Human platelet membranes were solubilized in 1% TritonX-100 and subjected to crossed immunoelectrophoresis in 1% agarose, employing a rabbit anti-human platelet membrane antibody in the second dimension.

If membranes are extracted in the absence of divalent cation chelating agents, the major antigen, designated 10, remains completely intact. If EDTA or ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid is employed in the extraction buffer, or citrate, EDTA, or ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid in the electrophoresis well, the major antigen dissociates into four different antigens: 13 and 18 which react with Concanavalin A and have similar electrophoretic mobilities, and 15 and 16 which are more anodal and have similar electrophoretic mobilities. This process is completely reversed by addition of excess CaCl₂ to the extraction buffer or the electrophoresis well. Similar results were obtained in the absence of chelating agents following dialysing for 60–200 h against "Ca²⁺-free" buffer prepared with BioRex 70. Autoradiography of platelet membranes prepared from iodinated washed platelets revealed incorporation of 125I into 10, 14, and 18, demonstrating their surface location. The major antigen reacts with antiserum against GPIIb and GPIIIa. After dissociation of the complex, anti-GPIIb reacts with 15–16, whereas anti-GPIIIa reacts with 13–18.

Platelet membranes from five patients with Glanzmann’s thrombasthenia have been studied in the presence of 2 mM EDTA; two have also been studied without EDTA. All four components of the major antigen are missing. These data indicate that the major antigen which is absent in Glanzmann’s thrombasthenia is composed of components containing four different immunoprecipitates which are held together by Ca²⁺: 15–16 which react with anti-GPIIb, and 13–18 which react with anti-GPIIIa. It is suggested that patients with Glanzmann’s thrombasthenia have a deficient membrane binding site for Ca²⁺ or a deficient membrane (glyco)protein which anchors these components to the membrane.

In a previous publication on crossed immunoelectrophoresis of human platelet membranes (1), we fairly consistently observed surface antigens in normal subjects. These included the major antigen, designated 10 by us (16 by Hagen et al. (2)), 1F (identified as fibrinogen), 12A (identified as albumin), 4, 5, 7–8, 9, 13, 14, and 18; as well as nine other antigens not observed as consistently. Four antigens (1F, 10, 13, and 18) reacted with Concanavalin A, indicating that they were glycoproteins (whereas at least six antigens did not).

Of particular interest was the association of the glycoprotein antigens 13 and 18 with 10. When the cathodal arm of 10 was missing or partially absent, antigens 13 and 18 were increasingly present. Furthermore, 10, 13, and 18, as well as 1F, were missing in three patients with Glanzmann’s thrombasthenia (a disorder in which platelets do not aggregate with physiologic aggregating agents). Absence of the major antigen has also been reported by Hagen et al. (3) in two additional patients. We suggested that 10, 13, and 18 might exist as a complex within the membrane. It is now apparent that these three antigens, as well as two other antigens, 15 and 16, do indeed exist as a complex, which is held together by Ca²⁺. Antigens 13 and 18 have similar electrophoretic mobilities and react with an antibody against platelet membrane glycoprotein IIIa. Antigens 15 and 16 are more anodal, also have similar electrophoretic mobilities, and react with an antibody against glycoprotein IIb.

Our present communication demonstrates that the separation of 10 into 13, 18, 15, and 16 on CIE is due to the presence of EDTA in the extraction buffer. Similar results can be obtained with EGTA or citrate. The separated antigens can be reassociated by addition of Ca²⁺ to the extraction buffer or the immunoelectrophoresis well. All four separated antigens are missing in five patients with Glanzmann’s thrombasthenia.

**EXPERIMENTAL PROCEDURES**

**Methods**

Preparation of Platelet Membranes—Platelet-rich plasma was obtained from the New York Blood Center and centrifuged at 2,800 × g for 15 min at 4 °C. The platelet pellet was washed in a human Ringer’s solution (4) containing 2 mM EDTA, 10 mM benzamidine, 100 µg/ml of soybean trypsin inhibitor, and 1% ammonium oxalate as described previously (1). Platelet membranes were prepared as described previously (1) by four cycles of freezing and thawing, followed by sonication and centrifugation on a cushion of 30% sucrose dissolved in 0.01 M Tris buffer, pH 7.4, containing 1 mM EDTA, 0.15 M NaCl, 10 mM benzamidine, and 100 µg/ml of soybean trypsin inhibitor. The membranes were washed once in 0.15 M NaCl, 10 mM benzamidine, 100 µg/ml of soybean trypsin inhibitor, centrifuged at 100,000 × g for 1 h at 4 °C, and solubilized.

*Antiserawere exchanged by our group and Hagen et al. (2). The major antigen designated 10 by Shulman and Karpatkin (1) and 16 by Hagen et al. was shown to be the same.

