Isolation and characterization of lipid–protein particles containing platelet factor 3 released from human platelets

Helena SANDBERG,* Lars-Olov ANDERSSON* and Stefan HÖGLUND†

*KabiVitrum AB, Research Department, Biochemistry, S-112 87 Stockholm, Sweden, and

†Department of Biochemistry, University of Uppsala, S-751 23 Uppsala, Sweden

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Lipid–protein particles with platelet factor 3 measured by the Stypven clotting-time test [Hardisty & Hutton (1966) *Br. J. Haematol.* **12**, 764–776] have been isolated from platelet-release supernatant. Starting material was washed platelets, which were released by treatment with collagen. Purification of the particles from other components in the release material was accomplished by gel filtration on Sepharose CL-4B followed by affinity chromatography on poly-L-lysine–Sepharose CL-4B gel. Chemical characterization showed that the particles were composed of 40% protein, 42% phospholipids, 13% cholesterol and 5% triacylglycerols. The phospholipid composition was 38% phosphatidylcholine, 25% phosphatidylethanolamine, 9% phosphatidylserine, 2% phosphatidic acid and 26% sphingomyelin. No carbohydrate was detected. Electron-microscopic studies revealed the presence of membranous particles with diameters between 70 and 170 nm.

It has been known for a long time that phospholipid components are involved in the normal blood-coagulation process. They participate in at least two different steps. Factor X has been shown to be activated by a complex of Ca^{2+} and phospholipid containing factor IX_a and factor VIII (Lundblad & Davie, 1964; Schiffman *et al.*, 1966; Hougie *et al.*, 1967; Hemker & Kahn, 1967; Esnouf & MacFarlane, 1968; Hemker *et al.*, 1970). Milstone (1964) and later many others (Papahadjopoulos & Hanahan, 1964; Esnouf & Jobin, 1965; Barton *et al.*, 1967; Hemker *et al.*, 1967; Barton & Hanahan, 1969) have shown that phospholipids are involved in the activation of prothrombin. The role of the phospholipids in coagulation appears to be to provide a binding surface on which many coagulation factors are bound and kept together in favourable positions for the proper hydrolytic splitting reactions to take place. Since many coagulation factors are present in blood at very low concentrations, the binding to phospholipid is an important mechanism for increasing their effective concentrations for reaction with each other. The vitamin K-dependent coagulation factors, i.e. factors II, VII, IX and X, are bound to phospholipid vesicles only in the presence of Ca^{2+} . These factors contain γ -carboxyglutamic acid residues, and Ca^{2+} is prob-

ably bound to these residues. It is possible that these factors are bound to the phospholipid surface, with Ca^{2+} ions functioning as bridges between γ -carboxyglutamic acid residues of the coagulation factors and phosphate or other Ca^{2+} -binding ligands of the phospholipid. The factors V and VIII are also bound to phospholipid, but the mechanisms of their binding are not known. They are probably different from those of the vitamin K-dependent factors.

Despite the essential role of the phospholipid components in normal blood coagulation, very little is known about their nature, structure and function. In coagulation assays one usually employs phospholipid extracts from brain. They substitute quite well for the native phospholipid components, but they are probably different from them in several respects. It has been known for a long time that most of the active phospholipid components participating in normal blood coagulation *in vivo* originate from, or are bound to, the platelets (Born, 1972; Walsh & Biggs, 1972). Recently it was shown that more than 95% of the phospholipid-related activity in normal plasma is derived from the platelets (Sandberg & Andersson, 1976). The high-density lipoproteins and, to some extent, even the low-density lipoproteins can partly substitute for the platelet-derived phospholipid components, but this contribution is negligible under normal conditions when platelets are present. The phospholipid-related coagulant activity of the platelet is usually called 'platelet

Abbreviations used: SDS, sodium dodecyl sulphate; PF 3, platelet factor 3.

factor 3' (PF 3) and is usually measured by the Stypven time-clotting test (Hardisty & Hutton, 1966), which is based on the activation of factor X and V by Russell's-viper (*Vipera russelli*) venom (Stypven). There has been some controversy as to whether PF 3 is a distinct component that is released from the platelets in connection with their aggregation or if it is an activity that is associated with the platelet membrane itself and becomes available during platelet aggregation (Marcus, 1969). Several reports (Horowitz & Papayouanou, 1967; Wolf, 1967; Hardisty & Hutton, 1966) show that considerable activity remains in the supernatant after sedimentation of the platelets.

