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Isolation and Partial Characterization of Platelet α-Granule Membranes

John Van der Meulen, Wendy Furuya, and Sergio Grinstein
Department of Cell Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Canada M5G 1X8

Summary. Porcine α-granules, prepared by a modification of pre-existing methods, were found to be essentially homogeneous by transmission electron microscopy. Freeze-fractured samples of isolated granules revealed numerous intramembranous particles on the EF (exoplasmic fracture) surface and to a lesser extent on the PF (protoplasmic fracture) surface whereas the PS (protoplasmic) surface was relatively smooth. The granules appear to be sealed, as evidenced by: a) the retention of their electron dense core material; b) the inability of impermeant labels to react with the granule contents, and c) the finding that the intragranular proteins are refractory to mild hydrolysis by externally added proteases. Membranes were isolated by alkali extraction of the granules and used for biochemical characterization. Approximately 87% of the protein, but only insignificant amounts of phospholipid were removed by this procedure, which yielded membrane vesicles devoid of the dense core. The membranes contain one major and several minor polypeptides of molecular weights ranging from 28,000 to 230,000, as determined by polyacrylamide gel electrophoresis. The major polypeptide contains carbohydrate residues. The exposure of specific proteins on the cytoplasmic surface of the granule membrane was determined by a combination of surface-specific labeling and proteolysis of intact granules, followed by membrane isolation and analysis. In sealed granules, only a limited number of bands are modified by the reagents whereas most of them are affected following granule lysis, indicating asymmetry in their transmembrane disposition. The fraction eluted by alkali extraction was also analyzed and found to contain nine major polypeptides of molecular weights ranging from 230,000 to 43,000. These are compared to the weights of the macromolecules believed to be secreted from α-granules, as determined by radioimmunological techniques.

Key words platelets – secretory granules · α-granules · membrane isolation

Introduction

Following vascular injury, platelets adhere to exposed collagen and subendothelial structures and undergo a series of functional and morphological alterations, including secretion and its structural counterpart, degranulation. Three basic types of products are released from stimulated platelets: low molecular weight compounds (mainly serotonin, ADP, ATP and Ca^{++}), lysosomal hydrolases, and a group of heterogenous macromolecules, many of which have been only incompletely characterized. The latter include platelet factor 4, β-thromboglobulin, a cell growth factor, fibronectin, factor V, thrombospondin, α_2-antiplasmin and fibrinogen. Each group of exported molecules is thought to be stored in a distinct secretory organelle: serotonin, Ca^{++} and the adenine nucleotides are localized in the dense granules [12, 13, 16]; hydrolases are thought to be stored in lysosomes [5, 11] and the polypeptide macromolecules are found in a third type of organelle, the α-granule [11, 27, 28, 48, 49]. Studies on patients with the "gray platelet" syndrome and with storage pool disease have shed some light on the compartmentation of the released products. Dense granules are virtually absent in platelets from patients with storage pool disease, and biochemical determinations have shown these platelets to be deficient in both serotonin and adenine nucleotides [22, 24]. In contrast, serotonin, adenine nucleotide and hydrolase levels are normal in "gray platelets", which are, however, markedly deficient in thrombospondin, β-thromboglobulin, fibrinogen, fibronectin and platelet factor 4 [17, 30, 36, 45]. This correlates with the marked reduction in α-granules observed in these patients, which otherwise display normal numbers of dense bodies and mitochondria [45].

Morphological evidence indicates that secretion of α-granule contents probably occurs by a type of compound exocytosis [18]. The granules first migrate to the center of the platelet, fusing with one another producing large (>0.5 μm diameter) membrane-bound masses. The latter migrate towards the platelet periphery where they presumably fuse with the plasma membrane whereupon their contents are released. Compared to the morphological information, very little is known about

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the molecular basis of α-granule secretion. In fact, to our knowledge, the structure and composition of their membranes have not been systematically explored. This is probably due, at least in part, to the technical difficulties involved in reproducibly obtaining sufficient quantities of pure material for biochemical characterization.

We have adapted and slightly modified pre-existing techniques [8, 16, 40] for the isolation of α-granules from large volumes of porcine blood, and developed means of isolating their membranes. The latter were studied both morphologically and biochemically, and the results are analyzed in the framework of the secretory response.

Materials and Methods

In general, each type of experiment was performed at least three times and representative individual experiments are illustrated.

Platelet Isolation

Platelet isolation was carried out at room temperature (22–24 °C). Typically, 4 liters of fresh porcine blood were processed in each experiment, and all the quantities cited in this protocol refer to this starting volume. Nine volumes of blood were collected into plastic bottles containing one volume of 85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose. The blood was centrifuged at $800 \times g$ for 25 min, and the resulting platelet-rich plasma was carefully siphoned off and recentrifuged at $3{,}000 \times g_{av}$ for 25 min. The pellet was resuspended in Ca^{++}-free Tyrode's solution (mM 137 NaCl, 2.7 KCl, 2 $MgCl_2$, 12 $NaHCO_3$, 0.42 NaH_2PO_4, 50 glucose and 0.35% bovine serum albumin) and the remaining contaminating red cells were sedimented at $40 \times g_{av}$ for 20 min on a bench-top centrifuge. The supernatant was decanted and the platelets were centrifuged at $1{,}000 \times g_{av}$ for 20 min and finally resuspended in 160 ml of Ca^{++}-free Tyrode's for ATP depletion.

ATP Depletion

As reported by Fukami et al. [16] for dense granules, we found that the yield and purity of α-granules was much improved by metabolically depleting the platelets prior to homogenization. Accordingly, 80 mg of 2-deoxy-D-glucose and 800 μg of rotenone (dissolved to 1 mg/ml in absolute ethanol) were added to the platelet suspension, which was then incubated for 40 min at 37 °C with gentle agitation, and then returned to room temperature. Ten mg of chymotrypsin and 40 mg of ATP were added and incubation was continued for 5 min at room temperature[1]. Proteolysis was then stopped by addition of a large molar excess of phenylmethylsulfonyl fluoride (PMSF), and centrifugation at $1{,}000 \times g_{av}$ for 20 min.

Platelet Disruption and Fractionation

The pellet was resuspended in 150 ml of ice-cold Tris resuspension buffer (TRB), which contained (mM): 250 sucrose, 1 EDTA, 0.15 PMSF, 10 Tris-HCl, pH 7.4. In agreement with Broekman et al. [8], we found nitrogen cavitation to be an efficient method for reproducible cell disruption. Platelets in TRB were subjected to three 10-min cycles of cavitation at 1,200 psi in a precooled Parr bomb (Parr Instruments, Moline, Ill.). The homogenate was spun at $1{,}000 \times g_{av}$ for 20 min at 4 °C to remove intact or incompletely disrupted platelets. The supernatant was recentrifuged at $10{,}000 \times g_{av}$ for 20 min at 4 °C. The resulting pellet was resuspended in a final volume of 25 ml of TRB and layered onto discontinuous sucrose gradients. The gradients (1.2 to 2.0 M sucrose in 0.2 M steps, made up in 10 mM Tris-HCl; 1 mM EDTA, pH 7.4) were centrifuged at $82{,}500 \times g_{av}$ for 3 hr at 4 °C. α-granules, recovered from the 1.8–2.0 M sucrose interphase, were gradually diluted in TRB, and sedimented at $13{,}500 \times g_{av}$ for 15 min and resuspended for further analyses in 2–4 ml TRB.