* The abbreviations used are: CIE, crossed immunoelectrophoresis; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Solubilization of Platelet Membranes with Triton X-100—Platelet membranes were adjusted to approximately 5 mg/ml of protein after extraction with 1% (v/v) Triton X-100 containing 0.07 M Tris, 0.02 M sodium barbital buffer, pH 8.6, 10 mM benzamidine, 100 µg/ml of soybean trypsin inhibitor with or without a divalent cation chelating agent (EDTA or EGTA). The solution was stored at −20°C until use.

The platelet membranes of five patients with Glanzmann’s thrombasthenia, M. C., M. Mo., N. Mo., L. M., and M. Ma., were prepared in a similar fashion with blood anticoagulated with acid/citrate/dextrose; citrate/phosphate/dextrose, or EDTA. Rabbit antipeptide membrane antibody was utilized in crossed immunoelectrophoresis as described previously (1).

Iodination of Washed Platelets—This was performed by the lactoperoxidase method, as described by Phillips (5). Stained agarose slides were dried by air, stored next to Kodak x-ray film for 5 days, and processed as suggested by the manufacturer.

Materials

These were obtained as described previously (1). EDTA and lactoperoxidase were obtained from Sigma. BioRex was obtained from Bio-Rad. Na[125I] (17 Ci/mg) was obtained from New England Nuclear. Anti-human platelet glycoprotein antibodies against GPIIb and GPIIIa were kindly supplied by Dr. Ralph Nachman of Cornell Medical Center, New York (6).

RESULTS

All platelet preparations were washed in a human Ringer’s solution containing 2 mM EDTA.

Effect of EDTA, EGTA, or Sodium Citrate—If the membrane extraction buffer contained 1 mM EDTA, the CIE pattern obtained (Fig. 1b) was similar to that previously described (1). Antigens 13, 18, 15, and 16 were visible. If the extraction buffer did not contain EDTA (Fig. 1a) or contained 1 mM EDTA plus 4 mM CaCl₂, the major antigen remained intact (Fig. 1e). Other antigens are relatively unchanged. Similar results were obtained with EGTA, but more pronounced (Fig. 1f). Addition of 4 mM CaCl₂ to the extraction buffer containing 1 mM EGTA prevented dissociation (data...
not shown), whereas addition of 4 mM MgCl₂ had no effect (same pattern as in Fig. 1/). Extraction in the presence of 4 mM CaCl₂ (in the absence of EDTA or EGTA) had no effect on the pattern of Fig. 1a (data not shown). The assembled major antigen (extracted in the absence of chelating agent) could also be dissociated by addition of 2 mM EGTA to the electrophoresis well (data not shown). Similar results were noted when 25 mM sodium citrate was added to the well (Fig. 1g).

When the major antigen was dissociated with EDTA, it could be reassociated by addition of 2 mM CaCl₂ to the electrophoresis well, Fig. 2, a and b. Similar results were obtained with 2 mM MnCl₂, MgCl₂, or ZnCl₂ (data not shown).

In an attempt to determine the minimum amount of chelating agent required per mg of membrane protein, 5 mg/ml of platelet membrane was extracted at varying EGTA concentrations. The earliest changes were noted at 0.05 and 0.1 mM EGTA (data not shown). This represented as little as 0.5-1.0 nmol of EGTA/50 μg of membrane protein in the electrophoresis well.

**Effect of Dialysis against “Ca²⁺-free” Buffer**—To determine whether the major antigen could be dissociated without divalent cation chelating agents, the platelet membranes were extracted in a Ca²⁺-free buffer, prepared from buffer passed through a BioRex 70 cation exchange resin, and dialyzed against the same buffer for 60-200 h. Although no change was noted after 60 h (Fig. 3a), changes were noted after 180 h of dialysis (Fig. 3b), which were partially reversed by addition of 200 nmol of CaCl₂ to the electrophoresis well (Fig. 3c). Following 204 h of dialysis, further splitting of the major antigen was noted. However, this could not be reversed by addition of 200 nmol of CaCl₂ to the well, by incubation of 50 μg of the membrane preparation with 200 nmol of CaCl₂ at 37 °C for 15 min, or by dialysis for 72 h against the same buffer with 4 mM CaCl₂. Control membranes maintained at 4 °C for 204 h gave a normal CIE pattern similar to Fig. 3a.

**Effect of Antibodies against GPIIb and GPIIIa on the Associated and Dissociated Complex**—A rabbit anti-human platelet membrane antibody from a different rabbit than that previously employed was used for the studies on Fig. 4. Antibodies against GPIIb and GPIIIa were placed in an intermediate spacer gel. In the absence of EGTA (associated complex), anti-GPIIb antibody reacted with the cathodal arm of the major antigen (retarded its upward movement); whereas anti-GPIIIa reacted with both the anodal and cathodal arms (Fig. 4, a-d). In the presence of 10 mM EGTA (dissociated complex), anti-GPIIb reacted with 15-16 and had no effect on 13-18 (Fig. 4e and f). Anti-GPIIIa reacted with 13-18 and residual 10, and had no effect on 15-16 (Fig. 4e and g).

