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COMPARISON OF PROTEIN AND LIPID COMPOSITION OF THE HUMAN PLATELET α-GRANULE MEMBRANES AND GLYCEROL LYSIS MEMBRANES

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Platelet glycerol lysis membranes and α-granule membranes were compared with respect to protein and lipid composition. Crossed immunoelectrophoresis using antibodies against whole platelets, and sodium dodecyl sulphate polyacrylamide gel electrophoresis, revealed the presence of the glycoproteins IIb and IIIa, myosin and an antigen termed G4 in both membrane fractions. The glycoproteins Ia, Ib and IIIb, in addition to β_2-microglobulin and actin, appeared specific for the glycerol lysis membranes, whereas two antigens, termed G8 and G18, were observed only in the α-granule membranes. The localization of glycoprotein IIa was inconclusive. Comparison with the surface-located proteins revealed that the glycerol lysis membranes represented a reasonable approximation to a plasma membrane preparation. Radioactively labelled immunoprecipitates obtained after crossed immunoelectrophoresis of ^{125}I-labelled platelets were cut out and applied to sodium dodecyl sulphate electrophoresis on polyacrylamide slab gels. Autoradiography of the dried gels revealed that antigen G4 represented a protein with an average molecular weight of 146000 in its unreduced state and 132000 in its reduced state. Antigen G18 represented a protein of molecular weight 130000–135000 in the reduced as well as unreduced state. Quantitation of protein and lipids showed that the α-granule membranes contained about one-third as much cholesterol and 2-times as much protein in relation to phospholipids as compared to the glycerol lysis membranes. No significant difference between the two membrane preparations was found as regards the composition of their phospholipids.

Introduction

The release of intracellularly stored material is a central process in the platelet response to stimulating factors. The predominent storage organelles are the so-called α-granules and the dense bodies. The α-granules appear to be the main source of those proteins which are secreted during the release reaction [1]. The function of the various α-granule proteins is not fully understood. However, functions related to haemostasis are indicated by the finding that the bleeding disorder known as the Gray platelet syndrome is characterized by a strongly reduced number of α-granules [2–5]. The α-granule components may participate in haemostasis in more ways. Released, soluble proteins may alter the surface of the releasing platelet [6–9], neighbouring platelets, other blood cells or endothelial cells, or interact with plasma proteins. The non-releasable α-granule membrane proteins which are transferred to the surface membrane by exocytosis [1] may alter surface-related platelet functions. We have previously characterized the protein composition of isolated α-granules and analyzed the distribution of proteins between their membranes and soluble contents [1,10]. In the present study, α-granule membranes are compared to platelet glycerol lysis

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membranes, which appear to resemble the plasma membranes in more respects.

Materials and Methods

Chemicals. Agarose, type HSA was from Litex, Glostrup, Denmark; Triton X-100, molecular weight standards for sodium dodecyl sulphate polyacrylamide gel electrophoresis, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and cholesterol, from Sigma Chemical Company, St. Louis, U.S.A.; human fibrinogen and human albumin from Kabi, Stockholm, Sweden. Thin-layer chromatography plates, Silica H, were from Merck, Darmstadt, F.R.G., Nycotest cholesterol, enzymatic reagent for determination of cholesterol, from Nyegaard & Co., A/S, Oslo, Norway and dye-reagent concentrate for protein determinations, from Bio-Rad Laboratories, Richmond, U.S.A.

Radioimmunoassay kits. The radioimmunoassay for the determination of β-thromboglobulin was obtained from Amersham International, U.K. The radioimmunoassay for the determination of platelet factor 4 was from Abbott Laboratories, U.S.A.

Antibodies. Antibodies to whole human platelets were obtained as described by Hagen et al. [11] and antibodies specific to the glycoprotein IIb-IIIa complex were obtained as described previously [12]. Antibodies to human albumin, human fibrinogen and human factor VIII-related antigen were obtained from Behringwerke AG, F.R.G. Antibody to β_2-microglobulin was from Dako, Copenhagen, Denmark.

Blood. Blood was collected from healthy, registered blood donors and anticoagulated with citrate-phosphate-dextrose solution (27.7 mM citric acid/ 142 mM disodium citrate/ 25.5 mM NaH_2PO_4/204 mM D-glucose) to a final concentration of 12.3%.

Platelets. Platelets were isolated and washed as described elsewhere [13].

Glycerol lysis membranes. These membranes were prepared according to Barber and Jamieson [14]. Washed platelets were layered upon 0–40% glycerol gradients containing 20 mM Tris-HCl buffer (pH 7.4)/0.154 M NaCl/5 mM D-glucose/ 0.6 mM EDTA and centrifuged at $1500 \times g_{av}$ for 30 min plus $5400 \times g_{av}$ for 10 min. The sedimented platelets were suspended in 2 ml of 40% glycerol containing the components listed above, and the suspension was rapidly diluted 10-fold with 0.27 M sucrose/2 mM EDTA. The suspension was layered upon 2 ml 30% sucrose/2 mM EDTA and centrifuged for 3 h at $60000 \times g_{av}$ (Rotor 40, Beckman Instruments). The interphase which contained the membranes were collected, sedimented by centrifugation for 2 h at $114000 \times g_{av}$ (Rotor SW 55Ti, Beckman Instruments) and finally washed by resuspension in 0.27 M sucrose and centrifugation as above.

α-Granules. The isolation of α-granules was as described elsewhere [13].

α-Granule membranes. These were prepared by ultrasonication of suspensions of isolated α-granules (3 mg protein/ml) in 0.27 M sucrose for six periods of 10 s each with 30-s intervals for cooling on an ice/NaCl mixture. The membranes were sedimented by centrifugation at $75000 \times g_{av}$ for 2 h (Rotor SW 55Ti, Beckman Instruments) and washed by resuspension in 0.27 M sucrose followed by centrifugation as above.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as described by Hagen et al. [11]. The membranes were solubilized in 0.038 M Tris/0.1 M glycine buffer (pH 8.7)/1% Triton X-100 to about 0.5 mg protein/ml.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed according to Laurell [15] as described by Weeke [16], modified by the inclusion of 0.5% Triton X-100 in the samples and the gels.

Surface labelling of platelets with ^{125}I. ^{125}I-labelling was performed with the lactoperoxidase-catalyzed iodination according to Phillips [17].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Electrophoresis was performed according to Laemmli [18] using a stacking gel of 3.5% acrylamide and a separating gel of 7.5% acrylamide. The ratio between bisacrylamide and acrylamide was 1 : 40 in both gels.

