteins; thus they may be used in Sepharose conjugates as affinity systems by which glycoproteins may be purifed from crude cell extracts [18—20]. Platelets from 3—4 units of fresh blood were solubilized in 0.05% SDS in 15 mM sodium phosphate/250 mM NaCl, pH 7.2, and applied to wheat germ agglutinin-conjugated Sepharose equilibrated with the same buffer (see Methods). Fig. 1 shows SDS-acrylamide gels of a crude platelet particulate fraction and the *N*-acetylglucosamine eluate from wheat germ agglutinin-conjugated Sepharose. The Schiff-stained crude platelet starting material shows six bands with molecular weights estimated to be between 90 000 and 150 000 [17], and a faint band at approx. 200 000. The *N*-acetylglucosamine eluate contains a principal band at aprpox. 150 000. The Schiff-stained gel also shows a small amount of material of slightly lower molecular weight. In order to establish the outward orientation of the glycoprotein isolated on wheat germ agglutinin, 2—4 units of fresh platelets were tritiated by mild periodate oxidation and [^{3}H]borohydride reduction (as described in Methods) before application to the affinity column. Fig. 2C shows that the glycoprotein of $M_r \approx 150\,000$ is labelled under these conditions. The ^{3}H cpm pattern also shows slight labelling in lower molecular weight material which corresponds to a comparable region of faint staining seen in the scan of the Schiff-stained gel (Fig. 2B). Thus, while the wheat germ agglutinin has high affinity for the glycoprotein 150 000 and is able to separate it effectively from solubilized whole platelets, small amounts of lower molecular weight glycoproteins are also bound and eluted with *N*-acetylglucosamine.

We next approached the question of whether the glycoprotein isolated on wheat germ-conjugated Sepharose might extend from the surface of the platelet through the bilipid layer to make reactive sites available on the cytoplasmic surface of the membrane. Fresh platelets derived from two or three units of blood were incubated with neutralized $H_3{}^{32}PO_4$ for 90 min at 37° C in a Tris-buffered balanced salt solution at pH 7.2 as described in Methods. After incubation the platelets were washed twice, solubilized in SDS and applied to the wheat germ-Sepharose column. Fig. 2D shows that the glycoprotein of $M_r \approx 150\,000$ eluted from the column was radiolabelled with ^{32}P. While small

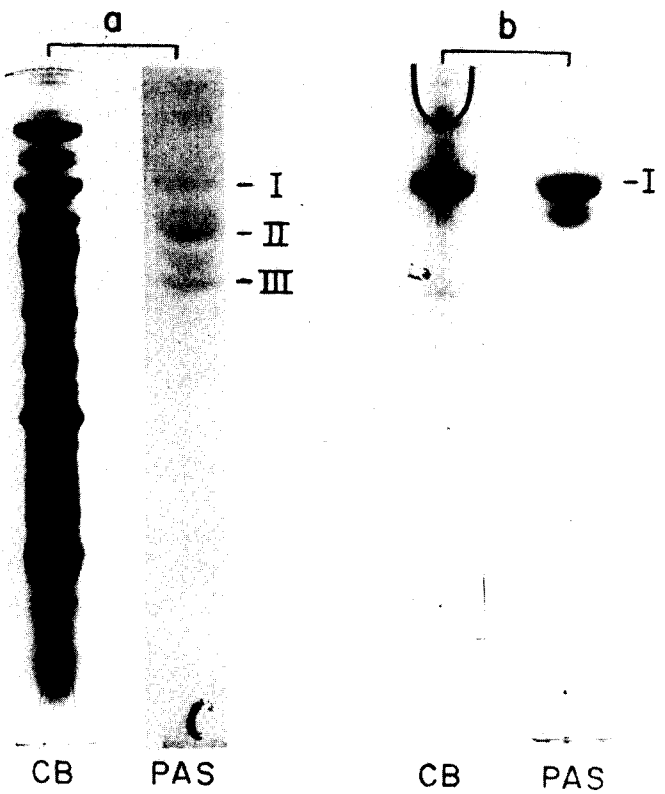

Fig. 1. SDS-acrylamide gel electrophoresis illustrating purification of GP I from whole platelets by wheat germ agglutinin-Sepharose affinity chromatography. Fresh platelets were solubilized in 0.05%SDS in 15 mM sodium phosphate/250 mM NaCl buffer, pH 7.2, and applied to the wheat germ agglutinin-Sepharose column equilibrated with the same buffer. After washing of the column, glycoprotein was eluted with 0.1 M *N*-acetylglucosamine. Dialyzed, lyophilized eluate was solubilized in 3% SDS, 10 mM Tris, 2 M urea, 1 mM EDTA, and 3% β-mercaptoethanol and heated to 100°C for 3 min prior to electrophoresis on 5.6% acrylamide gels containing 1% SDS. (a) Crude platelet sonicate, and (b) *N*-acetylglucosamine eluate, stained for protein (Coomassie blue) and carbohydrate (periodic acid-Schiff's reagent).

amounts of phospholipid may remain bound to membrane proteins during the affinity column purification, and could account for the ^{32}P labelling observed, the electrophoretic conditions used have been shown to result in complete dissociation of phospholipid from protein (Ref. 21 and see Discussion).