In the present study a particle having PF 3 has been isolated from the supernatant of collagen-released and aggregated platelets. The final preparation was found to contain membranous particles with diameters between 70 and 170 nm and mainly composed of lipid. Some of the present results have already been presented in a preliminary form (Sandberg, 1979).

Experimental procedure

Materials

SDS, dithiothreitol, Tris (Trizma base), galactose oxidase (from *Dactylium dendroides*) and neuraminidase (from *Clostridium perfringens*) were obtained from Sigma, St. Louis, MO, U.S.A. EDTA, silica-gel H, and thin-layer plates, precoated with silica-gel 60 were from Merck, Darmstadt, Germany. Concanavalin A and Sepharose gels 2B, 4B, CL-2B and CL-4B were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Standard protein kit for molecular-weight determination using polyacrylamide-gel electrophoresis was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. CNBr was from Fluka AG, Buchs, Switzerland. Agarose for electrophoresis was obtained from Litex, Glostrup, Denmark. Plastic film for agarose electrophoresis was purchased from Du Pont de Nemours and Co. Inc., Wilmington, DE, U.S.A. Polyacrylamide (Cyanogum) was obtained from Fischer Scientific Co., Pittsburg, PA., U.S.A. Coomassie Brilliant Blue G-250 was procured from Serva, Heidelberg, Germany. Bovine albumin was obtained from Armour Pharmaceutical Co., Chicago, IL, U.S.A. Glucose was from Mallinckrodt, St. Louis, MO, U.S.A. Chromogenic peptide substrate S-2238 was obtained from KabiVitrum, Stockholm, Sweden. Human thrombin was prepared at KabiVitrum and had a specific activity of 2650 N.I.H. (National Institutes of Health) units/mg. Collagen (tendon) was obtained from Hormon Chemie, Munich, Germany. Poly-(L-lysine) hydrobromide (mol.wt. 1500) was purchased from Miles-Yeda Ltd., Kanakee, IL, U.S.A., and Stypven from Wellcome

Research Laboratories, Beckenham, Kent, U.K. Phospholipids, cholesterol and triacylglycerols were supplied by Supelco, Bellefonte, PA, U.S.A. NaB^3H_4 (40.5 Ci/mmol) was from Amersham, Arlington Heights, IL, U.S.A. All other chemicals used were the purest commercial available.

Methods

Isolation of platelets. Fresh human acid-citrate/dextrose-treated blood from fasting and healthy donors was centrifuged within 1 h of collection at 200g for 10 min at 20°C. The upper two-thirds of plasma were pipetted off and re-centrifuged at 200g for 10 min at 20°C in order to sediment the remaining red and white cells. The platelets were then sedimented at 1100g for 10 min at 20°C. The platelet pellet was suspended in Tyrode's buffer, pH 6.2, containing 0.137 M-NaCl, 2.7 mM-KCl, 11.9 mM- NaHCO_3 , 0.36 mM- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.0 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 mM-EDTA, 0.1% glucose and 0.35% bovine albumin. The platelets were sedimented again by centrifugation at 1100g. The supernatant was discarded and the platelets were washed again by repeating this step with the exception that EDTA was omitted in the Tyrode's buffer. Finally, the platelets were suspended in Tyrode's buffer, pH 7.35, containing 0.137 M-NaCl, 2.7 mM-KCl, 11.9 mM- NaHCO_3 , 0.36 mM- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1.0 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0 mM- CaCl_2 , 0.1% glucose and 0.35% bovine albumin. The final volume of the platelet suspension was one-fifth of the starting plasma volume. The final platelet concentration was usually between 1.5×10^6 and 2.2×10^6 platelets/ mm^3 .

Platelet count. The platelet count was made by using a microscope and a Bürker chamber.