The membrane preparations were solubilized in 62.5 mM Tris-HCl buffer (pH 6.8)/10% glycerol/ 2% sodium dodecyl sulphate. Unreduced samples were incubated at 100°C for 5 min in the presence of 5 mM *N*-ethylmaleimide, whereas reduced samples were obtained by incubation with 2% 2-mercaptoethanol.

For the analyses of antigens in immunoprecipitates, ^{125}I-labelled immunoprecipitates were cut out from stained immunoplates by comparison to autoradiograms. The stained and dried immunoplates were soaked in distilled water for 1 min and identical precipitates from 3–5 immunoplates were scraped off and pooled. The agarose films were incubated with the sample buffer as described above.

The samples containing membrane proteins were applied to cylindrical gels (diameter, 5 mm), whereas heated samples containing the solubilized immunoprecipitates were applied to individual wells on polyacrylamide slab gels (1.5 mm). Electrophoresis was performed until a Bromophenol blue marker reached the edge of the gel. The gels containing the membrane proteins were first stained for glycoproteins with the periodic acid-Schiff reagent and then with Coomassie brilliant blue G-250 as described elsewhere [19]. The slab gels containing the labelled immunoprecipitates were stained with Coomassie brilliant blue, dried at 80°C under vacuum in a Bio-Rad slab gel dryer, and finally exposed to X-ray films as described in the next section. Densitometric scans of polyacrylamide gels and X-ray films were obtained using a Beckman CDS Gel Scanner.

Autoradiography. Immunoplates and dried polyacrylamide slab gels were exposed to Kodak X-Omatic films in Kodak Casette C1 equipped with X-Omatic regular intensifying screens for 1–5 days.

Assays

Total protein. Total protein was measured using the Bio-Rad assay according to the principle of Bradford [20]. The components of the assay were mixed in the following order: 25 μl sample, 25 μl 0.2% Triton X-100 and 2.5 ml protein assay reagent diluted to 25% with distilled water. The absorbance was read at 595 nm after 5 min. Bovine immunoglobulin fraction (Bio-Rad) served as standard.

Fibrinogen, factor VIII-related antigen, albumin and the glycoprotein IIb-IIIa complex. These were quantified by rocket immunoelectrophoresis. For albumin and fibrinogen, purified proteins were used as standards. The amounts of factor VIII-related antigen and the glycoprotein IIb-IIIa complex in the α-granule-derived material were calculated as a percentage of the total amount in isolated α-granules (100% fraction). A series of dilutions of the α-granule extract was electrophoresed simultaneously and served as the basis for the comparisons.

β-Thromboglobulin and platelet factor 4. These were measured by radioimmunoassay according to the manufacturers' instructions. The samples were diluted 500–1000-times in 0.01 M Tris-HCl buffer (pH 8.0)/0.15 M NaCl prior to the analyses.

Cholesterol. Cholesterol was measured using a commercial enzymatic kit. Reconstituted enzymatic reagent was preincubated at 37°C for 5 min. 20 μl of the membrane suspensions were added and incubation was continued for 10 min. The absorbances were read at 500 nm. Cholesterol dissolved in isopropanol served as standard.

Phospholipid analyses. Extraction of phospholipid was performed according to Rose and Oaklander [21]. Membranes were suspended in 0.27 M sucrose to 0.5 mg protein/ml. 6.0 ml isopropanol were added to 1.0 ml of the membrane suspension and the mixture was shaken for 1 h at 20°C. 3.0 ml chloroform were added and the mixture was shaken for another hour, this being followed by centrifugation at $2500 \times g_{max}$ for 10 min. The supernatant was collected, the solvents were removed by evaporation, and the lipids were dissolved in 1 ml chloroform/methanol (2 : 1, v/v) and washed twice with 0.05 M KCl to remove inorganic phosphate. The organic lipid extract was dried by the addition of a few crystals of water-free Na_2CO_3, the organic solvents were removed by evaporation and the lipids were finally dissolved in 100 μl chloroform/methanol (9 : 1).

The phospholipids were separated by thin-layer chromatography as described by Dryer [22]. Silica H plates were preactivated at 120°C for 1 h. The solution used for chromatography was chloroform/ methanol/ glacial acetic acid/ H_2O (50 : 25 : 7 : 3). 25 μl of the lipid solution was applied to each plate and the solvent was allowed to migrate to about 1 cm from the top of the plate. Commercially obtained lipids (sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine) were solubilized in the same manner as the samples and used as references. The plates were finally dried and exposed to iodine vapour to visualize the spots

containing the various phospholipids. These were scraped off for quantitation of their phosphate content as described by Dittmer and Wells [23]. 10 μl of solubilized lipid standards or material scraped off from the silica plates were heated with 0.4 ml $HClO_4$ (70%) at 200°C for 20 min. Inorganic phosphate was determined by the addition of 4.6 ml of 0.22% ammonium molybdate and 0.2 ml Fiske-SubbaRow reagent (0.5 g 1,2,4-amino-naphtholsulphonic acid plus 30 g sodium bi-sulphite and 6 g sodium sulphite in 250 ml distilled water). The mixtures were heated for 10 min on a bath of boiling water and the absorbances were read at 830 nm after cooling. The recovery of phospholipids after separation on the silica plates was measured to 90–95%.