** Autoradiography of Washed Intact Platelets**—Platelets were iodinated and membranes prepared and solubilized in 2 mM EDTA as described under “Methods.” Fig. 5 indicates the autoradiogram obtained from the CIE pattern. Antigens 10, 13, and 18 are clearly visible, indicating their surface location.

** Patients with Glanzmann’s Thrombasthenia**—The major antigen, 10, as well as 13, 18, 15, and 16 are absent from the platelet membranes of three patients with Glanzmann’s thrombasthenia when utilizing membrane extraction buffer containing 2 mM EDTA. Two additional patients were studied with and without 2 mM EDTA in the extraction buffer. In both sisters, N. Mo. and M. Mo., the major antigen as well as its four components are absent, Fig. 6, a-c.

** DISCUSSION**

CIE has only recently been used in the study of platelet membranes (1-3). It has several advantages over SDS-PAGE: membrane proteins are not completely denatured so that intrinsic biologic activity can often be assayed; it is more sensitive than SDS-PAGE; it is quantitative, since the peak areas of individual immunoprecipitates are proportional to antigen/antibody ratios; comparative studies of antimembrane antibody absorbed with whole cells enable relative surface location studies; and lectins can be employed as spacer gels, providing information regarding lectin binding of surface antigens.

The employment of CIE, rather than SDS-PAGE, allowed us to postulate an association between the major antigen 10 and 13 and 18 (1); when the cathodal arm of 10 was missing or partially absent, antigens 13 and 18 were increasingly present. In our previous report we suggested that this might be due to proteolysis since membranes stored in the absence of protease inhibitors and membranes treated with chymotrypsin had more of this shift of the major antigen 10 to 13 and 18. Our present data indicate that this shift can also be modulated by Ca²⁺ chelating agents, and that under these conditions it is
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**Fig. 4.** Effect of antibodies against GPIIb and GPIIIa on the major antigen complex. *a.* associated complex extracted in the absence of EGTA, with buffer in spacer gel; *b.* same as *a.* with 50 μl/ml of rabbit anti-GPIIIa in spacer gel; *c.* same as *a.* different membrane preparation; *d.* same as *c.* with 60 μl/ml of rabbit anti-GPIIb in spacer gel; *e.* dissociated complex with 10 mM EGTA in the electrophoresis well and buffer in the spacer gel; *f.* same as *e.* above, with 200 μl/ml of rabbit anti-GPIIb in spacer gel; and *g.* same as *e.* with 150 μl/ml of rabbit anti-GPIIIa in spacer gel.

Thus, extraction (solubilization) of platelet membrane in the presence of EDTA or EGTA results in splitting of the major antigen into 13 and 18, glycoproteins which react with Concanavalin A (1), and two other membrane antigens, 15 and 16. If platelet membranes are extracted in the absence of Ca$^{2+}$, the major 13 or 18 can be dissociated into two fractions, 13 and 18, which can be recombined as shown in the figure.

**Fig. 5.** Autoradiogram and Coomassie brilliant blue CIE patterns of platelet membranes solubilized in the presence of 2 mM EDTA. Washed platelets were incubated with Na$^{125}$I, lactoperoxidase and H$_2$O$_2$ as described (5). Platelet membranes were prepared as described under "Methods." *a.* Coomassie pattern, *b.* autoradiogram.
of chelating agents or in the presence of EDTA or EGTA and an excess of Ca\(^{2+}\), the major antigen, 10, remains intact, and 13, 18, 15, and 16 are not visible. Furthermore, the dissociated complex can be reassociated by addition of Ca\(^{2+}\) to the electrophoresis well. In intact platelets the Ca\(^{2+}\)-membrane complex is not easily accessible to Ca\(^{2+}\)-chelating agents since washing of the platelets with a buffer containing 2 mM EDTA, or incubating them for 10 min at 37 °C in the presence of 5 mM EDTA does not result in dissociation of the antigen; dissociation requires EDTA in the Triton-extraction buffer.

These findings suggest that the loss of platelet aggregability which occurs after incubation with EDTA (7) may not be caused by permanent dissociation of the major antigen.

These data therefore indicate that four surface membrane antigens are held together by divalent cation. (Surface components were determined with intact platelets by antibody adsorption and enzyme treatment experiments (1) and by iodination experiments.) Since EGTA was more potent than EDTA, and the splitting of the major antigen by EGTA could be reversed by Ca\(^{2+}\) but not Mg\(^{2+}\), it is most likely that the divalent cation is Ca\(^{2+}\). The possibility that EDTA per se may be responsible is unlikely because extraction in EGTA, exposure to citrate, or dialysis against Ca\(^{2+}\)-free buffer are also capable of splitting the major antigen.