B. Purification of platelet glycoproteins by lithium diiodosalicylate-phenol extraction

While the affinity column system effectively purifies one of the major glycoproteins from crude platelets, we thought it essential to develop a method for extraction of all the major glycoprotein species in preparative amounts for later separation and characterization. We have attempted this using lithium diiodosalicylate, a chaotropic salt which is capable of solubilizing particulate cell fractions and in doing so, releases glycoproteins from membranes in water-soluble form. 30—50 units of outdated platelets (more than 72 h after collection) were

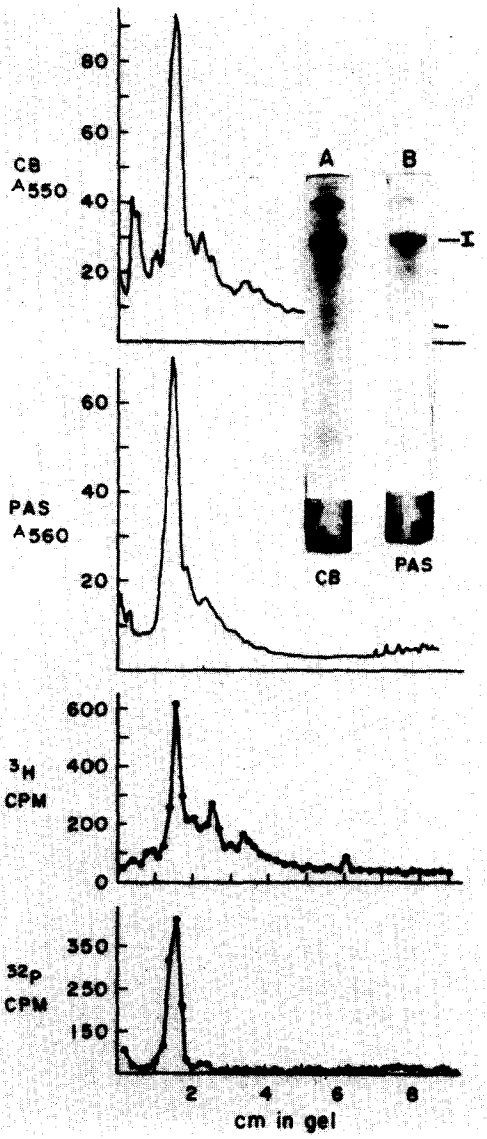

Fig. 2. SDS-acrylamide gel electrophoresis of glycoprotein from platelets labelled with NaB^3H_4 or $^{32}P_i$, purified by wheat germ-Sepharose affinity chromatography and electrophoresed as described in Methods and Fig. 1. (A) Scan of gel stained for protein. (B) Scan of gel stained for carbohydrate. (C) cpm of 1 mm slices of gels loaded with glycoprotein from $^3HBH_4$-labelled platelets. (D) cpm of 1 mm slices of gels loaded with glycoprotein from platelets labelled with $^{32}P_i$ in the presence of 20 mM unlabelled phosphate.

freed of red cells and sonicated; glycoprotein was extracted by the lithium diiodosalicylate-phenol procedure described in Methods. Electrophoresis of the lyophilized extract on SDS-5.6% acrylamide gels under conditions of sulfhydryl reduction is shown in Fig. 3A. The Schiff-stained gel shows three glycoproteins