Extraction Procedures for Membrane Isolation

α-granules were sonicated in TRB using a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Inc., Plainview, N.Y.) at a setting of 30. Four 15-sec cycles were used, taking care to keep the sample on ice at all times. Granules in TRB were hypotonically shocked by resuspension in 20 volumes of ice-cold water. For high ionic strength extraction, α-granules were sedimented and then resuspended in 1.0 M KI, 250 mM sucrose, 10 mM Tris, pH 7.4, and incubated for 10 min at room temperature. Acidic extraction was achieved by resuspending granules in 250 mM sucrose 1 mM EDTA, adjusted to pH 2, with HCl followed by 10 min incubation at room temperature. Alkali extraction was performed incubating the granules at room temperature for 10 min in 250 mM sucrose, 1 mM EDTA adjusted to pH 12 with NaOH. In all cases, the extracted granules were recovered by centrifugation ($100{,}000 \times g$ for 30 min) and the pellet and supernatant fractions were saved for analysis. Unless otherwise indicated, "isolated membranes" refers to the pellet obtained from alkali extracted granules.

Labeling Procedures

Iodination of granules (3–5 mg/ml) or membranes (0.5–1 mg/ml) in 250 mM sucrose, 1 mM EDTA, pH 7.4, was performed using Iodogen [14], as described by Fraker and Speck [15]. One ml of suspension was added to a scintillation vial freshly coated with 10 μg of Iodogen, and 300 μCi of carrier-free Na [^{125}I] was added, followed by a 20-min incubation at 4 °C. The reaction was terminated by removal of the sample from the vial and by a 40-fold dilution with TRB followed by a 30-min centrifugation at $13{,}500 \times g_{av}$ in the case of granules or $120{,}000 \times g_{av}$ for membranes. The labeled organelles were then washed threefold with TRB as above, and used for further extraction and/or electrophoresis.

Carbohydrate chains of granule glycoproteins and glycolipids were labeled with [^{14}C]-aniline using a procedure developed by Schweizer et al.[2] Briefly, the method involves periodate oxidation of vicinal hydroxyl groups, followed by formation of a Schiff base with [^{14}C]-aniline. Reduction of the Schiff base is achieved with $NaCNBH_3$. Washing, extraction and analysis were as described.

Proteolysis

For trypsin or chymotrypsin digestion, granule (2–4 mg/ml) or membrane (1–2 mg/ml) suspensions in TRB were incubated

[1] This step was initially included in the preparation based on the report by Fukami et al. [16] that a brief proteolytic treatment of the ATP-depleted platelets improved subsequent separation of the subfractions on the sucrose gradient. We found, empirically, that addition of ATP and chymotrypsin was essential for high, reproducible yields.

[2] Schweizer, E., Angst, W., and Lutz, H.V. (Eidgenössische Technische Hochschule, Zürich), Modification of glycoproteins on intact cells with an arylalkylamine for crosslinking studies (*submitted for publication*).

with the specified amount of the protease for the indicated times at room temperature. A molar excess of phenylmethylsulfonyl fluoride was added to terminate the reaction. The samples were washed once and used for extraction and/or electrophoresis.

Marker Assays

Bis (*p*-nitrophenyl)-phosphate phosphodiesterase was measured by the method of Linhardt and Walter [31]. Glucose-6-phosphatase was assayed as reported [25]. Cytochrome *c*-oxidase was measured by the method of Hodges and Leonard [23]. Hexosaminidase was assayed by the fluorimetric method of Lowden et al. [32]. Serotonin uptake, used as a marker for dense granules, was performed as described [16] with the following minor modifications: platelets from 2 liters of blood were resuspended in 50 ml of homologous plasma and incubated with 5 μCi of [^{14}C]-serotonin (53 mCi/mmol, Amersham) for 30 min at room temperature. The platelets were then sedimented and fractionation proceeded as described above. Aliquots of the homogenate and of the fractions were used for radioactivity and protein determinations.

The radioimmunoassay for β-thromboglobulin (Amersham), which has been used as a marker for α-granules from human platelets [1, 19], was found not to react with either porcine platelet homogenate or purified porcine α-granules. Controls using human platelets in identical media gave the expected results. Fibrinogen was assayed by the staphylococcal clumping test [2] using the Sigma kit and human fibrinogen as a standard. The immunodiffusion plate system (M-Partigen-Fibrinogen, Behring) was used to confirm the results of the clumping assay.

Electrophoresis and Miscellaneous Methods

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli [29]. The stacking gel contained 5% acrylamide. Unless otherwise specified 10% acrylamide was used in the separating gel. Staining with Coomassie blue or the periodic-acid-Schiff (PAS) method was by standard procedures. Gels with ^{125}I-labeled samples were dried on a BioRad Model 224 gel slab dryer and directly used for autoradiography. [^{14}C]-labeled gels were treated with EN^{3}HANCE (New England Nuclear) as specified by the manufacturer prior to drying and fluorography. Kodak XR-7 film was used in both instances. Scanning of PAS-stained gels (550 nm) or of autoradiograms (600 nm) was performed with a Gilson Spectrophotometer Model 250, adapted with a Model 2520 gel scanning unit. Protein was determined by the method of Bradford [7] using the BioRad reagent. Phospholipid phosphorus was measured by the technique of Bartlett [4]. Aquasol II (New England Nuclear) was used for liquid scintillation counting in a Model 2405 Packard counter.

Electron Microscopy

For transmission microscopy, the samples were fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.0. Post-fixation was in 1% OsO_4, followed by dehydration through graded ethanol solutions and into propylene oxide, and finally embedding in Araldite-Epon. Samples were thin-sectioned using a Porter-Blum MT2 Ultramicrotome, stained with uranyl acetate and lead citrate and the grids were examined using a Philips EM201 microscope at 60 kV.

For freeze-etching, membranes of granule suspension in TRB were fixed by addition of four volumes of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After 12 hr on ice the samples were sedimented and the pellet washed with 0.1 M phosphate buffer, pH 7.4. Samples were fractured on a Balzers BA 360 freeze-fracturing apparatus at −100 °C and etched for 20 sec. The replica was generated by shadowing with platinum-carbon at a 45° angle to a depth of 5 nm. Specimens were examined on Philips electron microscopes models 201 or 300.

Materials

Trypsin, chymotrypsin, porcine fibrinogen, phenylmethylsulfonyl fluoride, rotenone, adenosine triphosphate and 2-deoxy-D-glucose were all from Sigma. Iodogen was from Pierce. [^{14}C]-aniline hydrogen sulfate (98.8 mCi/mmol) and Na [^{125}I] were from Amersham. [^{14}C]-serotonin was from New England Nuclear.