Results

Preparation of α-granule membranes

Isolated α-granules were suspended in isotonic sucrose and ultrasonicated in order to disrupt the vesicles. The membrane fragments thus produced were sedimented by ultracentrifugation and the supernatant containing the liberated, soluble material, and the pellet containing the membranes were analysed for total protein and a series of α-granule-specific proteins. As shown in Fig. 1, the various α-granule proteins were liberated at different rates. Some of the proteins, i.e., β-thromboglobulin and albumin were rapidly liberated and only small amounts were associated with the membranes after 1 min of sonication. Platelet factor 4, fibrinogen and factor VIII-related antigen were liberated more slowly and were difficult to separate from the α-granule membranes. Thus, after 3 min of sonication, nearly 20% of factor VIII-related antigen, which is a releasable protein, was still associated with the α-granule membranes. Sonication at 20°C did not change the result. Inclusion of EDTA or divalent cations to 1 mM concentration or increasing the ionic strength with NaCl or KCl above 20 mM led to the immediate formation of aggregates during sonication and thus did not facilitate the removal of the releasable proteins from the α-granule membranes. Measurement of the glycoprotein IIb-IIIa complex showed that only small amounts of these integral mem-

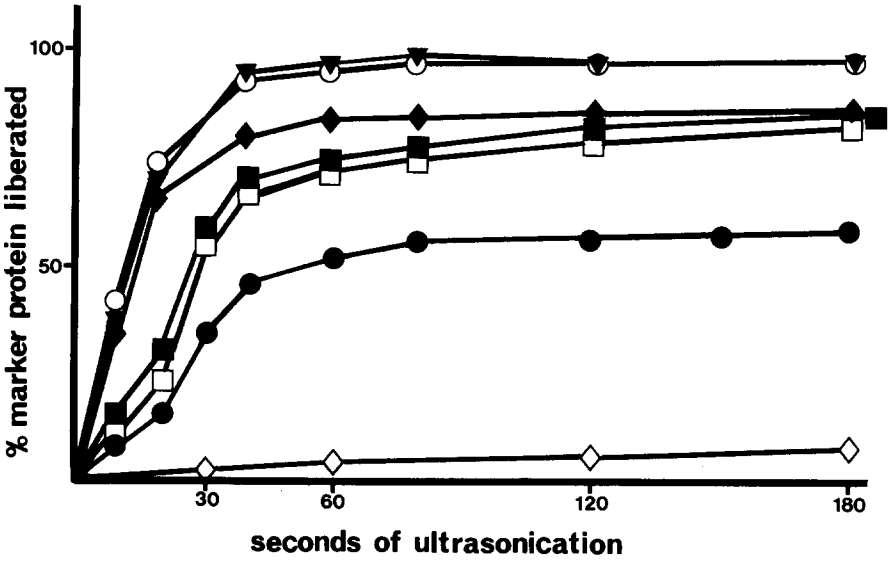

Fig. 1. Ultrasonication of α-granules. Time-course of liberation of components. ●———●, Total protein; ◇———◇, glycoprotein IIb-IIIa complex; □———□, factor VIII-related antigen; ◆———◆, platelet factor 4; ■———■, fibrinogen; ▼———▼, albumin; ○———○, β-thromboglobulin. Isolated α-granules were suspended in 0.27 M sucrose and ultrasonicated for periods of 10 s with 30-s intervals for cooling in an ice/NaCl mixture. Aliquots were drawn in two parallels after various times of ultrasonication and centrifuged at 75000 × g_{av} for 2 h. Analyses were performed on the supernatant and the pellet. The results are expressed as percentage liberated material recovered in the supernatant, of the total in the supernatant plus the pellet. The results are the means of two experiments.

brane proteins were liberated, indicating that solubilization of such proteins during ultrasonication was negligible.

Characterization of glycerol lysis membranes

The glycerol lysis technique is frequently used for the isolation of platelet plasma membranes. In order to examine the reliability of this method, in this respect the proteins in glycerol lysis membranes were compared to the surface-located proteins of intact platelets by crossed immunoelectrophoresis using antibodies against whole platelets. Platelets which had been surface labelled with ^{125}I were applied to electrophoresis either alone (Fig. 2A) or with the addition of glycerol lysis membranes (Fig. 2B). After electrophoresis, the washed and dried immunoplates were exposed to X-ray films. In the figures the immunoprecipitates are numbered as described previously [10,11]. The pattern obtained with glycerol lysis membranes is shown in Fig. 3A. The autoradiograms (Fig. 2C,D) reveal that most of the labelled immunoprecipitates were altered in the addition experiment (Fig. 2B,D) compared to the pattern obtained with whole platelets only (Fig. 2A,C). The altered immunoprecipitates are interpreted as representing the proteins which were present also in the glycerol lysis membranes. These are No. 13 (corresponding to glycoprotein Ib [23–26]), No. 16 (corresponding to the glycoprotein IIb-IIIa complex [24,27]), No. 17 and G4. Although labelled, precipitate G18 and a precipitate indicated by arrows in Fig. 2C and D were not altered in the addition experiment. In addition there was a marked labelling of fibrinogen (No. 24) and a weak labelling of albumin (No. 6), which both represent soluble proteins which are merely believed to be associated with the α-granules and not with the platelet surface. However, other α-granule proteins like factor VIII-related antigen (No. 9), platelet factor 4 (No. 1) and the protein corresponding to precipitate No. 8 [1,10] were not labelled, showing that the labelling of fibrinogen and albumin was not due to penetration of the ^{125}I to the cellular interior. Examination of the pattern obtained with glycerol lysis membranes (Fig. 3A)