Platelet aggregation. The platelets were aggregated in silicone-treated glass cuvettes at 37°C with a Payton dual-channel aggregometer stirring at 900 rev./min. Collagen or thrombin was used to induce platelet aggregation. The concentrations of agents yielding optimal platelet aggregation were used. Aggregation was allowed to proceed for 3–5 min. When large volumes of platelets (>5 ml) were aggregated, this was done in a water bath at 37°C placed on a magnetic stirrer.

Preparation of matrix-bound poly-(L-lysine) and concanavalin A. Poly-(L-lysine) and concanavalin A were coupled to Sepharose CL-4B by the method of Axén *et al.* (1967). The substitutions were 1 mg of poly-(L-lysine)/ml of gel and 8 mg of concanavalin A/ml of gel.

Preparation of synthetic phospholipid vesicles. Phospholipid vesicles composed of phosphatidylserine and phosphatidylethanolamine were made in a sonicating water bath. A mixture of 0.1% (w/v) of phosphatidylserine/phosphatidylethanolamine (2:3, w/w) was sonicated for 5 min in Michaelis buffer,

pH 7.4, composed of 0.026 M-sodium acetate, 0.026 M-sodium barbiturate and 0.108 M-NaCl.

Coagulation assays. PF 3 was assayed by using the Stypven clotting test (Hardisty & Hutton, 1966). Platelet-free plasma was made by centrifuging platelet-rich plasma at 20000g for 40 min. A mixture of 0.1 ml of platelet-free plasma, 0.1 ml of sample containing PF 3 and 0.1 ml of Stypven diluted 1:20000, was incubated for 1 min at 37°C. Then 0.1 ml of 0.025 M-CaCl₂ was added and the clotting time was recorded with a clotting-time-measuring apparatus (Fibrometer). As there is no defined activity unit for PF 3 and no international standard, we have arbitrarily defined 100 units of PF 3 as the amount of rabbit brain phospholipid added to the test mixture giving a Stypven clotting time of 15 s. A stable freeze-dried preparation of rabbit brain phospholipid was used as a reference. Factor IX and VIII were determined by the method of Veltkamp *et al.* (1968) and factor X by the method of Denson (1961). Factor V was determined by the method of Biggs (1972) and prothrombin by the method of Norén (1970).

Determination of lactate dehydrogenase. Samples were assayed for lactate dehydrogenase activity by using a commercially available assay kit (KabiVitrum, Stockholm, Sweden).

Polyacrylamide-gel electrophoresis. Polyacrylamide-disc-gel electrophoresis was performed in an SDS-containing multiphasic buffer system 4217-II-IV as described by Wyckoff *et al.* (1977). Samples were prepared by incubation at 65°C for 40 min in buffer containing 1% SDS, 1 mM-EDTA and, in most cases, 45 mM-dithiothreitol. The gel-staining procedure used was a modification [F. Dombrose (Department of Pathology, University of North Carolina), personal communication] of a method originally described by Blakesley & Boezi (1977). SDS was removed from the gels by placing them in a solution composed of 7.5% (w/v) acetic acid and 25% (w/v) propan-2-ol and then placed in a solution of 0.04% (w/v) Coomassie Brilliant Blue G-250 and 3.5% (w/v) HClO₄ (Reisner *et al.*, 1975) at 55°C for 1–3 h. To increase the intensity of the stained protein bands the gels were transferred to 3.5% (w/v) acetic acid at 55°C for 30 min (Holbrook & Lever, 1976). For destaining, 3.5% (w/v) acetic acid was used. The standards used for molecular-weight determinations were β -galactosidase (*M_r* 116 500), phosphorylase *b* (*M_r* 94 000), bovine albumin (*M_r* 68 000) and ovalbumin (*M_r* 43 000).

Electrophoresis on agarose gel. Plastic film (10 cm × 10 cm) was coated with a 1.5 mm-thick layer of 1% agarose and used for electrophoresis in 0.02 M-sodium barbital buffer, pH 8.6. The electrophoresis was for 4 h at 50 V and 5 mA. Human platelet-free plasma was run in parallel with the samples as a reference. After the run the protein

bands were precipitated with picric acid and stained with Coomassie Brilliant Blue.