Results

Density Gradient Fractionation

Because we used a modification of pre-existing isolation procedures [8, 16, 40], we examined the different sucrose gradient fractions extensively by electron microscopy to ascertain the homogeneity of the isolated α-granules. A typical density gradient showed five distinct bands, and a pellet of variable size. The uppermost band, Fraction I (frequently split into a doublet) consisted largely of membrane vesicles, some of which contained variable amounts of amorphous material (panel *A* in Fig. 2). Fraction II (Panel *B*) consisted largely of mitochondria and a few dense core organelles which could represent lysosomes (*see below*), whereas fractions III and IV contained mostly α-granules, but a few mitochondria and incompletely lysed platelets were regularly observed (Panel *C*). Fraction V was always finely aggregated, particularly when the gradients were heavily loaded. These aggregates were easily dispersed upon washing and resuspension. Fraction V was composed almost exclusively of α-granules, with very little contamination with other organelles or platelet remnants (Panel *D*, Fig. 2). The pellet (Panel *E* in Fig. 2) was enriched in dense or bull's-eye granules.

The identity of the components in the different fractions was confirmed by marker enzyme studies. Table 1 shows the results of these studies. Phosphodiesterase, a plasma membrane marker in pig platelets [42], remained largely in the $10{,}000 \times g$ supernatant, so that the pellet (P in Table 1) which was resuspended and applied to the gradient, had a diminished relative activity. When compared to the pellet, phosphodiesterase activity was relatively enriched in fractions I and II and decreased in the remaining fractions. The activity of glucose-6-phosphatase activity (an endoplasmic reticulum marker) was detected throughout the gradient with relative specific activities lower than that of the homogenate. The mitochondrial marker (i.e. cyto-

Table 1

	P	Fraction					
		I	II	III	IV	V	VI
Marker enzyme		(0.25–1.2)[a]	(1.2 –1.4)	(1.4 –1.6)	(1.6 –1.8)	(1.8 –2.0)	(>2.0)
Phosphodiesterase	0.55±0.13	0.92±0.12	0.61±0.12	0.51±0.14	0.32±0.06	0.41±0.21	ND
Glucose-6-phosphatase	1.04±0.04	0.77±0.1	0.62±0.11	0.44±0.18	0.44±0.08	0.63±0.16	ND
Cytochrome oxidase	5.69±1.9	10.54±6.1	15.66±6.1	3.03±0.6	1.35±0.7	1.59±0.9	ND
Hexosaminidase	2.70±0.9	2.13±0.3	2.39±0.9	1.81±0.7	1.34±0.1	1.84±0.4	ND
Fibrinogen	2.08±0.4	0.45±0.1	1.44±0.3	3.74±1.2	4.97±0.1	4.97±0.1	(3.94)[b]
Serotonin	3.6 ±1.2	0.06±0.06	0.05±0.04	0.26±0.2	3.1 ±1.7	5.51±0.3	129.3 ±2.9
Protein	13.6 ±1.3	0.21±0.01	0.20±0.01	0.41±0.1	2.3 ±1.2	3.6 ±1.1	0.04±0.03

[a] Numbers in parentheses indicate the molar concentration of sucrose above and below the interphase. Band VI is the pellet at the bottom of the 2 M layer. The numbers indicate the relative specific activity, i.e. specific activity of the fraction/specific activity of homogenate. The protein values are percent of the homogenate. The values are the mean ±SE of at least three experiments performed in duplicate. P = 10,000 × *g* pellet. ND = not determined. Enzyme assays were not performed in fraction VI because the small amounts of material available made the determinations unreliable. The enzyme activities of the 10,000 × *g* supernatant, which are complementary to those found in the pellet, are not reported to avoid redundancy.

[b] One determination only. The specific activities of the enzymes in the homogenate were (in μmoles/hr mg protein): phosphodiesterase = 0.267; cytochrome oxidase = 0.929; glucose-6-phosphatase = 0.272 and hexosaminidase 0.027. The fibrinogen content of the homogenate was 9.5 μg/mg protein.

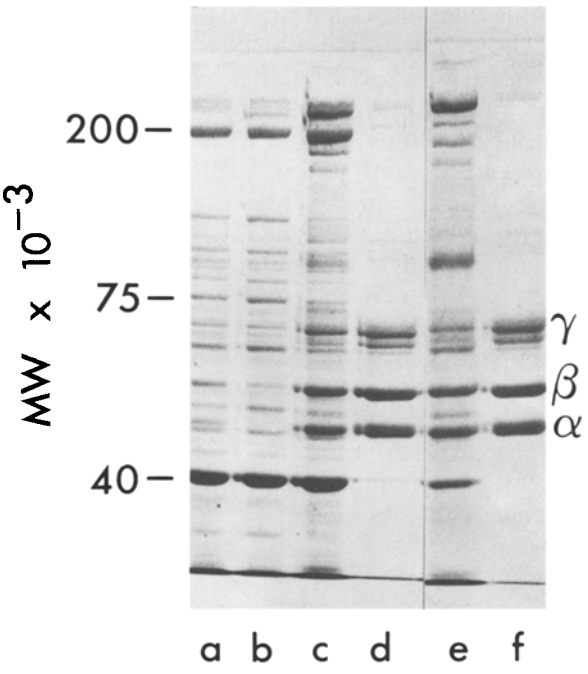


Fig. 1. Fibrinogen enrichment in the α-granule fraction. Porcine platelet homogenate (*a*), the 10,000 × *g* supernatant (*b*) and pellet (*c*) and a sample of fraction V from the gradient (*e*) were electrophoresed in 7.5% acrylamide gels with SDS. Purified porcine fibrinogen was used as a reference (*d* and *f*). The molecular weight scale is in kilodaltons. The position of the fibrinogen chains is indicated

chrome oxidase) was found preferentially in fractions I and II, and hexosaminidase, a lysosomal marker, was spread throughout the gradient, being slightly more abundant in fractions I and II. Figure 1 shows evidence that fibrinogen, known to be found in α-granules [19, 28], is markedly enriched in the fractions that morphologically correspond to α-granules. Samples of the homogenate, and of different fractions were electrophoresed in polyacrylamide gels, and purified porcine fibrinogen was used as a standard. Clearly, the 10,000 × *g* pellet and the final α-granule preparation are considerably enriched in fibrinogen content. More quantitative results were obtained using the staphylococcal clumping test. As indicated in Table 1, fractions III-V, which are composed largely of α-granules, are enriched about fivefold in fibrinogen. Similar results were obtained using the fibrinogen immunodiffusion assay. The latter assay is considerably less sensitive and more expensive than the clumping method, and was therefore used only for confirmation.

Radioactive serotonin uptake was used as a marker of dense granules [16, 19]. In agreement with the electron-microscopical results, dense granules were found in significant quantities only in the pellet (band VI in Table 1), which was enriched over 100 times in serotonin. This value is higher than those reported by several authors [16, 19, 40], but is similar to that found by Rendu et al. [39].