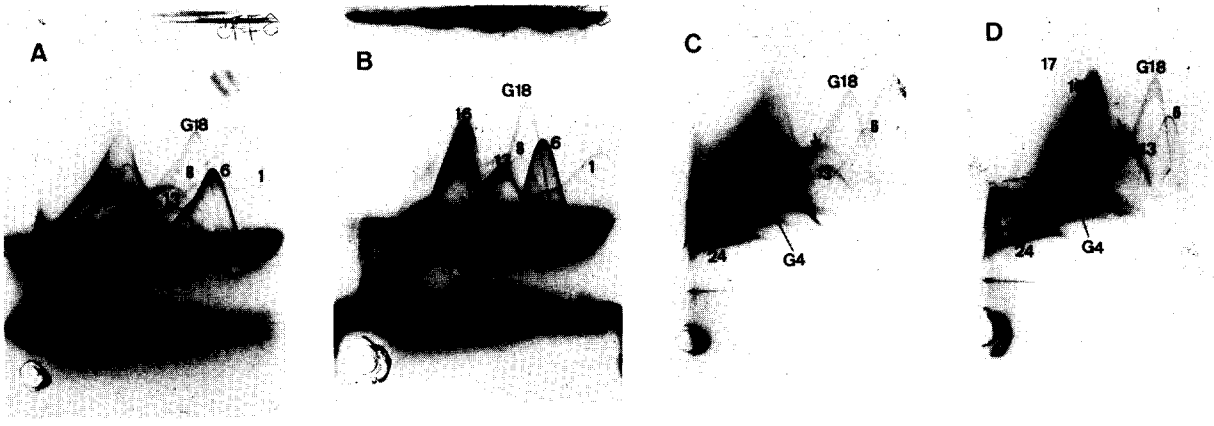

Fig. 2. Comparisons of surface-exposed platelet proteins and proteins in glycerol lysis membranes by crossed immunoelectrophoresis against anti-platelet antibodies. (A) ^{125}I-labelled platelets, stained immunoplate; (B) ^{125}I-labelled platelets plus glycerol lysis membranes, stained immunoplate; (C) autoradiogram of the immunoplate in A; (D) autoradiogram of the immunoplate in B. The corresponding pattern of glycerol lysis membranes is shown in Fig. 3A. The exact position of immunoprecipitate No. 17 in (D) cannot be verified, since the antigen has migrated partly out of the gel. Identified immunoprecipitates: No. 1: Platelet factor 4. No. 6, albumin; No. 9, factor VIII-related antigen, No. 13, glycoprotein Ib; No. 16, glycoprotein IIb-IIIa complex; No. 24, fibrinogen. 20–40 μg of protein solubilized in 0.038 M Tris/0.1 M glycine buffer (pH 8.7) containing 1% Triton X-100 were applied to 1% agarose gels. Electrophoresis in the first dimension was performed at 10 V/cm for 1 h. An antibody-free intermediate gel was inserted between the first-dimension gel and the second-dimension gel containing the antibodies (600 μg protein/ml) to obtain good resolution. The second-dimension electrophoresis was performed overnight at 2 V/cm. The electrode buffer contained 0.038 M Tris/0.1 M glycine buffer (pH 8.7). All agarose gels contained the same buffer and 0.5% Triton X-100. Platelets were radiolabelled with ^{125}I using lactoperoxidase-catalyzed iodination. The autoradiograms were obtained by exposure of the immunoplates to X-ray films.

shows the presence of immunoprecipitates Nos. 13, 16, 17 and G4, but not G18. In addition, a precipitate termed No. 20, which previously has been shown to represent β_2-microglobulin [28], and precipitates representing the releasable proteins albumin (No. 6) and factor VIII-related antigen (No. 9) were seen. The identity of these immunoprecipitates was established by inclusion of monospecific antibodies in intermediate gels in parallel electrophoreses.

Comparison of α-granule membranes and glycerol lysis membranes by crossed immunoelectrophoresis

Platelet glycerol lysis membranes and α-granule membranes were solubilized and compared by crossed immunoelectrophoresis (Fig. 3A, B). Comparison of the patterns was facilitated by parallel electrophoresis of a mixture of the two samples (Fig. 3C). The patterns obtained with both membrane preparations contained less dominant immunoprecipitates representing albumin (No. 6) and factor VIII-related antigen (No. 9). The predominant immunoprecipitate No. 16 (glycoprotein IIb-IIIa complex) and G4 were present in both patterns. The immunoprecipitates No. 13 (glycoprotein Ib), 17 and 20 (β_2-microglobulin) represent antigens which were present only in the glycerol lysis membranes. No immunoprecipitates corresponding to glycoprotein Ib (No. 13) or β_2-microglobulin (No. 20) were seen when α-granule membranes or intact α-granules were analyzed by crossed immunoelectrophoresis with anti-glycocalicin or anti-β_2-microglobulin in the intermediate gels (data not shown).

As evident from Fig. 3, the proteins representing the precipitates No. 17, G4 and G8 are different antigens and G8 and G18 are specific for the α-granule membrane preparation. The identity of the proteins corresponding to No. 17, G4 and G18 will be dealt with in the next section, whereas it was not possible to elucidate the characteristics of G8. Although the G4 antigen was seen in both the membrane preparations analyzed, the bulk of this antigen appears to be associated with the α-granule membranes as judged from the areas covered by the immunoprecipitates. In addition to the proteins already mentioned, the α-granule preparations contained some antigens corresponding to releasable proteins. These were platelet factor 4 (No. 1), thrombospondin (No. 14) [10] and fibrinogen (No. 24) (Fig. 1). Other differences in antigen composition between the two types of

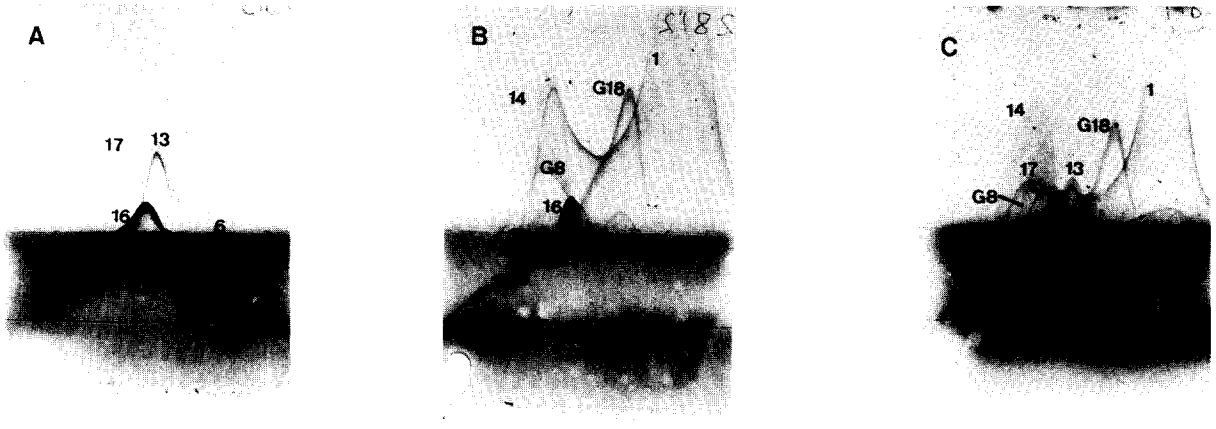

Fig. 3. Comparison of proteins in glycerol lysis membranes and α-granule membranes by crossed immunoelectrophoresis using antibodies to whole platelets. (A) Glycerol lysis membranes; (B) α-granule membranes; (C) glycerol lysis membranes plus α-granule membranes. Identified immunoprecipitates: No. 1, platelet factor 4; No. 6, albumin; No. 9, Factor VIII-related antigen; No. 13, glycoprotein Ib; No. 14, thrombospondin; No. 16, glycoprotein IIb-IIIa complex; No. 20, β_2-microglobulin; No. 24, fibrinogen. Glycerol lysis membranes were prepared by loading the platelets with 40% glycerol by centrifugation through a glycerol gradient, followed by lysis by suspension in an isotonic medium. The membranes were isolated by centrifugation in a discontinuous density gradient. α-Granule membranes were isolated by ultrasonication of a suspension of isolated α-granules followed by ultracentrifugation in order to sediment the membranes. Other experimental conditions were as in Fig. 2.