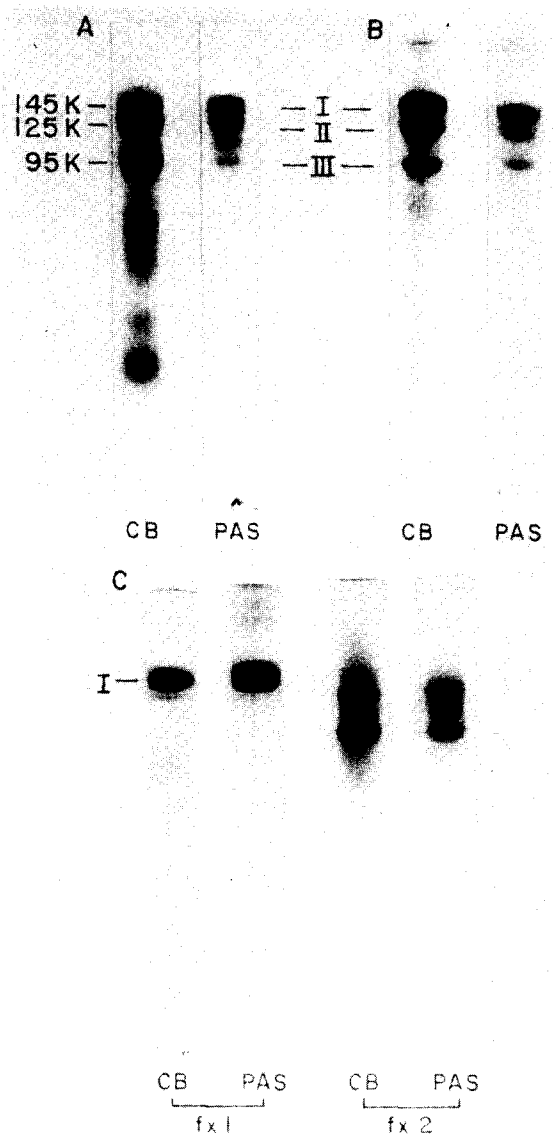

Fig. 3. SDS-acrylamide gel electrophoresis of lithium diiodosalicylate-phenol-extracted platelet glycoprotein. Conditions for solubilization and electrophoresis of sample as in Fig. 1. (A) Lithium diiodosalicylate-phenol extract of platelet sonicates. (B) Glycoprotein fraction recovered from Sephadex G-200 filtration of lithium diiodosalicylate-phenol extract in 1% SDS, 10 mM Tris, 1 mM EDTA, and 0.05% β-mercaptoethanol. (C) Glycoprotein fractions recovered from gel filtration of lithium diiodosalicylate-phenol extract on Sepharose 6B in the same buffer as in (B). Samples were stained for protein (Coomassie blue) and carbohydrate (periodic acid-Schiff's reagent).

with estimated molecular weights of approx. 145 000, 125 000 and 95 000 which were seen in all preparations. These components were associated with comparable Coomassie blue-stained bands as well as non-glycoprotein contaminants primarily of lower molecular weight. We shall term these compo-

nents GP I, GP II, and GP III, respectively. An additional Schiff-staining component migrating between GP II and III is seen here which did not appear in all preparations even after sulfhydryl reduction, and which was associated with a faint or absence stain for protein *.

Fifty units of platelets provide 1—2 g of sonicate, which yields approx. 25 mg of lithium diiodosalicylate extract, approximately 30% of which is protein as estimated by the Lowry procedure. This extraction procedure has been performed many times with comparable gel patterns, whether the platelets used were fresh, stored 24—72 h at room temperature, and when cells were stored at —20° C prior to lithium diiodosalicylate extraction.

When lithium diiodosalicylate-extracted glycoproteins are applied to Sephadex G-200 equilibrated with 1% SDS, the glycoproteins emerge near the void volume, and are separated from lower molecular weight contaminants as shown in Fig. 3B. When the extracted glycoprotein is applied to Sepharose 6B in 1% SDS, GP I is isolated (Fig. 3C fx 1). In later fractions, GP II and III elute together (Fig. 3C fx 2); we have not yet been able to separate these components satisfactorily by gel filtration. Note the present in this fraction of the Schiff-staining band between GP II and III discussed above.

Fig. 4 illustrates a combination of the lithium diiodosalicylate extraction and wheat germ affinity column purification methods applied to [^{3}H]borohydride-labelled cells. Tritiated platelets from six units of fresh blood were extracted with lithium diiodosalicylate, and the ^{3}H-labelled glycoprotein extract applied to the wheat germ-Sepharose column. It can be seen that under these conditions all three major glycoproteins are recovered although examination of the gels of the 'through' and 'wash' before application of *N*-acetylglucosamine show that wheat germ clearly has the greatest affinity for GP I.

C. Effect of lipid extraction on electrophoresis of glycoproteins

Membrane proteins which have been solubilized in detergents may have small amounts of lipid bound to them in aqueous solutions after detergent has been removed. This may affect their subsequent behavior on electrophoresis. Lithium diiodosalicylate-extracted glycoproteins were treated with ethanol and chloroform/methanol as described in Methods and electrophoresed on SDS-acrylamide gels after each step. No change in the migration patterns of glycoproteins I, II, and III was observed at any point during the lipid extraction.