In general, the organelle distribution is similar to those reported for human [8, 16] and pig [40] platelets in sucrose gradients. Fraction V was used for most of the following experiments. In some cases, however, fractions IV and V were pooled together and the results obtained were not significantly different. Although fraction III had a comparable purity, it contained very little material, and was therefore never used.

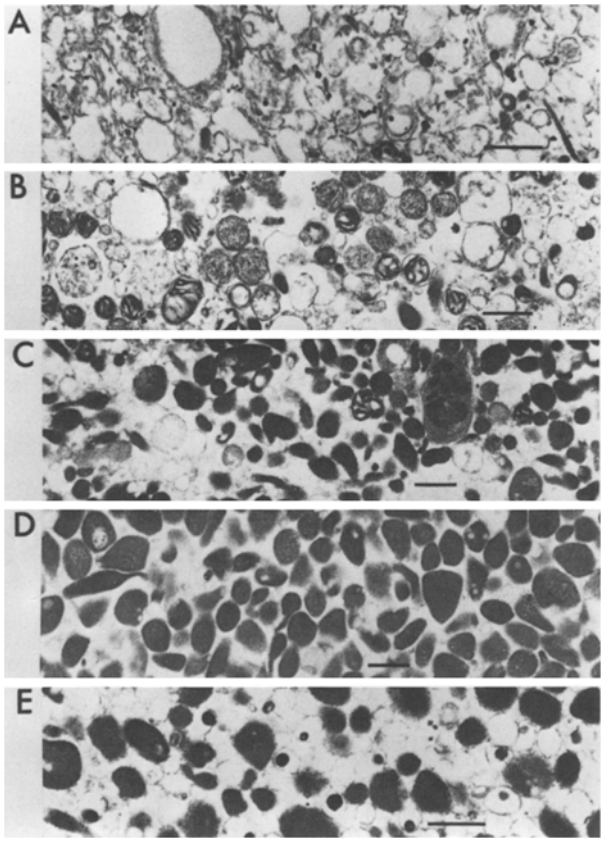


Fig. 2. Electron micrographs of samples harvested from the sucrose gradients. The individual bands were sedimented and the resulting pellets fixed and processed for electron microscopy. Fraction I (*A*) contains mostly membranous vesicles of varying size, some showing attached filamentous material. Fraction II (*B*) is formed almost exclusively by mitochondria. Fraction IV (*C*) shows mostly α-granules, with some mitochondria and incompletely disrupted platelets. Fraction V (*D*) is composed almost exclusively by α-granules. Dense (bull's eye) granules are occasionally observed. The pellet (Fraction VI, *E*) contains a mixture of dense and α-granules. Bars = 0.5 μm

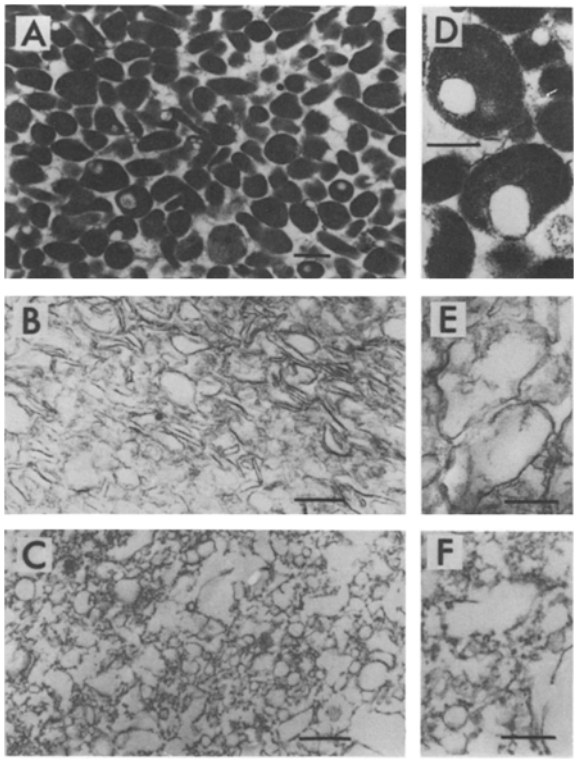
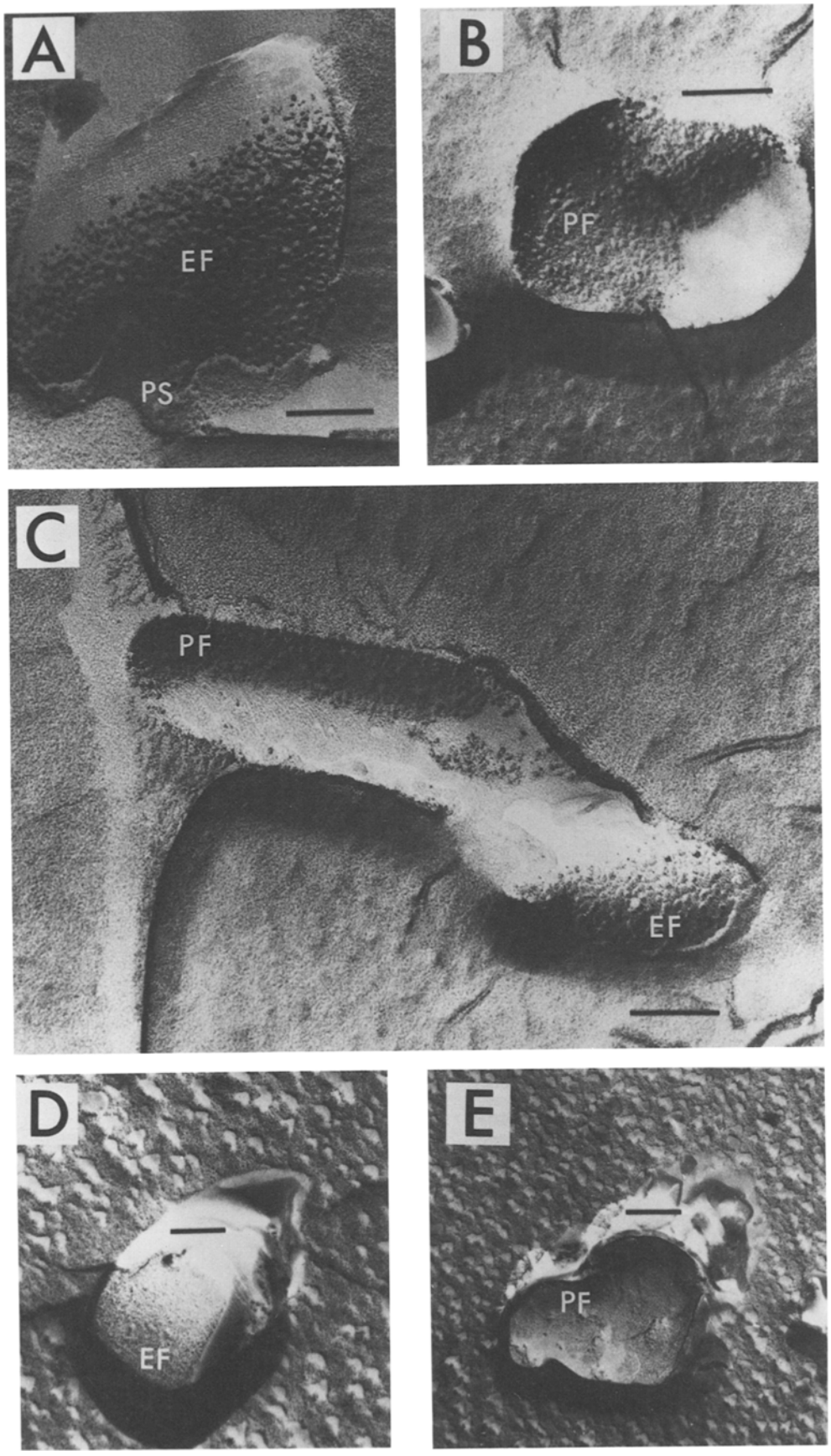