Electron-microscopic analysis. Specimens were prepared either from material fixed with charcoal-treated glutaraldehyde (Sabatini *et al.*, 1963) overnight at 4°C in 0.05 M-cacodylate buffer at pH 7.0, or from material which had not been fixed. Samples applied to carbon-coated grids were dialysed against 0.1 M-ammonium acetate, pH 7.0, and were subsequently 'contrasted' with 2% ammonium molybdate or 2% phosphotungstic acid at pH 7.0. Other specimens were rinsed with 0.1 M-sodium acetate/acetic acid at pH 4.5 before contrasting with 1% uranyl acetate, pH 4.5. The specimens were examined in a Philips 300 or 400 electron microscope with an accelerating voltage of 60 kV. Photographs were taken in through focal series, with special precautions taken to minimize beam exposure (Williams & Fischer, 1970). The diameters of the objects were measured by a profile projector directly on the photographic negatives.

Amino acid analysis. Hydrolysis with 6 M-HCl was carried out for 24 h at 110°C in evacuated glass tubes. The hydrolysate was assayed with a Bio-Cal BC 200 analyser equipped with an Introfonic DRS-110 A integrator.

Carbohydrate analysis. Carbohydrate was analysed by using two methods: the phenol/H₂SO₄ method (Dubois *et al.*, 1956) and the neuraminidase/galactose oxidase/NaB³H₄ method (Gahmberg, 1976). When the latter method was used, PF 3 material (approx. 1 mg) from the affinity-chromatography step in 1 ml of phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.4) was incubated for 10 min at 37°C with neuraminidase (0.025 unit/ml) and galactose oxidase (3 units/ml). The enzymes were removed from the PF 3 material upon gel filtration on a column (0.9 cm × 20 cm) packed with Sepharose CL-4B. The PF 3 material was then incubated with NaB³H₄ (0.5 mCi) for 10 min at 22°C and rechromatographed on Sepharose CL-4B.

Lipid determination. Lipid extraction was performed by using the method of Folch *et al.* (1957). Phosphorus analysis (Bartlett, 1959) of the purified material with PF 3 and the extracted lipid indicated that less than 1% of the phospholipid phosphorus was lost during extraction.

Total lipids were determined by the method of Bragdon (1960).

Cholesterol and triacylglycerols were determined by quantitative densitometric t.l.c. using copper acetate reagent (Fewster *et al.*, 1969). For this purpose were used glass plates, 20 cm × 20 cm, coated with a 0.25 mm-thick layer of silica-gel 60 (precoated plates from Merck). The applied amount of cholesterol or triacylglycerol in standards and samples ranged from 0.5 to 5 μ g. The solvent system

used for the chromatography was light-petroleum (b.p. 35–80°C)/diethyl ether/acetic acid (90:10:1, by vol.). After chromatography the plates were sprayed with a 3% cupric acetate solution in aq. 8% H_3PO_4 and placed in an oven at 180°C for 10 min. Absorbances of the charred spots were measured with a Shimadzu dual-wavelength t.l.c. scanner (model CS-900) equipped with a BBC Goerz Servogor 320 recorder.

The phospholipid distribution was determined by t.l.c. Glass plates measuring 20 cm × 20 cm were coated with 0.5 mm layer of silica-gel H, previously washed by the method of Marcus *et al.* (1962). Before use the plates were activated at 110°C for 60 min. Chromatography was done in an equilibrated chamber containing the solvent system chloroform/methanol/acetic acid/water (50:25:7:3, by vol.). The fractionated lipids were made visible with iodine and the outlined spots were identified by comparing them with phospholipid references that had been run concurrently. They were then scraped off into borosilicate glass tubes and phosphorus analysis was carried out by the method of Parker & Peterson (1965).

Results

Centrifugation and gel-filtration studies

Fresh platelet-rich citrate plasma was used as the starting material to prepare a platelet concentrate by centrifugation as described in the Materials and methods section. The suspension of platelets washed by centrifugation twice in Tyrode's buffer was aggregated by adding collagen (4 µg/ml). After complete aggregation, which usually occurred after 3–3.5 min, the PF 3 in the suspension was determined by using the Stypven clotting-time assay. The values obtained were between 300 and 500 units/ml.