D. Sulfhydryl reduction and carboxymethylation of glycoproteins

During the course of the work described above, it was noted that in some lithium diiodosalicylate-extracted preparations GP I appeared heterogeneous, and resolved poorly from GP II. This occurred in preparations derived from

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* While different terminologies have been proposed to describe platelet glycoproteins, the simple designation I, II, and III in order of decreasing molecular weight seems adequate and functional for the current work, and corresponds generally, by molecular weight estimates, to the groups of glycoproteins termed I, II and III (with subtypes a, b, c, etc.) by others. In attempting to match our isolated glycoproteins with those seen by others in membrane preparations after sulfhydryl reduction, we conclude that our GP I, II, and III probably correspond to GP Ib_{α}, IIb_{α}, and IV of Phillips and Poh Agin [29] and to GP Iab, IIbc, and IIIb (on 5% acrylamide) of George [1]. The minor component migrating between GP II and III in our preparations probably corresponds to Phillips' GP III and George's IIIa.

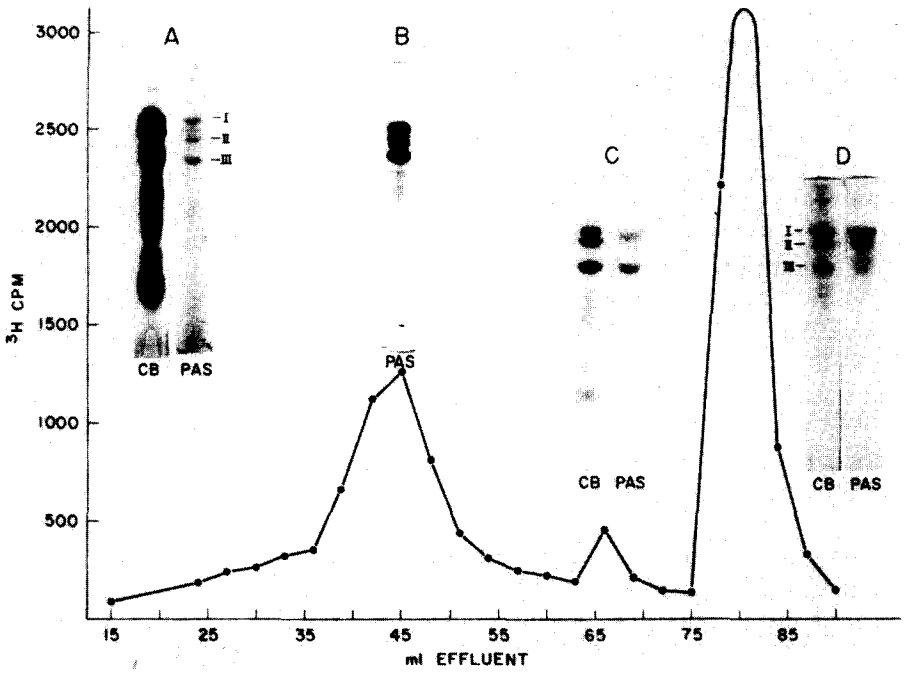

Fig. 4. Wheat germ-Sepharose affinity chromatography of tritiated lithium diiodosalicylate-extracted glycoprotein. Fresh platelets were tritiated with periodate and Na^3HBH_4 as described in Methods. A lithium diiodosalicylate-phenol extract of the tritiated platelets was solubilized in 15 mM sodium phosphate, 250 mM NaCl buffer containing 0.05% SDS and applied to the wheat germ-Sepharose column in the same buffer. The column was washed and glycoprotein eluted with 0.1 M *N*-acetylglucosamine. Lyophilized column fractions were solubilized and electrophoresed as described in Fig. 1. (A) Lithium diiodosalicylate-phenol starting material. (B) 'Through' sample (components not adherent to wheat germ-Sepharose). (C) 0.05% SDS column wash. (D) *N*-Acetylglucosamine eluate. Gels are stained for protein (Coomassie blue) or carbohydrate (periodic acid-Schiff's reagent).

both fresh and outdated platelets and was not affected by the presence or absence of a proteolytic inhibitor (PMSF) during the purification procedure. Alteration of electrophoretic conditions (e.g. decreasing SDS concentration in gels and buffer from 1% to 0.2%, omission of urea, or addition of a stacking gel) changed migration patterns only slightly and did not substantially improve the resolution of GP I in these preparations. However, omission of mercaptoethanol in the gel samples did alter the electrophoretic behavior of the glycoproteins as has been observed by others [1,2,22,23]. The effect of sulfhydryl reduction and carboxymethylation on the electrophoretic behavior of purified glycoprotein preparations is shown in Fig. 5. Comparing the Coomassie blue-stained gels in Fig. 5a and b, it can be seen that in the absence of reducing agent (Fig. 5a) a broad band is present in the GP II region and discrete GP I is not seen. Addition of β-mercaptoethanol (Fig. 5b) appears to slow the migration of a glycoprotein component which now forms a band in the GP I region incompletely resolved from GP II. This effect of mercaptoethanol has been observed repeatedly, in both disc and slab gels. No further change in migration of the glycoproteins is observed if the samples are heated to 100°C for 3 min