Of particular interest is the report of Hagen et al. (3) that the major antigen, when run on SDS-PAGE, is composed of two proteins with molecular weights similar to glycoproteins IIb and IIIa. Our four antigens have two electrophoretic positions on CIE in the first dimension and therefore could represent anywhere from two to four different (glyco)proteins. Antisera against GPIIb and GPIIIa reacted with the associated major antigen complex, as well as with specific components of the dissociated complex. Anti-GPIIIb reacted with 15–16 and anti-GPIIIa reacted with 13–18. These data indicate that GPIIb and GPIIla are part of the major antigen complex, confirming and extending the observation of Hagen et al. (3) whose proof was by molecular weight determination. The presence of four immunoprecipitates rather than two could represent heterogeneity of carbohydrate moieties or other undefined glycoproteins (8) which could conceivably bind to GPIIb or GPIIla. While our work was submitted,2 Kunicki et al. (9), employing crossed immunoelectrophoresis, published similar observations on the presence of a Ca\(^{2+}\)-major antigen complex, composed of GPIIb and GPIIIa. Glycoproteins IIb and IIIa are missing or diminished in patients with Glanzmann’s thrombasthenia (10, 11) and react with Concanavalin A (8). These data are thus compatible with our observation (1), as well as that of Hagen et al. (3), that the major antigen is missing in Glanzmann’s thrombasthenia and reacts with Concanavalin A (1). The major antigen was absent in the platelet membranes of two patients when their platelets were extracted in the absence as well as the presence of EDTA. In retrospect, this could have been predicted from Fig. 10b of our previous report (1), wherein antibody absorption studies with platelets from a patient with Glanzmann’s thrombasthenia resulted in the disappearance of all antibodies detecting platelet membrane antigens, except 10, 13, 18, 15, 16, and 1F (fibrinogen). It is likely that the defect in Glanzmann’s thrombasthenia is more basic than the relative absence or complete absence of glycoproteins IIb and IIIa as determined by SDS-PAGE. We would like to postulate that the defect may consist of the absence or impairment of a binding site for the Ca\(^{2+}\)-membrane complex of 13, 18, 15, 16, and perhaps, 1F. Indeed, Peerschke et al. (12) have recently reported 50% impaired binding of external Ca\(^{2+}\) to platelets of patients with Glanzmann’s thrombasthenia. The relationship of platelet surface fibrogen to this complex is unclear. Fibrogen is also decreased or absent in Glanzmann’s thrombasthenia, but it is clearly a separate immunoprecipitate on CIE of normal platelet membranes in the absence of EDTA. Therefore, it does not appear to be associated with the Ca\(^{2+}\)-membrane complex of 13, 18, 15, 16, and perhaps, 1F. Indeed, Peerschke et al. (12) have recently reported 50% impaired binding of external Ca\(^{2+}\) to platelets of patients with Glanzmann’s thrombasthenia. The relationship of platelet surface fibrogen to this complex is unclear. Fibrogen is also decreased or absent in Glanzmann’s thrombasthenia, but it is clearly a separate immunoprecipitate on CIE of normal platelet membranes in the absence of EDTA. Therefore, it does not appear to be associated with the Ca\(^{2+}\)-membrane complex.

The possible role of Ca\(^{2+}\) in platelet membranes (12–15) or other membranes is of interest. Ca\(^{2+}\) appears to be required for cellular adhesion (16–19). For example, cells dissociate from each other in calcium-free solutions and reassociate following addition of Ca\(^{2+}\). It has been suggested that Ca\(^{2+}\) functions as an adhesive between cells by chemically binding membrane components of one cell to those of another (16). Ca\(^{2+}\) also appears to be required for membrane "hardening" and resiliency (16). In this respect, Ca\(^{2+}\) appears to affect membrane permeability; lack of Ca\(^{2+}\) increases the permeability of various tissues to water (17, 20, 21), electrolytes (22, 23) and other intracellular contents (22, 24). Of further interest is the observation that membrane components are lost from Haemophilus parainfluenzae by treatment with EDTA; these components can be recovered from the medium (25).

How does this relate to the calcium complex of IIb and IIIa in the platelet membrane? It is conceivable that platelets adhere to each other via Ca\(^{2+}\) bridging between these com-

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plexes and fibrinogen, following membrane perturbation. Indeed, it is of interest that platelets from patients with Glanzmann’s thrombasthenia which do not have this membrane Ca$^{2+}$ complex also do not aggregate with each other when exposed to physiologic aggregating agents; yet they do undergo the release reaction and do appear to have membrane receptors (26, 27). We would therefore like to suggest that this complex of the platelet membrane may be required for platelet-platelet bridging and aggregation.

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