Fig. 3. Electron micrographs of thin sections of α-granules and their membranes. *A*) Purified α-granules from band *D* of the sucrose gradient. Note the homogeneity of this fraction, which contains oval electron-dense granules many of which show one or sometimes two clear eccentric structures (bar = 0.5 μm). *B*) Pellet obtained after alkaline extraction of isolated α-granules. This pellet contains tightly packed membrane vesicles from which the dense contents have been almost completely stripped (bar = 0.5 μm). *C*) Pellet from acid-extracted granules. Vesicles of varying size with substantial amounts of adhering core material are observed. Occasional incompletely lysed granules are also found (bar = 0.5 μm). Frames *D*–*F* (bar = 0.25 μm) are enlargements of *A*–*C*, respectively

Morphology

Granules in fraction V were found to be mostly round or oval, but a few were elongated or racket shaped (Figs. 2 and 3). Their average diameter was 400 nm and many of them showed one or more "vacuoles", i.e. areas that are substantially less electron dense than the general matrix. These vacuoles were always peripherally located and in some cases appeared clearly to be membrane bound (Fig. 3). In some preparations, a small fraction of the granules appeared to be lysed, i.e. devoid of most of their electron-dense core. Because these lysed membranes are unlikely to comigrate with the intact granules during density fractionation, we feel that "lysis" probably occurred at a later stage, either during washing of the gradient fractions, or during fixation. In this regard, we have found that granules prepared as described in Materials and Methods spontaneously lyse when transferred to isotonic salt media [43*a*].

Fracture of α-granule membranes results in visualization of the concave outer (PF) leaflet (Fig. 4*B* and *C*), which is dotted with intramembranous particles 4 to 7 nm in diameter. These are evenly distributed, and are superimposed on a background of more numerous smaller particles. The convex fractured (EF) surface (Fig. 4*A* and *C*) is almost uniformly covered by large particles of homogenous size (8 to 10 nm in diameter), also on a background of finer particles. The outer (PS)


A
EF
PS
B
PF
C
PF
EF
D
EF
E
PF

granule membrane, exposed after etching the preparation, is essentially smooth (Fig. 4*A*).

Polypeptide Composition

Figure 5 compares the polypeptide composition of purified α-granules, determined by polyacrylamide gel electrophoresis, with that of the starting platelet homogenate (lanes *a* and *b*). The α-granules contain at least 9 major and many minor polypeptides, and except for minor variations (*see below*), the patterns were remarkably reproducible ($n=26$). The molecular weights of the major bands range between 43,000 and 250,000 daltons. The largest variability was observed in the case of two bands, mol wt 43,000 and 200,000, presumably actin and myosin, respectively. The amounts of these components that copurified with the granules varied among preparations and decreased upon repeated washing in sucrose-Tris-EDTA buffer. An additional abundant component(s) comigrates with the tracking dye (mol wt <20,000). This material could be further resolved in 15% polyacrylamide gels (Fig. 5*h*). In addition to the components observed in 10% gels (lane *b*), a major band of mol wt 20,000 and a doublet of mol wt 14,000 to 16,000 were also discerned. Several minor bands of mol wt between 10,000 and 21,000 were also present. At least eight of the major polypeptides seen in 10% gels stain positively with the periodate-Schiff (PAS) procedure and are therefore probably glycoproteins (Fig. 6*B*). A band of mol wt 38,000, which is only faintly stained by the Coomassie stain, is also PAS positive. The major mol wt 20,000 band detected in 15% gels was only lightly PAS-positive, whereas intense staining was found in the mol wt 14,000 to 16,000 region (Fig. 6*h*).

Membrane Isolation

Several methods were assayed for the separation of the granule membranes and dense cores, with variable results (Table 2). The core was found to be firmly attached to the membranes so that mechanical disruption by sonication released little material to the supernatant, and a reticular matrix was seen to remain attached to the broken mem-

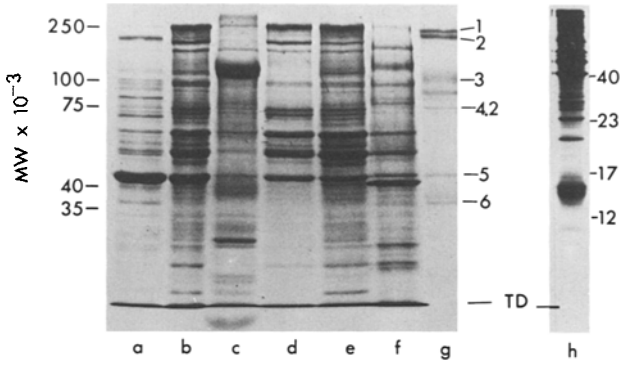


Fig. 5. Polypeptide composition of α-granules and their membranes. Lanes *a–g*: samples were reduced and electrophoresed in 10% polyacrylamide gels as described in Materials and Methods, stained by the PAS method (Fig. 6) and finally suprastained with Coomassie blue. Lane *a*: whole platelet homogenate after nitrogen cavitation. Lane *b*: isolated α-granules. Lane *c*: pellet obtained after alkali extraction and centrifugation of isolated granules. Lane *d*: supernatant fraction from alkali-extracted granules. Lane *e*: pellet from acid-extracted granules. Lane *f*: supernatant fraction from acid-extracted granules. Lanes *a–f* had 95 μg protein. Lane *g*: human red cell ghosts. The major ghost polypeptides, which were used as mol wt standards (*see* legend to Table 3), are numbered according to [41]. Lane *h*: electropherogram of intact α-granules in a 15% polyacrylamide gel. The mol wt scale is in kilodaltons. TD = tracking dye (bromphenol blue)