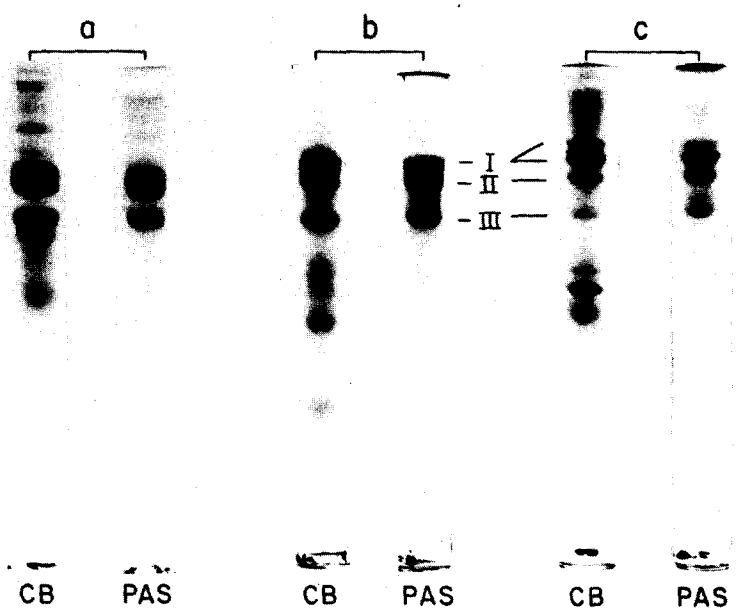

Fig. 5. SDS-acryladmide gel electrophoresis of partially purified platelet glycoprotein: effect of sulfhydryl reduction and stabilization of sulfhydryls by carboxymethylation. Lithium diiodosalicylate-extracted glycoprotein fractions were solubilized in 3% SDS, 1 mM EDTA, 10 mM Tris (a) in the absence of β-mercaptoethanol and (b) in the presence of 3% β-mercaptoethanol and electrophoresed as described in Methods and Fig. 1. Sample (c) was carboxymethylated prior to electrophoresis as described in Methods. The migration pattern shown in (c) was the same whether the sample was electrophoresed in the presence or absence of β-mercaptoethanol. All samples are stained for protein (Coomassie blue) and carbohydrate (periodic acid-Schiff's reagent).

prior to electrophoresis, if the mercaptoethanol concentration is increased to 10%, or if 20 mM dithiothreitol is substituted as reducing agent.

In order to observe the electrophoretic pattern of the glycoproteins after stabilization of sulfhydryl groups in their reduced form, carboxymethylation was performed after denaturation of glycoprotein in 8 M urea and excess β-mercaptoethanol. Electrophoresis of carboxymethylated glycoproteins (Fig. 5c) shows resolution of GP I into two components and further resolution of GP II. This pattern was seen whether the carboxymethylated sample was electrophoresed in the presence or absence of reducing agent. The electrophoretic behavior of glycoprotein which had been carboxymethylated in the absence of reducing agent was similar to that in (a) and (b), evidence that the changes observed with carboxymethylation were primarily due to stabilization of cysteine residues. The apparent molecular weights in 5.6% acrylamide of the two 'GP I' components are 165 000 and 145 000.

E. Orientation of lithium diiodosalicylate-extracted glycoproteins within the membrane

In order to establish the outward orientation of the glycoproteins isolated by lithium diiodosalicylate extraction, ten units of platelets obtained less than 24 h after collection were tritiated by the periodate-[^{3}H]borohydride reaction

as described in Methods. Tritium labelling was limited almost totally to the glycoproteins in the 90—150 000 range as demonstrated by fluorograms of ^3H sonicates electrophoresed on SDS-acrylamide under reducing conditions (not shown). Thus, the periodate oxidation procedure does not appear to induce significant cell leakiness or lysis which would result in a generalized labelling pattern. Fig. 6 shows SDS-acrylamide slab gels of tritiated glycoproteins after lithium diiodosalicylate extraction and carboxymethylation. The fluorogram shows labelling of GP I, II, and III but not of glycoprotein 165 000. The same results were obtained using fresh platelets as starting material.