Centrifugation at +4°C of the suspension with aggregated platelets was performed at different centrifugal forces. At forces between 1500 and 15000 g for 15 min, the PF 3 found in the supernatant was about the same, i.e., depending on preparation, between 15 and 35% of the activity in the aggregated suspension. This indicates that at least a substantial fraction of the PF 3 can exist separately from the platelet. At higher forces the yield of PF 3 in the supernatant decreased. Thus at 20000 g, the activity yield obtained in the supernatant was between 8 and 13% and at 100000 g between 3 and 6%.

The yield of PF 3 in the supernatant after aggregation also depended, for instance, on the amount of time the suspension of aggregated platelets was allowed to stand before centrifugation. In a series of experiments using platelets from the same donor, a comparison was made of the activity

yield obtained in the supernatant after leaving the suspension of platelets for different periods of time before centrifugation. When the centrifugation was performed after $\frac{1}{2}$ h, 24% of the total activity was found in the supernatant, and after 2 h, 18%, compared with 29% when the suspension was centrifuged immediately. No decrease in activity was found in a control sample of release supernatant during storing for at least 2 h. It appears as if the component having PF 3 either slowly becomes bound to the aggregated platelets or forms aggregates that are more easily sedimented.

As there was a possibility that the PF 3 found in the release supernatant was due to cell-membrane fragments formed as a result of cell lysis possibly having taken place during the handling of the platelets, the cytoplasmic enzyme marker lactate dehydrogenase was measured. Supernatants obtained after removal of collagen-aggregated platelets by centrifugation at 5000 g for 15 min were assayed. However, in most cases no lactate dehydrogenase activity could be detected and was never in any case greater than 0.2% of the activity in the supernatant from the corresponding three-times-frozen-thawed whole suspension of released platelets. This indicates that no, or very little, cell lysis occurred and that the PF 3 appearing in the supernatant is due to a specific process.

Since after release and centrifugation the supernatant could still contain a few platelets (although not seen under the optical microscope), a few experiments were performed in which the supernatant was filtered through a 0.8 µm Millipore filter. Activity determinations before and after the filtration showed that the activity loss was negligible (155 units/ml before and 140 units/ml after filtration). Thus most of the material exhibiting PF 3 passed unchanged through the membrane and was obviously not associated with any residual platelets.

Material containing PF 3 remaining in solution after centrifugation at 5000 g for 15 min of collagen-aggregated platelets was studied by gel filtration. When the material was run on Sepharose CL-4B the activity eluted at the void volume of the column. On Sepharose CL-2B it eluted as a broad peak close to the void volume of the column. These observations suggest that the entity responsible for the supernatant PF 3 has a large Stokes radius. When non-cross-linked Sepharose gels were used, adsorption of PF 3 seemed to occur, as low recoveries and retardation effects were observed. These effects could be counteracted by using a buffer of high ionic strength.

To study the effect of thrombin-induced platelet aggregation, 0.3 N.I.H. unit of thrombin per ml was added to a platelet suspension. After removal of the aggregated platelets by centrifugation, the supernatant was subjected to gel filtration on Sepharose

CL-4B, where PF 3 eluted at the void volume of the column. The gel filtration separated PF 3 from the residual thrombin as tested by using the thrombin-specific chromogenic substrate S-2238 (Claesson *et al.*, 1977). About twice as much supernatant PF 3 was obtained when thrombin was used as aggregation inducer as compared with collagen.

Isolation of supernatant PF 3

Platelets isolated from 3 litres of freshly drawn acid-citrate/dextrose-treated blood were aggregated by adding collagen. After centrifugation of the platelet aggregates at 5000g for 15 min, the supernatant solution containing 15–35% of the total PF 3 of the platelet suspension was subjected to gel filtration on Sepharose CL-4B. EDTA (2 mM final concn.) was added to the gel-filtration buffer in order to dissociate any possible complex of PF 3 and clotting factors. Fig. 1 shows the elution pattern obtained. Fractions containing PF 3 were pooled and further purified by affinity chromatography on poly(L-lysine)-Sepharose CL-4B gel. Salt-gradient elution was performed and the elution profile obtained is shown in Fig. 2. The PF 3 was eluted in a

small number of fractions in a concentration about three times higher than in the gel-filtration eluate. The fractions were dialysed for 1 h against Michaelis buffer, pH 7.4, containing 2 mM-EDTA, before testing for activity. Table 1 shows the yields and degree of purification. The calculations are based on protein measurements made by using amino acid analyses. This gives a value of 138-fold purification for the final material. However, as shown below, protein accounts for only one-third of the PF 3 material, which means that the actual purification is about 50-fold. In a series of five purification runs, the yield varied between 47% and 68%, and the specific activity between 29 200 and 35 000 units/mg of protein.