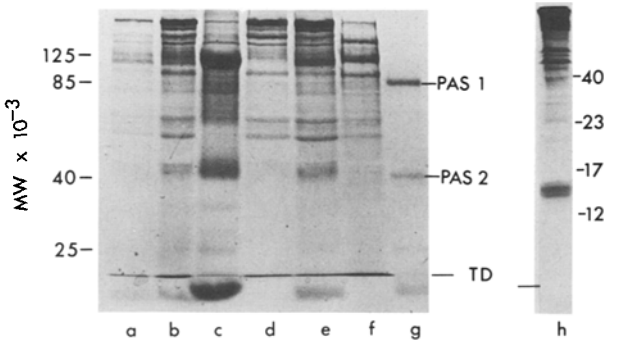


Fig. 6. Glycopeptide composition of intact α-granules and their membranes. Fractions were obtained as in Fig. 5 and stained by the PAS method. Lanes *a–g*: 10% polyacrylamide gel. Lane *a*: whole platelet homogenate. Lane *b*: purified α-granules. Lane *c*: alkali-extracted pellet. Lane *d*: supernatant from alkali extraction. Lane *e*: acid-extracted pellet. Lane *f*: supernatant from acidic extraction. Lanes *a–f* had 95 μg protein. Lane *g*: human red cell ghosts. The position of the PAS 1 and 2 bands is indicated. Lane *h*: electropherogram of intact α-granules in a 15% gel. The mol wt scales are in kilodaltons. TD = tracking dye (bromphenol blue)

Fig. 4 (*facing page*). Freeze-etching of purified pig α-granules and their alkali-isolated membranes. *A*) The EF (exoplasmic fracture) and a small area of the PS (protoplasmic surface) leaflet (bottom) of intact isolated granules are shown. Numerous large intramembranous particles are seen on the EF surface, whereas the PS surface appears smooth (bar = 0.1 μm). *B*) The PF (protoplasmic fracture) leaflet of intact isolated α-granules shows intramembranous particles of varying size on a finely granulated background (bar = 0.1 μm). *C*) Both the convex and concave surfaces of intact granules are shown (bar = 0.1 μm). *D* and *E* are replicas of alkali-extracted membranes. Notice that the large particles on the fracture faces have disappeared and only the coarse background remains (bar = 0.1 μm)

Table 2. Elution of α-granule components during lysis[a]

Extraction procedure	% protein	% phospholipid phosphorus
Hypotonic shock	37	<10
KI	73	ND
pH 2	50	<10
pH 12	87	<10

[a] α-granules were extracted under the above conditions as described in Materials and Methods. Samples of the suspension were taken before and after centrifugation at 90,000 × *g* for 30 min and used for protein and phospholipid phosphorus determinations. The numbers refer to the percent remaining in the supernatant after sedimentation of the membranes. 10% of the original phospholipid phosphorus was the smallest amount that could be reliably measured with the method employed. ND = not determined. The results are the mean of 2–6 determinations.

branes by electron microscopy (*not illustrated*). Following hypotonic lysis and centrifugation at 100,000 × *g* for 30 min, up to 37% of the granule protein remained in the supernatant. However, recovery of the sedimented fraction was very difficult since a diffuse sticky film of material, rather than a pellet, was obtained, making morphological and biochemical analyses impossible. For these reasons, more drastic methods which perturb protein-protein interactions were attempted. Treatment of the granules with 1 M KI released 73% of the protein, but as was the case for hypotonically treated membranes, recovery was difficult and incomplete. Incubation of the granules in acidic solutions (pH 2) lysed the granules, solubilizing up to 50% of the protein, with essentially complete recovery of phospholipids in the pellet (Table 2). Electron micrographs of the sedimented material (Fig. 3*C* and *F*) showed lysed membranes with considerable amounts of adherent amorphous material, presumably representing remnants of the granular contents. Occasionally, incompletely broken granules with most of their cores were also observed. A Coomassie blue-stained gel of the material that remains membrane-bound after acidic extraction is shown in Fig. 5*e*. For comparison, the polypeptide pattern of the solubilized fraction is also included (5*f*). PAS-stained samples are also presented (Fig. 6*e* and *f*). Few differences are observed between the two fractions, consistent with the incomplete content solubilization detected morphologically. Several high molecular weight components are substantially enriched in the pellet. The bands of the solubilized and sedimented fractions are not perfectly complementary when compared to the starting material, indicating that some hydrolysis and/or aggregation occurred upon extraction.

Extraction in isotonic alkaline media proved to be the method of choice for the isolation of intrinsic membrane components. Incubation at pH 12 for 10 min at room temperature was found to yield rapid and reproducible separations, and therefore this protocol was chosen for the experiments described hereafter. Upon addition of the alkaline medium, the initially turbid granule suspension clarified and after centrifugation a translucent pellet, containing 13% of the initial protein but over 90% of the phospholipids (Table 2), was obtained. The pellet was tightly packed and displayed rather uniform vesicles and flattened sacs with only traces of the original contents of the granules (Fig. 3*B* and *E*). As has been observed for zymogen granules [9], alkali-extracted α-granule membranes were almost entirely free of intramembranous particles when viewed after freeze-fracture (Fig. 4*D* and *E*). This is true for both the EF and PF leaflets.

The Coomassie blue pattern of membranes extracted at pH 12 was greatly simplified when compared to the original granules (compare 5*b* and 5*c*). One major band (mol wt 125,000) and several minor bands remained attached to the membranes. The former could occasionally be resolved into two bands. Another large polypeptide, mol wt 230,000, sedimented with the pellet to varying extents (compare Figs. 5 and 8) and could be largely removed by repeated washing. No additional bands could be resolved in the membrane fraction in 15% polyacrylamide gels (*not shown*).

The pellet and supernatant fractions of alkali extraction were also stained with the PAS technique (Fig. 6*c* and *d*). Most of the bands in the pellet proved to be glycoproteins. As expected, the complementary glycoproteins were found in the soluble fraction. The glycoprotein composition of the membranes was also analyzed with the more sensitive [^{14}C]-aniline labeling method. A densitometric scan of one such fluorograph is illustrated in Fig. 7. Predictably, the 230,000 and 125,000 dalton bands were also the primary labeled components, but in addition about four or five minor peaks could also be detected.

Surface Labeling of Intact Granules

Surface specific reagents were employed to investigate the exposure of α-granule membrane proteins to the cytoplasmic (external) milieu [14]. The granules were labeled by iodination using the sparingly soluble chloroamide Iodogen. The labeling effi-