In order to determine whether any of the glycoproteins isolated by lithium diiodosalicylate extend onto the internal surface of the membrane as suggested by the phosphorylation of glycoprotein of $M_r \approx 150\,000$ eluted from wheat

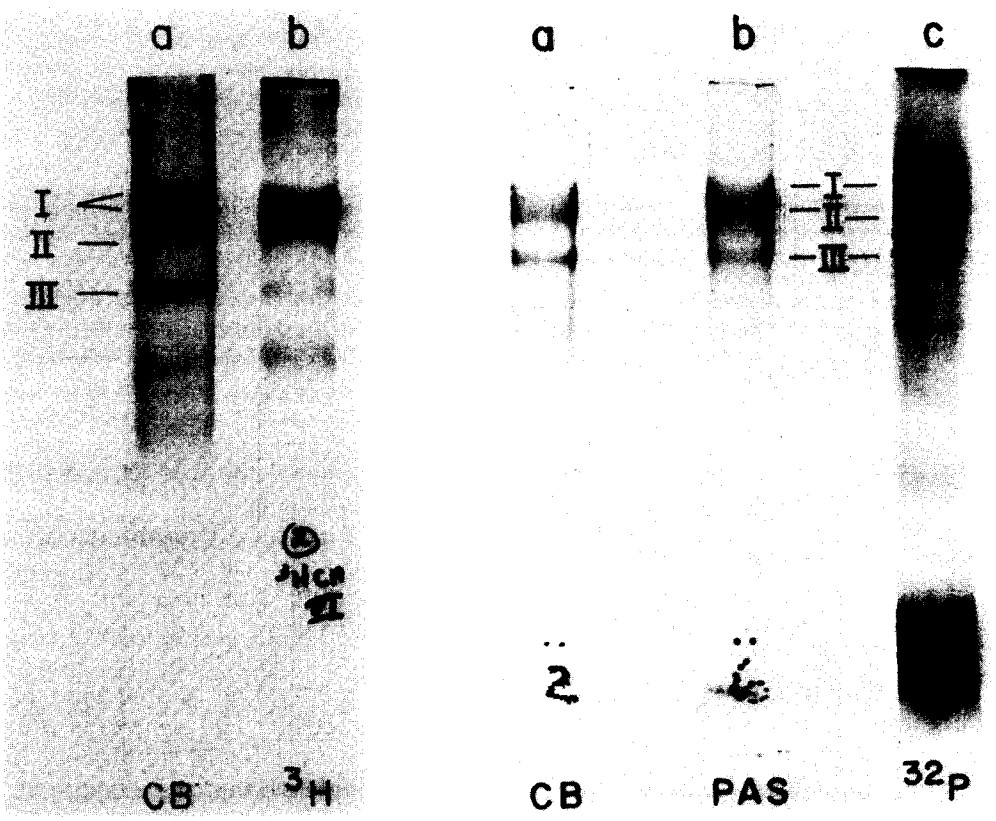

Fig. 6. SDS-acrylamide slab gel electrophoresis of lithium diiodosalicylate-phenol-extracted carboxymethylated platelet glycoproteins after periodate oxidation and NaB^3H_4 reduction of intact platelets. Solubilization of samples and electrophoretic conditions were as described in Fig. 1. Gels were stained and photographed, then treated with Me_2SO/PPO prior to autoradiography as described in Methods. (a) Coomassie blue stain. (b) Autoradiograph.

Fig. 7. SDS-acrylamide slab gels of lithium diiodosalicylate-phenol-extracted platelet glycoprotein after incubation of fresh platelets with neutralized $H_3{}^{32}PO_4$ (without carrier phosphate) at 37°C for 90 min. Solubilization of samples and conditions for electrophoresis were as described in Fig. 1. Gels were stained for (a) protein, and (b) carbohydrate; (c) is an autoradiograph of the same preparation.

germ-conjugated Sepharose, fresh platelets derived from six units of blood were incubated with [^{32}P]orthophosphate as described in Methods, washed twice and glycoprotein was extracted by the lithium diiodosalicylate-phenol method. Fig. 7 shows that the major glycoproteins isolated are phosphorylated. Carboxymethylation was not performed on this preparation, thus GP I and II are not completely resolved, and the glycoprotein 165 000 is not seen. The heavy band of radioactivity towards the bottom of the gel represents phospholipid which copurifies with the glycoprotein but which dissociates under the electrophoretic conditions used (Ref. 21, and see Discussion).

Discussion

The recent surge of interest and publication in the field of platelet membrane glycoproteins has led to the description of increasing numbers of glycoprotein species and has, at the same time, produced a complicated and sometimes conflicting nomenclature. Interpretation and correlation of these studies has been hampered somewhat by the fact that in most instances the glycoproteins have not been separated from the platelet membrane, so that parallel gels stained for protein are not evaluable.