The poly(L-lysine)-Sepharose CL-4B gel contained poly(L-lysine) of a mol.wt. of about 15000. Poly(L-lysine) of this molecular weight was chosen because it bound PF 3 such that it was possible to

Fig. 1. *Sepharose CL-4B chromatography of 155 ml of platelet-release supernatant*

The column (5 cm × 100 cm) was equilibrated in Michaelis buffer, pH 7.4, containing 2 mM-EDTA. Fractions (9 ml) were collected at a rate of 100 ml/h.

▲, PF 3; ●, A_{280} .

Fig. 2. *Affinity chromatography on poly(L-lysine)-Sepharose CL 4B*

Fractions from the Sepharose CL 4B step containing material with PF 3 were pooled and then 180 ml containing 0.7 g of protein were applied to a poly(L-lysine)-Sepharose CL-4B column (2.6 cm × 9 cm). The elution was performed with a linear gradient consisting of 100 ml of Michaelis buffer, pH 7.4, with 2 mM-EDTA and 100 ml of the same buffer containing 1 M-NaCl. Fractions (2.4 ml) were collected. ▲, PF 3; ●, A_{280} ; ···, [NaCl].

Table 1. *Purification of PF 3 particles isolated from platelet-release supernatant*

Fraction	Vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold) (based on protein measurements)
Aggregated suspension*	175	2712.5	—	—	—	—
Platelet-release supernatant*	155	62.0	14 570	235	100	1.0
Gel-filtration pool	180	0.70	12 240	17 486	84.0	72.9
Affinity-chromatography pool	28	0.27	8960	33 185	61.5	138.3

* The contribution to the protein content by albumin in the Tyrode's buffer has been subtracted in all calculations.

elute under mild non-denaturing conditions. A number of higher-molecular-weight poly-(L-lysine) fractions were tested, but they bound PF 3 so tightly that detergents had to be used for the elution. The data obtained indicated that the tightness of the binding was proportional to the length of the poly-(L-lysine) chain.

Characterization of the purified material

The gel-filtration experiments described above show that the PF 3 active component(s) is (are) large-sized. Electron microscopy of the purified material after fixation with glutaraldehyde and negative staining demonstrated the presence of membranous particles with diameters ranging between 70 and 170 nm (Plate 1). A mean diameter of 110 nm was calculated from the measurements of 380 particles. Some preparations were not fixed with glutaraldehyde but only rinsed with volatile buffer before negative staining. Compared with fixed preparations, similar results were obtained. Most particles appeared somewhat flattened, regardless of whether the material was fixed or not. In unstained specimens, round particles resembling phospholipid droplets (Höglund, 1980), were observed. Round, somewhat irregular particles were observed in specimens stained by potassium phosphotungstate or ammonium molybdate at pH 7.0 (Plate 1). Morphological features were most obvious on studies on uranyl acetate-stained particles at pH 4.5, peripheral projections of material associated with the particles being seen (Plate 1). The diameters of these projections were around 10 nm. The morphological analysis suggested a flattened membranous particle or an enveloped particle with some material inside the envelope (Plate 1). On storage at +4°C for several weeks it was observed that the PF 3 particles were slowly aggregated. This process was accompanied by gradually decreasing activity.

Electron-microscopic studies of platelet-release supernatant showed the presence of particles of about the same size and shape, thus indicating that the fractionation procedures did not affect the morphology of these particles.

Pooled fractions from the affinity-chromatography step on poly-(L-lysine)-Sepharex CL-4B were concentrated for agarose electrophoresis. On electrophoresis using plastic film covered with 1% agarose, the material was found to migrate as a single homogeneous spot with a mobility like that of an α_1 -globulin (Fig. 3).