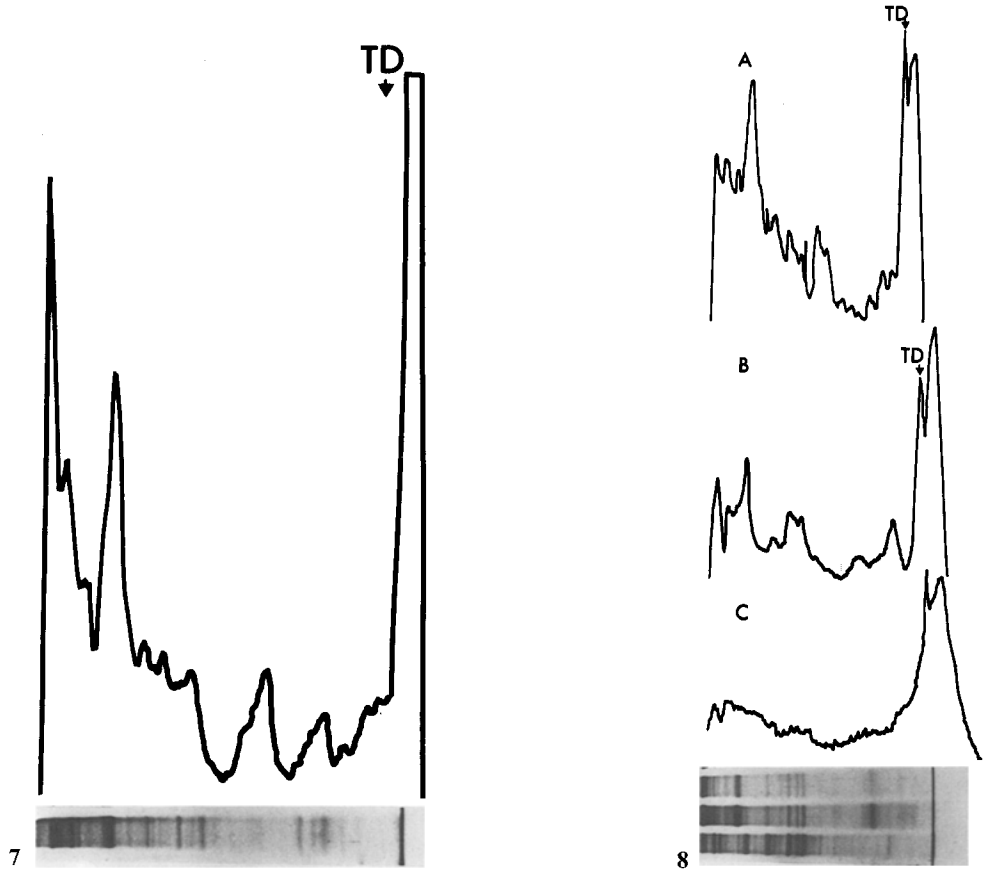


Fig. 7. Glycopeptide composition of isolated α-granule membranes. Membranes prepared by alkali extraction were labeled with $NaIO_4$-[^{14}C] aniline as described. The samples were electrophoresed, and the gels were stained, photographed and finally used for fluorography. A scan of one such fluorogram is illustrated, together with a photograph of the corresponding Coomassie blue-stained sample. TD = tracking dye

Fig. 8. Labeling of α-granules and their membranes with ^{125}I plus Iodogen. *A*) Membranes isolated by alkali extraction were labeled and electrophoresed. A scan of a typical autoradiogram is illustrated. *B–C*) Intact isolated α-granules were labeled, washed and finally subjected to alkali extraction. The pellet (*B*) and supernatant (*C*) fractions obtained were electrophoresed and scans of the resulting autoradiograms are shown. The corresponding Coomassie blue-stained patterns are also illustrated for reference. *Top:* labeled membranes; *middle:* membranes isolated from labeled granules; *bottom:* supernatant from alkali-extracted labeled granules. Note that a smaller amount of protein was loaded on *A* as compared to *B* and *C*

ciency of this reagent is demonstrated in Fig. 8*A*: when leaky membranes isolated by alkali extraction of the granules are iodinated, essentially every band detectable by protein staining is also radioactively labeled. In contrast, when intact granules are treated under comparable conditions, different results are obtained. The specific activity of the labeled granules was always at least 20-fold lower than that of labeled isolated membranes. The specificity of the labeling became even more apparent when membranes and contents of labeled granules were separated by alkali extraction (Fig. 8*B* and *C*). Several membrane bands, including the major glycoproteins, were found to be labeled by Iodogen (Fig. 8*B*), although to a much lesser extent than in isolated membranes (Fig. 8*A*; note that the amount of protein loaded on *A* is smaller than that on *B*). On the other hand, the soluble fraction which contains mostly intragranular proteins, was only marginally labeled (Fig. 8*C*), consistent with the surface specificity of the iodination technique.[3]

Proteolysis of α-Granules

The topography of the granule surface was further analyzed by means of proteolytic treatment, fol-

[3] A precise determination of the fraction of radioactivity bound to membranes and contents could not be obtained directly, due to the presence of free ^{125}I in the granules even after 3 washes. More extensive washing resulted in damage to the granules and loss of material. Quantitation could, however, be obtained by slicing fixed gels and integrating the peaks. In these experiments the membrane fraction had a specific activity over 20 times higher than that of the contents.

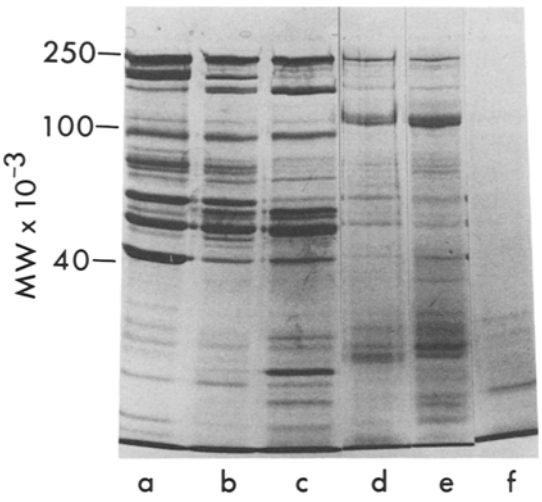


Fig. 9. Proteolysis of α-granules. Intact α-granules were treated with 0 (*a*), 5 μg/ml (*b*) or 50 μg/ml (*c*) trypsin for 30 min. After terminating the reaction, the granules were washed once and used for electrophoresis. Lanes *a–c* contained 75 μg protein. Similarily treated granules were also used for membrane isolation by alkaline extraction. Membranes from untreated granules (*d*) or from granules pretreated with 50 μg/ml trypsin (*e*) are shown. Finally, membranes isolated from untreated granules were exposed to 50 μg/ml trypsin for 30 min, washed and electrophoresed (*f*). Samples *d–f* contained 75 μg protein. All the samples were from the same granule preparation, and were electrophoresed and stained simultaneously

lowed by electrophoretic analyses of the samples. Figure 9 shows the effects of trypsin digestion of α-granules and their membranes. When treated with low trypsin concentrations, most of the granule polypeptides were unaffected (Fig. 9*b*). Major changes were only noted in the 200,000 and 43,000 dalton polypeptides, probably myosin and actin, which are expected to be bound on the external granule surface. Higher trypsin concentrations resulted in a more noticeable digestion (Fig. 9*c*) of the granules. Nevertheless, membranes isolated from these granules were similar to those of untreated granules (Fig. 9*d* and *e*), suggesting that most of the membrane glycoproteins are not readily accessible. In contrast, exposure to trypsin *after* isolation of the membranes led to extensive protein degradation (Fig. 9*f*). Similar results were obtained using papain or chymotrypsin (*not shown*). Taken together, these results suggest that at least a fraction of the granules are sealed, and their contents inaccessible to the medium.