Initially, three or four major glycoprotein species were described with molecular weights ranging between approximately 90 000 and 150 000, based on one dimensional SDS-acrylamide gel electrophoresis of crude membrane fractions stained for carbohydrate with Schiff stain [24–26]. Surface labelling of platelets with ^{125}I prior to preparation and electrophoresis of membranes established the surface orientation of GP II and III [1,3,25,27,28]. More recently, labelling of surface carbohydrate moieties by treatment of whole platelets with galactose oxidase [29,30] or periodate [30], followed by borotritide reduction, has resulted in labelling of at least five components in membrane preparations [29] and as many as eleven components in solubilized whole platelets [30].

In a study by George [1], sulfhydryl reduction of iodinated membrane preparations before electrophoresis was found to change the mobility of all three major glycoprotein groups. Phillips and coworkers [2], using two-dimensional electrophoresis of ^{125}I-labelled membranes, counted six glycoprotein species, all but one of which changed its electrophoretic migration after sulfhydryl reduction. These authors concluded from their observations that the reduction process involved cleavage of both inter- and intramolecular disulfide bonds.

In addition to the complexity of the findings summarized above, current studies also indicate that there are several species of glycoprotein included in the 'GP I' region as defined on SDS-acrylamide gels. The studies by Okumura and coworkers [3,4] suggest that GP I is composed of an integral membrane component and a 'releasable' components (glycocalicin) which have similar electrophoretic mobility. Other studies suggesting the 'GP I' is heterogeneous are based on two-dimensional electrophoresis [2] and lectin binding studies [22]. Solum and coworkers [5], comparing electrophoresis in SDS of whole platelets, membranes, and granule fractions solubilized under reducing conditions, concluded that 'GP I' consists of three components including glycocalicin, an integral membrane protein, and a granule glycoprotein of slightly higher molecular weight than the membrane components.

The present studies were initiated in the expectation that purification of platelet surface glycoproteins will be a major step toward clarifying some of the complex observations summarized above conerning the origin, number, orientation, and sulfhydryl composition of these molecules.

The approaches to purification of the glycoproteins were those that had previously been used successfully in purification of red cell membrane glycophorin, including extraction with the chaotropic salt lithium diodosalicylate [31] and lectin affinity column chromatography [19]. Nachman et al. [20] first applied the lithium diiodosalicylate extraction method to platelet membranes after iodination of whole platelets and obtained a radiolabelled glycoprotein of $M_r \approx 100\,000$. In the current study, the lithium diiodosalicylate extraction procedure consistently isolated three glycoprotein species from platelet sonicates under standard sulfhydryl-reducing conditions but with further resolution of a fourth component after carboxymethylation (see below). Varying amounts of non-glycoprotein contaminants were also extracted which could be removed by gel filtration in SDS. In addition, a slow moving glycoprotein component ($M_r \approx 210\,000$) was seen in small amounts, as well as the occasional observation of a Schiff-staining band between GP II and III; this material stained very faintly on parallel Coomassie blue-stained gels (Fig. 3A and C; Fig. 5C). (See footnote p. 451.)

It was noted after many lithium diiodosalicylate-phenol extracts had been prepared that GP I was frequently smeared on both Coomassie blue and Schiff-stained gels, and appeared to consist of two or more components. We determined that this was not related to the time of platelet storage before use, nor to the presence or absence of proteolytic enzyme inhibitors during sonication and extraction of glycoprotein, nor was it affected by varying the electrophoretic conditions (see Results, section D). However, the presence of sulfhydryl-reducing agents in the sample before electrophoresis did affect resolution of the components in the GP I region and the heterogeneity observed was thought to be due to incomplete and variable sulfhydryl reduction of glycoprotein components even after heating samples to 100° C for 3 min in 10% β-mercaptoethanol. As shown in Fig. 5, irreversible sulfhydryl reduction of glycoprotein preparations by carboxymethylation in the presence of β-mercaptoethanol resolves the GP I region on SDS-acrylamide gels into two components with apparent molecular weights of approximately 145 000 and 165 000. Sulfhydryl reduction and carboxymethylation had no effect on the migration of GP II and III. These findings differ from those obtained by others in membrane preparations and other subcellular fractions [1,2,22,23], and may reflect either, (1) interactions between glycoprotein and non-glycoprotein components in crude subcellular preparations which do not occur in isolated glycoprotein fractions, or (2) the presence in these preparations of glycoprotein components not extracted by our methods.