TABLE I

PROTEINS IN GLYCEROL LYSIS MEMBRANES AND α-GRANULE MEMBRANES FROM HUMAN PLATELETS

Parentheses indicate proteins occasionally found in the membrane fractions.

Protein	Designation in crossed immuno-electrophoresis	Identified by polyacrylamide gel electrophoresis	Present in glycerol lysis membranes	Present in α-granule membranes
Platelet factor 4	1			+
Albumin	6	+	+	+
Factor VIII-related antigen	9		+	+
Glycoprotein Ia	17		+	
Glycoprotein Ib	13	+	+	
Glycocalicin		(+)	(+)	
Thrombospondin	14	+		+
Glycoprotein IIa			?	?
Glycoprotein IIb	} 16	+	+	+
Glycoprotein IIIa		+	+	+
Glycoprotein IIIb		+	+	
β_2-Microglobulin	20		+	
Fibrinogen	24	+	(+)	+
Myosin		+	+	+
Actin		+	+	
G4	G4		+	+
G8	G8			+
G18	G18			+

membrane were less pronounced and will not be dealt with here. The data are summarized in Table I.

Molecular weight estimation of antigens

Immunoprecipitate No. 16 has previously been shown to represent a complex between the glycoproteins IIb and IIIa [24,27], whereas immunoprecipitate No. 17 has been suggested to contain the glycoproteins Ia and IIa [29]. The molecular weights of the antigens corresponding to G4 and G18 have not previously been investigated. ^{125}I-labelled platelets were solubilized and applied to crossed immunoelectrophoresis followed by exposure of the immunoplates to X-ray films. The resulting autoradiograms and the stained immunoplates were compared and the various labelled immunoprecipitates were cut out, incubated with sodium dodecyl sulphate and applied to polyacrylamide slab gels. After electrophoresis, the gels were stained, dried and exposed to X-ray films. The resulting autoradiograms revealed the labelling of specific polypeptide bands. Densitometric scans of the various lanes of the X-ray films are shown in Fig. 4. Immunoprecipitate No. 13 showed the expected result, containing a polypeptide with molecular weight corresponding to glycoprotein Ib (Fig. 4A). Immunoprecipitate No. 16 showed the presence of polypeptides with molecular weights corresponding to those of the glycoproteins IIb and IIIa, as expected, but also showed the presence of a polypeptide with an apparent molecular weight of 160 000 in the reduced state (Fig. 4B). Assuming this to comigrate with glycoprotein IIb in the unreduced state, its migration corresponded to that of glycoprotein IIa. Immunoprecipitate No. 17 showed a labelled polypeptide band with apparent molecular weight 141 000 on the unreduced state and 165 000 in the reduced state. This would fit with the molecular weights of glycoprotein Ia.

The labelled polypeptide corresponding to immunoprecipitate G4 exhibited an apparent molecular weight of 146 000 in the unreduced state and 132 000 in the reduced state (average values). In the unreduced state, it migrated slightly differently from glycoprotein IIb. Immunoprecipitate G18 corresponded to a rather heterogeneous polypeptide band with a molecular weight of 130 000–135 000 in its reduced as well as its unre-

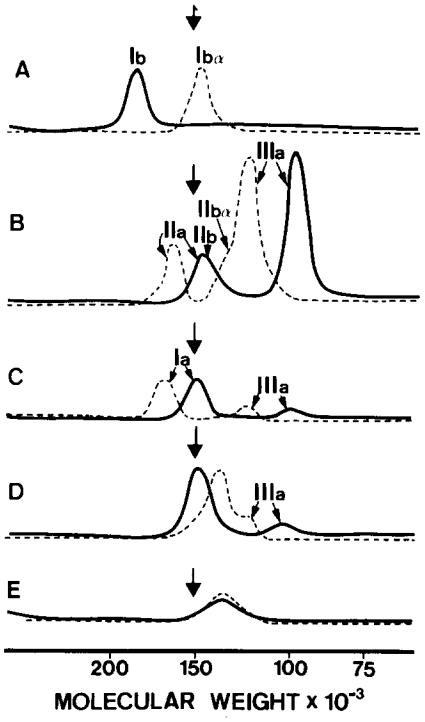

Fig. 4. Densitometric scans of autoradiograms obtained after sodium dodecyl sulphate polyacrylamide slab gel electrophoresis of ^{125}I-labelled immunoprecipitates and exposure of the dried slab gels to X-ray films. (A) Immunoprecipitate No. 13 (glycoprotein Ib); (B) immunoprecipitate No. 16 (glycoprotein IIb-IIIa complex); (C) immunoprecipitate No. 17; (D) immunoprecipitate G4; (E) immunoprecipitate G18, ———, unreduced samples; - - - - - -, samples reduced with 2-mercaptoethanol. The arrows indicate the position of the immunoglobulin-derived material. The immunoglobulins represented the only polypeptide bands which were significantly stained with Coomassie brilliant blue. Intact platelets were surface-labelled with ^{125}I, solubilized and applied to crossed immunoelectrophoresis. After exposure of the immunoplates to X-ray films, the labelled immunoprecipitates were cut out by comparison to the autoradiograms. Identical immunoprecipitates from 3–5 immunoplates were pooled, solubilized with sodium dodecyl sulphate and applied to polyacrylamide slab gels with or without reduction of the samples with 2-mercaptoethanol. After the electrophoresis, the slab gels were stained, dried and exposed to X-ray films. The individual electrophoretic lanes on the autoradiograms were scanned at 550 nm. The illustrations show a series which is typical for three individual experiments.

duced state. Occasionally other polypeptide bands were also seen in the autoradiograms. However, their occurrence was not reproducible. Labelled polypeptide bands corresponding to glycoprotein IIIa were routinely observed in the patterns obtained with the immunoprecipitates No. 17 and G4. These probably represent small contaminations of precipitate No. 16. The polypeptide bands representing the immunoglobulin-derived material were the only visible bands after staining with Coomassie brilliant blue. The positions of these bands are indicated in Fig. 4. None of these bands showed any sign of ^{125}I-labelling.