The PF 3 material was also analysed by polyacrylamide-gel electrophoresis. Electrophoresis in SDS-containing buffer of denatured and reduced samples on 8% (w/v) polyacrylamide gels showed two main bands, of M_r 123 000 and 53 000 (Fig. 3). Stained material was also observed at the electrophoretic front. However, the intensity of this band

Fig. 3. (a) SDS/polyacrylamide-gel electrophoresis of particles with PF 3 incubated in 1% SDS, 1 mM-EDTA and 45 mM-dithiothreitol, and (b) electrophoresis of particles with PF 3 and platelet-free plasma on agarose gel in 0.02 M-sodium barbital buffer, pH 8.6

was not related to the amount of protein applied and was therefore considered as an artefact. Denatured but non-reduced samples did not penetrate 5 or 8% polyacrylamide gels; most of the material remained in the application chamber after the run.

The amino acid analysis of the material with PF 3 showed that the amino acid composition of protein associated with the particles was similar to that of ordinary globular proteins. Neither glucosamine nor galactosamine was found, suggesting that no carbohydrate is associated with the material. Nor was any evidence for carbohydrate found by using the phenol/ H_2SO_4 method or the more sensitive neuraminidase-galactose oxidase/ NaB^3H_4 method. This agrees with the observation that no adsorption of PF 3 to concanavalin A-Sepharex occurred upon incubation with the purified PF 3.

As calculated from the amino acid and the lipid analyses, the PF 3 particles contained about 40% protein and 60% lipid. The chemical composition of the particles is shown in Table 2. Phospholipid constituted the main part of the lipid, about 70%, cholesterol and triacylglycerols representing about 21 and 9% respectively.

In order to determine whether the PF 3 material was contaminated with any of the coagulation factors that are thought to form complexes with PF 3 in the presence of Ca^{2+} during the coagulation

EXPLANATION OF PLATE 1

Isolated PF 3 particles demonstrated by uranyl acetate staining or potassium phosphotungstate staining (insert)
Arrows indicate either protrusions on the particle or occasionally curled, membranous morphology.

Table 2. *Chemical composition of particles with PF 3, isolated from platelet release supernatant*
Results are means and range for four experiments.

Components	Concentration	
	(%)	(w/w)
Protein	40.4	(38.3–41.7)
Cholesterol	12.8	(10.8–15.5)
Triacylglycerols	5.4	(4.5–6.5)
Phospholipids	41.5	(39.7–43.1)
Phosphatidylcholine	37.4	(34.0–41.4)
Phosphatidylethanolamine	24.6	(22.1–28.3)
Phosphatidylserine	8.7	(5.7–12.5)
Phosphatidic acid	2.1	(1.3–3.4)
Sphingomyelin	26.3	(22.9–28.3)

process, the PF 3 material was tested for factors VIII, IX, V, X, and II. As was to be expected, no coagulation-factor activity was found associated with the PF 3 material. It is probable that the EDTA present in the purification process effectively dissociates any complexes.

The specific activity of the purified PF 3 material was compared with that of phospholipid vesicles. A sonicated mixture of phosphatidylserine and phosphatidylethanolamine (2:3, w/w) was chosen for this purpose, since in comparison with other phospholipid mixtures this proved to be one of the most active ones when tested by the Stypven clotting-time test. The specific activity of the phospholipid vesicles was found to be 5800 units/mg, whereas the specific activity of the particles with PF 3 was 13700 units/mg of material. Thus the specific activity of the PF 3 particles was about twice as high as that of the phospholipid vesicles.

Discussion

PF 3 can be obtained from platelets in various ways. One way is to induce the release reaction with agents such as collagen or thrombin. Another way is to disrupt or damage the platelets by various methods such as freeze-thawing or sonication. Sonication of platelets produces much more PF 3 than the platelet-release process (Joist *et al.*, 1974). It is doubtful, however, if this activity has any biological relevance. This kind of drastic damage to the platelets probably does not occur during normal blood coagulation *in vivo*. It is likely that virtually all of the PF 3 in both normal and pathological coagulation *in vivo* is derived from the platelet-release reaction.