The second method used for isolation of the glycoproteins, that of wheat germ-conjugated Sepharose affinity chromatography, was based on the known binding of lectins by cell surface glycoproteins. The binding of lectins by intact platelets was first described in 1972 by Majerus and Brodie [32]. In these studies the binding of erythroagglutinating phytohemagglutinin produced typical aggregation and release with accompanying inhibition of adenyl cyclase

activity. More recently, Nachman et al. [33] have recovered GP I (approx. 150 000 daltons) and a slowly migrating glycoprotein (approx. 210 000 daltons) from wheat germ-conjugated Sepharose, while Clemetson et al. [22], in a study of several types of lectin affinity systems, describe elution of 'GP I_a' and 'GP I_b' from wheat germ agglutinin-Sepharose when the eluate is electrophoresed under non-reducing conditions and a single GP I after reduction. In the current study, chromatography on wheat germ agglutinin-Sepharose of platelets solubilized in SDS yield a glycoprotein of approx. 150 000 (in the presence or absence of sulfhydryl-reducing agents) and a small amount of glycoprotein 210 000 (see Fig. 2A) as described by Nachman et al. [33].

When lithium diiodosalicylate-phenol-extracted glycoprotein preparations were applied to the wheat germ agglutinin-Sepharose column (Fig. 4) GP II and III were bound and eluted with GP I by *N*-acetylglucosamine, although the binding of GP II and III was weaker than that of GP I. This is possibly due to the fact that when GP II and III are concentrated in relation to contaminating proteins, they compete more efficiently for binding to the wheat germ. In any case, combining the two purification methods produces a mixture of GP I, II, and III with almost complete removal of non-glycoprotein contaminants. This preparation was not carboxymethylated and thus we cannot comment upon whether it contained the 165 000 molecular weight component described above.

In order to study the orientation of the isolated platelet glycoproteins in relation to the platelet surface, intact platelets were tritiated by the periodate-borotritide method which, under the conditions used, results primarily in labelling of externally oriented sialic acid residues [34]. Glycoprotein of $M_r \approx 150\,000$ separated by wheat germ agglutinin-Sepharose was labelled by this method, as were glycoprotein 145 000, glycoprotein 125 000 and glycoprotein 95 000 isolated by the lithium diiodosalicylate-phenol procedure suggesting that these are all surface-oriented membrane proteins. As shown in Fig. 5, however, the glycoprotein 165 000 resolved by carboxymethylation of this preparation was not labelled, suggesting that it may be the granule protein described by Solum et al. [5] or that it is a membrane protein without sites available for surface labelling. Our findings to date suggest that glycoprotein of $M_r \approx 150\,000$ isolated on wheat germ agglutinin-Sepharose represents GP I isolated by lithium diiodosalicylate-phenol extraction, but this will require confirmation by carboxymethylation of wheat germ agglutinin-Sepharose-purified glycoprotein.

Studies of erythrocyte membranes suggest that several membrane proteins, including the major surface glycoprotein, traverse the bilipid layer of the membrane and bear reactive groups on both its inner and outer surfaces [35]. Phosphorylation of intact platelets by $^{32}P_i$ was undertaken in the current studies in order to determine whether glycoproteins known to have surface orientation could be labelled by this technique, which would suggest that a polypeptide tail of each glycoprotein molecule labelled extends onto the cytoplasmic surface of the membrane. Such membrane proteins might be capable of translating information received on the outer surface of the cell to soluble cytoplasmic enzyme systems which might then result in a specific response by the cell.

While incubation of platelets with $^{32}P_i$ is known to label many cytoplasmic

proteins, labelling of specific membrane proteins has not been reported. The work reported above (Figs. 2 and 7) shows that under the conditions used, phosphorylation of fresh intact platlets with $^{32}P_i$ results in labelling of glycoproteins purified by both wheat germ agglutinin-Sepharose and lithium diiodosalicylate-phenol techniques. While it is well known that purified membrane components may have phospholipid bound to them, it has been shown in phosphorylation studies of red cell membranes [21] that treatment with SDS dissociates even the most tightly bound phospholipid from membrane glycoprotein; thus the radiolabel migrating with the platelet glycoprotein on 1% SDS-acrylamide gels is thought not to be due to bound phospholipid. Taken together with the surface labelling studies reported here for the same glycoproteins, the results of the phosphorylation studies suggest a transmembrane orientation for these platelet glycoproteins. Further phosphorylation studies are planned to improve resolution of glycoprotein components (e.g. by carboxymethylation and use of the Laemmli gel system [36]), and also to confirm the presence of labelled phosphoserine and phosphothreonine in the glycoprotein preparations.

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