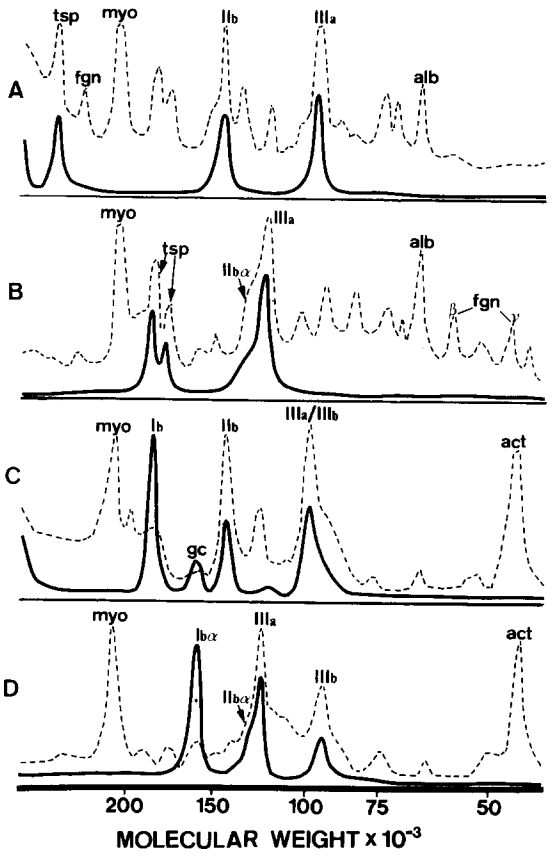

Fig. 5. Densitometric scans of sodium dodecyl sulphate polyacrylamide electrophoresis gels of: (A) α-granule membranes, unreduced sample; (B) α-granule membranes, reduced sample; (C) glycerol lysis membranes, unreduced sample; (D) glycerol lysis membranes, reduced sample. Reduction of disulphide bonds was performed with 2-mercaptoethanol. - - - - - -, Stained for total protein with Coomassie brilliant blue; ———, stained for glycoprotein with the periodic acid-Schiff reagent. The roman numericals refer to glycoproteins. myo, myosin heavy chain; tsp, thrombospondin; fgn, fibrinogen; alb, albumin; gc, glycocalicin; act, actin.

Characterization of the membrane preparations by sodium dodecyl sulphate polyacrylamide gel electrophoresis

The preparations of α-granule membranes and glycerol lysis membranes were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis either after reduction of the disulphide bonds or in their unreduced states. Cylindrical gels rather than slab gels were used for a better interpretation of the periodic acid-Schiff stain for the glycoproteins. After electrophoresis, the gels were first stained for glycoproteins and then for total proteins with Coomassie brilliant blue. The densitometric scans of the gels are shown in Fig. 5. The glycoproteins are numbered according to Phillips and Poh Agin [30] except that the glycoproteins III and IV are termed IIIa and IIIb, respectively. The glycoproteins thrombospondin, IIb and IIIa were present in the α-granule membranes (Fig. 5A, B), whereas the glycoproteins Ib, glycocalicin, IIb, IIIa and IIIb were present in the glycerol lysis membranes (Fig. 5C, D). Two bands which comigrated with myosin heavy chain and actin, respectively (comigration experiments not shown) were prominent in the Coomassie-stained gels containing the glycerol lysis membranes, whereas significant amounts of actin could not be observed in the α-granule membrane preparation.

Characterization of lipid composition

Glycerol lysis membranes and α-granule membranes were analyzed for total amounts of protein, cholesterol and phospholipids as well as for the composition of the phospholipids. The results revealed pronounced differences in the ratios between protein, cholesterol and phospholipids between the two membrane preparations (Table II), but no significant difference in composition of the phospholipids (Table III).

TABLE II

LIPID AND PROTEIN CONTENT IN PLATELET PLASMA MEMBRANES AND α-GRANULE MEMBRANES

Mean ± S.D.

	Plasma membranes	α-Granule membranes
Number of measurements	3	6
Phospholipid/protein (μmol/mg)	0.85 ± 0.06	0.38 ± 0.13
Cholesterol/protein (μmol/mg)	0.56 ± 0.03	0.20 ± 0.04
Cholesterol/phospholipid (μmol/μmol)	0.66 ± 0.08	0.24 ± 0.05

TABLE III

PHOSPHOLIPID COMPOSITION OF PLATELET PLASMA MEMBRANES AND α-GRANULE MEMBRANES

The values are expressed as a percentage of the total amount of phospholipid recovered after separation (mean ± S.D.).

	Plasma membranes	α-Granule membranes
Number of measurements	3	6
Sphingomyelin	13.4 ± 2.2	18.0 ± 6.1
Phosphatidylcholine	43.5 ± 1.6	36.3 ± 3.3
Phosphatidylserine + phosphatidylinositol	12.3 ± 2.4	15.9 ± 3.9
Phosphatidylethanolamine	30.7 ± 4.0	29.8 ± 6.8

Discussion

We have previously described a method for the isolation of α-granules from human platelets and documented the homogeneity of the preparations by various approaches [1,10,13]. The method for the isolation of the α-granule membranes has been adapted from procedures used for the isolation of membranes from similar vesicular subcellular structures [31,32]. Although some of the proteins which belong to the secretory pool of the α-granules were easily removed, others tended to stick rather tightly to the membranes. These peripherally associated proteins are considered as not belonging to the α-granule membranes and, although they may be specifically connected to the membranes, the considerations of protein-lipid ratios are hampered by their presence. Thus, the observation that the protein-lipid ratios were higher in the α-granule membranes than in the glycerol lysis membranes may be caused by the presence of adsorbed, secretable α-granule proteins.

The reliability of the qualitative considerations of the α-granule membrane proteins is based on previous work showing the purity of the α-granule preparations combined with work in which the α-granule secretable and non-secretable proteins were distinguished [1,10,13]. The non-secretable

proteins are considered as specific membrane proteins fitting the exocytosis theory for secretion of the α-granule contents [1] and will be dealt with in the forthcoming discussion.