Discussion

Although porcine platelets have been fractionated in the past [40, 42, 43], no specific markers were used for the identification of α-granules. The only exception, to our knowledge, is a recent report [10] in which β-N-acetylglucosaminidase was used as a marker. However, the same enzyme has been repeatedly used as a *lysosomal* marker in both pig [40] and human [1] platelets, so that its validity as an α-granule indicator is questionable. Fibrinogen, instead, is reported to be located largely in α-granules [19, 28], and could therefore be used as a specific marker. Three different methods (gel electrophoresis, immunodiffusion and the staphylococcal clumping assay) indicated that fibrinogen is enriched in the α-granule fraction. In all cases, plasma fibrinogen was used as a reference. (For discussion on the identity of plasma and platelet fibrinogens *see* James et al. [26].) Thus, because antibodies against human β-thromboglobulin (the commonly used α-granule marker in human platelets) failed to react with porcine globulin, determination of fibrinogen was a useful alternative.

Methodological limitations of the isolation procedure are probably the reason why the structure of platelet α-granule membranes has not been systematically explored. Methods for isolation of human α-granules have been reported in the past [8, 11] but the fact that improvements on these methods are still being introduced [19] is an indication that they are only partially satisfactory. In this report, pig α-granules prepared by a modification of pre-existing procedures [8, 16, 40] were analyzed biochemically and morphologically. This isolation procedure, which reproducibly yielded substantial amounts (20–25 mg protein/liter blood) of morphologically pure α-granules, was based on: (a) metabolic depletion of the platelets; (b) homogenization by nitrogen cavitation, and (c) fractionation by differential centrifugation followed by a discontinuous sucrose gradient. The granules obtained by this method are relatively stable in sucrose-Tris buffer but become increasingly fragile in media of higher ionic strength, an observation that has also been reported by Gogstad [19].

A number of proteins released during platelet activation have been identified as components of the α-granules, mostly by immunological methods [11, 17, 27, 28, 48, 49]. The subunit composition and molecular weight of the human form of most of these proteins has been reported, and is compared in Table 3 with the molecular weight of the predominant polypeptides of porcine α-granules, as determined by polyacrylamide gel electrophoresis under reducing and denaturing conditions. Allowing for possible species-related differences, all the reported components of α-granules can be accounted for by Coomassie-stained bands in the electropherogram.

Bands with molecular weights corresponding to those of actin and myosin were frequently observed in the α-granule fraction, but their amounts varied among preparations and decreased with repeated washing in Tris-sucrose medium. Whether these proteins are actual components of α-granules or become unspecifically adsorbed to their surface during isolation cannot be presently resolved. The presence of actin in a granular fraction of pig platelets was reported [43] and actin binding to other secretory vesicles, particularly chromaffin granules, has been studied in some detail [46]. A role for the contractile proteins in secretion has been repeatedly suggested [6, 46], but no evidence is available involving the acto-myosin complex in α-granule content secretion.

Due to the nature of the interaction of the core proteins with the membrane, harsh extraction methods need to be used to isolate the latter. These methods (high ionic strength or extreme pH extractions) are known to remove extrinsic membrane proteins, so that only intrinsic components remained in the isolated membranes analyzed in this study. Alpha-granule membranes displayed one major and several minor intrinsic polypeptides, some of which contain a carbohydrate moiety, as evidenced by both PAS staining and periodate-[^{14}C]-aniline labeling. Interestingly, the major glycoprotein comigrates with one of the principal plasma membrane glycoproteins (*unpublished observations*). Similar findings have been reported for human platelets [20]. Localization of the membrane components on either granule surface was attempted by conventional surface labeling and proteolysis techniques [13]. In order to differentiate between the internal and external aspects of the membrane, this approach involves the assumption that sealed vesicular structures are being studied. Several lines of evidence bear out this assumption: (a) electron micrographs show no major discontinuities in the granule profile and loss of dense material was observed only in a very small fraction of granules; (b) surface specific labeling with Iodogen failed to label the granule contents, and (c) low concentrations of proteases added to intact granules did not hydrolyse the content proteins, but produced extensive cleavage when added to solubilized contents (*not shown*). We have also found that the granules are osmotically sensitive, as determined turbidimetrically, implying that at least a fraction of them are sealed [43*a*].

Labeling of the external surface of intact granules was very limited in the case of ^{125}I plus Iodogen. Consistently, externally added proteases induced only minor changes in the electrophoretic profile of the membranes. This evidence points to a very limited exposure of protein chains on the cytoplasmic surface of the α-granules. This could be a characteristic feature of these membranes, but could also be the result of the extraction of loosely bound components during the lengthy isolation procedure, which is carried out mostly in media of low ionic strength and in the presence of EDTA, conditions that are known to remove extrinsic polypeptides.[4]

In summary, porcine α-granules isolated by the method reported in this communication proved to be largely sealed structures almost completely filled by an electron-dense core, except for a clear eccentric nucleus which appears itself to be membrane bound. As isolated in this report, the α-granule membrane bears no major extrinsic proteins on its cytoplasmic surface and, after isolation at pH 12, displays one major glycoprotein which could account for the numerous intramembranous

Table 3. Comparison of pig α-granule polypeptides with macromolecules released from human α-granules

Porcine polypeptides mol wt × 10^{-3} [a]	Released polypeptides	mol wt × 10^{-3}	Ref.
230	Factor VIII	~230	[35]
	Fibronectin	220–230	[47]
200			
180	Thrombospondin	185	[38]
100			
72			
70.5	Albumin	68	[21]
	Fibrinogen γ chain	63.5	[33]
60	Antiplasmin	60	[37]
52.5	Fibrinogen β chain	56	[33]
43	Fibrinogen α chain	47	[33]
20			
14–16	Platelet growth factor(s)	13–16	[3]
	β-thromboglobulin	8.9	[34]
	Platelet factor 4	7.8	[44]

[a] Apparent molecular weights were calculated from polyacrylamide gel migration patterns of the granules using human red blood cell ghosts, chymotrypsinogen, myoglobin, cytochrome *c* and the synthetic BDH markers (mol wt 2,512–16,949) as standards. The molecular weights of the major ghost polypeptides were considered as follows [41]: Band 1: 240,000; band 2: 215,000; band 3: 95,000; band 4.2: 72,000; band 5: 43,000; band 6: 35,000.

[4] Proteolysis of externally facing components cannot be entirely ruled out, but is unlikely because the results were similar whether or not protease inhibitors were present during homogenization and fractionation. Moreover, the presence of myosin – which is highly susceptible to proteolysis – in the granule fraction argues against this possibility. When present, myosin was readily hydrolyzed by externally added enzymes.

particles observed in freeze-fractured preparations of intact granules. The carbohydrate-bearing portions of these proteins probably face the intragranular milieu, since they are not cleaved by externally added proteases, nor are they significantly labeled by external galactosé oxidase plus B^3H_4 (Van der Meulen, *unpublished observations*). Some of the proteins constituting the dense granular core are anchored to the membrane, and remain attached to it unless protein-protein interactions are altered. This was achieved in our experiments by manipulating the pH of the incubation medium, but an equivalent perturbation must occur *in vivo* if successful secretion is to take place.

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