The question of whether the PF 3 made accessible during the platelet-release process is only a property of the platelet membrane or dependent on an entity that can exist separately from the platelet has

previously been studied on suspensions of released platelets by using centrifugation techniques. In most cases it has been found that most of the activity sediments with the platelets and a smaller portion stays in the supernatant. It has been suggested that the activity found in the supernatant is caused by platelet membrane fragments from lysed platelets (Joist *et al.*, 1974) and that PF 3 is not dependent on a specific component but a property of the platelet membrane (Marcus, 1969).

The results obtained in the present study show that one part of the PF 3 sediments with the platelets and that one part stays in the supernatant, a result in conformity with those obtained in most previous investigations (Horowitz & Papayouanou, 1967; Wolf, 1967; Hardisty & Hutton, 1966). The isolation and characterization of the material in the supernatant responsible for the PF 3 show that this activity does not originate from platelet membrane fragments but from these special kind of closely similar membranous particles having diameters between 70 and 170 nm. These particles could also be demonstrated in the platelet-release supernatant itself and are thus not artefacts produced by the purification procedure. In studies on the platelet-release process with sectioned platelets, Webber & Johnson (1970) have previously found a similar type of particle, although of a larger size and were able to demonstrate its migration from the interior of the platelet and its release into the surrounding solution.

The particles are mainly composed of lipids and, as might be expected, phospholipids constitute a large proportion of the total mass. The outer surface of the particles is probably covered to a large extent with the polar end groups of the phospholipids, but there are probably also some protein components exposed on the surface. The electron-microscopic pictures show that the surface of the particles has some small protrusions which may be protein immersed in the membrane. Polyacrylamide-gel electrophoresis under reducing and denaturing conditions demonstrated the presence of two main protein components. Amino-acid-analysis data for the whole particle indicated that about 40% of it is protein. No evidence for the presence of glycoproteins was found. This can also be viewed in relation to the observation that the particles are not bound to matrix-linked concanavalin A. This is different from the platelet surface membrane, which contains a number of glycoproteins and does bind concanavalin A.

How do the characteristics of this particle fit in with what can be expected of the entity(ies) responsible for the PF 3? The PF 3 is known to be dependent on the presence of phospholipids, especially phosphatidylserine and phosphatidylethanolamine, and, in conformity therewith, a large amount of phospholipid, including these latter, is

found in the particles. The phospholipid is exposed on the surface of particles, as indicated by the binding of poly-(L-lysine). It is thus evident that these particles do have the expected properties of the entity(ies) responsible for PF 3.

An important question arises as to whether the PF 3 material that sediments with the platelets is dependent on a different type of entity. Several possibilities exist, one of them being that there is only one type of particle which is bound to various extents to the platelet surface and thus becomes distributed both in the platelet sediment and the supernatant on centrifugation. Another possibility is that there is just one type of membranous structure having PF 3 formed in the platelet, but during the release reaction the main fraction fuses with the platelet membrane, whereas some membranous particles are ejected into the solution and become responsible for the supernatant PF 3. Some previously performed electron-microscopic studies (White & Krivit, 1966; Webber & Johnson, 1970) on the platelet-release process have produced results that would be compatible with such a model. A third possibility is that the supernatant PF 3 particles are quite different from the structure responsible for the PF 3 on the platelets.

In the present study PF 3 has simply been defined as the phospholipid-related procoagulant activity of platelets that is made accessible during the release process. It may be questioned, however, if the ability of released platelets to enhance the activation of prothrombin and factor X only is dependent upon a phospholipid surface of certain composition. Other components may also be essential parts of the biologically important structure. The recent discovery that there are specific platelet receptors for factors V and Va (Tracy *et al.*, 1979) and the finding of a platelet receptor for factor Xa (Miletich *et al.*, 1977, 1978a,b, 1979; Kane *et al.*, 1980) which can be blocked by antibodies against factor V are important observations in this connection. The released particles with PF 3 described here were not associated with any clotting-factor activities. This was not expected, since the isolation was performed in the presence of EDTA. However, preliminary studies on the released particles with PF 3 in the absence of EDTA suggest that the factor V-like coagulant activity of platelets, also called platelet factor 1 (Ware *et al.*, 1948), is released in a strong complex with those particles from stimulated platelets (Sandberg *et al.*, 1981).

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