The glycerol lysis technique [14] represents a widely used approach to the preparation of platelet plasma membranes. The comparison of surface-located platelet proteins and the proteins in the glycerol lysis membranes revealed a great extent of conformity. Most of the proteins that had incorporated significant amounts of ^{125}I after surface labelling of the platelets by the lactoperoxidase-catalyzed reaction were recovered in the glycerol lysis membranes. Fibrinogen, G18 and a minor, unidentified component constitute exceptions to this general rule. The glycerol lysis membrane preparations showed only one additional, non-labelled protein of an apparent specific membrane nature which was seen by crossed immunoelectrophoresis, namely β_2-microglobulin. Thus, with reference to crossed immunoelectrophoresis, the glycerol lysis membranes appear to contain the major part of the surface-located platelet proteins. Together with the observation that no prominent additional proteins were found in the glycerol lysis membranes, this indicates that the glycerol lysis technique represents a reasonable approach to the isolation of platelet plasma membranes. Although the conclusions reached above are obtained from considerations of protein distribution, the considerably higher content of cholesterol relative to phospholipids in the glycerol lysis membranes compared with that in the α-granule membranes is in accordance with the general concept of distribution of cholesterol between plasma membranes and intracellular membranes. A distribution of cholesterol similar to that seen in the present paper was reported also for platelet membranes of surface origin as compared to membranes of non-granular, intracellular origin [33]. One major point which needs to be mentioned is that a partial activation of the platelets may take place in the course of surface iodination, altering the pattern of surface-exposed membrane proteins. The presence of surface-exposed fibrinogen may be explained by some degree of activation of the platelets leading to release and formation of surface-receptors for fibrinogen. Since other α-granule proteins like platelet factor 4 and factor VIII-related antigen were not labelled after surface iodination of whole platelets, the possibility can be excluded that ^{125}I had penetrated the membranes and been coupled to intracellularly located proteins. A partial activation may also explain the behaviour of the G18 protein as discussed below. Hence it may not be absolutely correct to state that all the proteins which are normally labelled after surface iodination are part of the plasma membrane of non-activated platelets.

Comparison of the proteins of the glycerol lysis membranes and the α-granule membranes revealed the presence of the glycoproteins IIb and IIIa in both types of membrane. This is consistent with the dual subcellular location of these glycoproteins proposed previously [10]. Glycoprotein IIIb and β_2-microglobulin were found exclusively in the glycerol lysis membranes, which is consistent with their proposed surface location [30,34]. Glycoprotein Ib was seen in both the electrophoretic systems applied and appeared to be located only in the glycerol lysis membranes. Its absence from the α-granule membranes is in accordance with previous observations [10]. The antigen contained in immunoprecipitate No. 17 was shown to exhibit a migration in sodium dodecyl sulphate gel electrophoresis which corresponded to that of glycoprotein Ia [30]. This is in part consistent with the observations made by Kunicki et al. [29], but these investigators also recovered glycoprotein IIa in the same immunoprecipitate. In contrast to their findings, the results presented herein reveal the presence of a protein with the characteristics of glycoprotein IIa in immunoprecipitate No. 16. Previously, only the glycoproteins IIb and IIIa have been recovered in this immunoprecipitate [24,27]. Our results may mean either that glycoprotein IIa is a third member of this glycoprotein complex or that glycoprotein IIa was present in an immunoprecipitate which could not be distinguished from the dominant glycoprotein IIb-IIIa precipitate in our systems. In this respect it is interesting to note that in a recent work by Pidard et al. [35], glycoprotein IIa seems to accompany glycoprotein IIb and/or glycoprotein IIIa in sucrose density gradient centrifugations. Whichever explanation will prove to be correct, our results indicate that the glycoproteins Ia and IIa are present in sep-

arate immunoprecipitates. With respect to the subcellular location of these glycoproteins, glycoprotein Ia is then present only in the glycerol lysis membranes whereas the attempts to localize glycoprotein IIa were non-conclusive.

The protein corresponding to immunoprecipitate G4 appears to exhibit a dual subcellular location, whereas the G18 antigen was recovered exclusively in the α-granule membranes. The behaviour of G18 in this respect is not easily understood, since it became labelled after surface ^{125}I-iodination of intact platelets. However, since it was not recovered in the glycerol lysis membranes along with the other surface-located proteins, this would support a different location in the platelets. One possibility is that the labelling of this antigen after surface ^{125}I-iodination of platelets is due to a limited α-granule release in the course of isolation, washing or surface-labelling of the platelets. The same may be considered for G4, since quantitation based on the area covered by the immunoprecipitates indicates that the bulk of G4 was located in the α-granule membranes. Neither of these proteins is releasable and both appear to be glycosylated as judged from their altered electrophoretic mobilities after treatment of the platelets by neuraminidase (unpublished results). Consequently, their identities must be sought among the platelet membrane glycoproteins. Their apparent molecular weights in reduced and unreduced sodium dodecyl sulphate polyacrylamide gel electrophoresis are different from those of the glycoproteins Ia, Ib, IIa, IIb, IIIa and IIIb. The electrophoretic behaviour of G4 is very similar to that of glycoprotein IIb, but it exhibited a slightly lower electrophoretic mobility in its unreduced state. Furthermore, the G4-precipitate showed no merging with the glycoprotein IIb-IIIa precipitate, which excludes G4 from being identical to any of these glycoproteins. According to available knowledge, the electrophoretic properties of G4 resembles those of glycoprotein Ic [30,36]. The presence of this glycoprotein cannot be verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis of the membranes, since glycoprotein Ic does not stain with the periodic acid-Schiff reagent, nor with Coomassie brilliant blue. Consequently, better methodology must be applied to verify this possible identity.

None of the presently characterized major membrane glycoproteins of the platelets is a probable candidate for the G18 antigen. Since this glycoprotein did not alter its electrophoretic mobility by reduction, it differs from all the major glycoproteins in the actual range of molecular weights. Recently, McGregor et al. [37] have described a minor glycoprotein which they termed $GP^{4-4.5}_{132-135}$. This glycoprotein consequently exhibits a molecular weight which fits that of the G18 antigen. However, current knowledge does not include the electrophoretic migration in the reduced versus the unreduced state of this glycoprotein. Thus, firm conclusions as to the identity of the G18 antigen cannot be made at the present stage.

The presence of actin as well as myosin in platelet plasma membranes has been reported previously [33,38,39] and the present results showing considerable amounts of myosin and little or no actin in the α-granule membranes are in accordance with previous studies on total α-granules [1,10]. Additional information as to the distribution of contractile proteins in platelet membranes has been presented by Menashi et al. [33]. These investigators showed that a preparation of intracellular membranes of non-granular origin contained neither actin nor myosin. The different contents of the various proteins of the contractile system in the various platelet membranes, and particularly the large amounts of myosin in the α-granule membrane, may point to a specific function of the platelet contractile apparatus in the release of the α-granule contents.

The composition of phospholipids in the glycerol lysis membranes varies somewhat from that reported for platelet plasma membrane preparations by other groups [40,41] which in turn have presented results which show individual variations. Different isolation procedures for the membranes may account for these differences. The variation in the composition of phospholipids between the glycerol lysis membranes and the α-granule membranes is not significant. Theoretically, the α-granule membranes might be the source of the negatively charged, procoagulant phospholipids which became surface-exposed during activation of the platelets [40,42]. Since our data do not demonstrate increased amounts of such phospholipids in the α-granule membranes, there

is no immediate evidence for this theory. However, since the release proceeds by exocytosis [1] the possibility cannot be excluded that the inner phospholipid bilayer of the α-granule membrane which will be exposed on the platelet surface may be enriched in such phospholipids.

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References

1 Gogstad, G.O., Hagen, I., Korsmo, R. and Solum, N.O. (1982) Biochim. Biophys. Acta 702, 81–89
2 White, J.C. (1978) Am. J. Pathol. 66, 296–312
3 Raccuglia, G. (1971) Am. J. Med. 51, 818–828
4 Gerrard, J.M., Phillips, D.R., Rao, G.H.R., Plow, E.F., Walz, D.A., Ross, R. and Harker, L.A. (1980) J. Clin. Invest. 66, 102–109
5 Levy-Toledano, S., Caen, J.P., Breton-Gorius, J., Rendu, F., Cywiner-Golenzer, C., Dupuy, E., Legrand, Y. and Maclouf, J. (1981) J. Lab. Clin. Med. 98, 831–848
6 George, J.N., Lyons, R.M. and Morgan, R.K. (1980) J. Clin. Invest. 66, 1–9
7 Phillips, D.R., Jennings, L.K. and Prasanna, H.R. (1980) J. Biol. Chem. 255, 11629–11632
8 Chesney, C.M., Pifer, D. and Colman, R.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5180–5184
9 George, J.N. and Onofre, A.R. (1982) Blood 59, 194–197
10 Gogstad, G.O., Hagen, I., Korsmo, R. and Solum, N.O. (1981) Biochim. Biophys. Acta 670, 150–162
11 Hagen, I., Bjerrum, O.J. and Solum, N.O. (1979) Eur. J. Biochem. 99, 9–22
12 Gogstad, G.O., Hagen, I., Krutnes, M.-B. and Solum, N.O. (1982) Biochim. Biophys. Acta 689, 21–30
13 Gogstad, G.O. (1980) Thromb. Res. 20, 669–681
14 Barber, A.J. and Jamieson, G.A. (1970) J. Biol. Chem. 245, 6357–6365
15 Laurell, C.B. (1972) Scand. J. Clin. Lab. Invest. 29, suppl. 124, 21–37
16 Weeke, B. (1973) Scand. J. Immunol. 2, suppl. 1, 15–56
17 Phillips, D.R. (1972) Biochemistry 11, 4582–4588
18 Laemmli, U.K. (1970) Nature (London) 227, 680–685
19 Solum, N.O., Hagen, I. and Peterka, M. (1977) Thromb. Res. 10, 71–82
20 Bradford, M.M. (1976) Anal. Biochem. 72, 248–252
21 Rose, H.G. and Oaklander, M. (1965) J. Lipid Res. 6, 428–431
22 Dryer, R.L. (1970) in Fundamentals of Clinical Chemistry (Tietz, W.W., ed.), pp. 344–346, W.B. Saunders Co., Philadelphia
23 Dittmer, J.C. and Wells, M.A. (1969) Methods Enzymol. 14, 482–530
24 Hagen, I., Nurden, A., Bjerrum, O.J., Solum, N.O. and Caen, J.P. (1980) J. Clin. Invest. 65, 722–731
25 Solum, N.O., Hagen, I., Filion-Myklebust, C. and Stabaek, T. (1980) Biochim. Biophys. Acta 597, 235–246
26 Solum, N.O., Hagen, I. and Sletbakk, T. (1980) Thromb. Res. 18, 773–785
27 Kunicki, T.J., Pidard, D., Rosa, J.-P. and Nurden, A.T. (1981) Blood 58, 268–278
28 Hagen, I., Bjerrum, O.J., Gogstad, G.O., Korsmo, R. and Solum, N.O. (1982) Biochim. Biophys. Acta 701, 1–6
29 Kunicki, T.J., Nurden, A.T., Pidard, D., Russell, N.R. and Caen, J.P. (1981) Blood 58, 1190–1197
30 Phillips, D.R. and Poh Agin, P. (1977) J. Biol. Chem. 252, 2121–2126
31 Janson, T.L. and Helgeland, L. (1975) Biochim. Biophys. Acta 379, 598–605
32 Gogstad, G.O. and Helgeland, L. (1978) Biochim. Biophys. Acta 508, 551–564
33 Menashi, S., Weintroub, H. and Crawford, N. (1981) J. Biol. Chem. 256, 4095–4101
34 Ervin, P.-E. and Pertoft, H. (1973) J. Immunol. 111, 1147–1154
35 Pidard, D., Rosa, J.-P., Kunicki, T.J. and Nurden, A.T. (1982) Blood 60, 894–904
36 Sixma, J.J. and Sciphorst, M.E. (1980) Biochim. Biophys. Acta 603, 70–83
37 McGregor, J.L., Clemetson, K.J., James, E., Clezardin, P., Dechavanne, M. and Lüscher, E.F. (1982) Biochim. Biophys. Acta 689, 513–522
38 Taylor, D.G., Mapp, R.J. and Crawford, N (1975) Biochem. Soc. Trans. 3, 161–164
39 Puszkin, E.G., Moldonado, R., Spaet, T.H. and Zucker, M.B. (1977) J. Biol. Chem. 252, 4371–4378
40 Zwaal, R.F.A., Comfurius, P. and Van Deenen, L.L.M. (1977) Nature (London) 268, 360–362
41 Perret, B., Chap, H.J. and Douste-Blazy, L. (1979) Biochim. Biophys. Acta 556, 434–446
42 Zwaal, R.F.A. (1978) Biochim. Biophys. Acta 515, 163–205