Antigenic and Antiheparin Properties of Human Platelet Factor 4 (PF₄)

By Nrapendra Nath, Carolyn T. Lowery, and Stefan Niewiarowski

Platelet factor 4 (PF₄, a heparin-neutralizing protein) was isolated from washed human platelets. It was found to be homogeneous by SDS-polyacrylamide gel electrophoresis, immunodiffusion, and immunoelectrophoresis, when tested with monospecific antibody produced in rabbits. PF₄ is a heat-stable protein, but its antiheparin activity and antigenicity are destroyed by trypsin. The molecular weight of PF₄ as calculated by amino acid analysis is approximately 8000 and by SDS-polyacrylamide gel electrophoresis with β-mercaptoethanol, 7100 daltons. PF₄ migrated to the cathode at pH 8.6. The interaction of PF₄ with heparin resulted in the formation of a complex which migrated to the anode, as tested by immunoelectrophoresis. Incubation of purified PF₄ with its antibody at 37°C resulted in a loss of antiheparin activity. The presence of antiheparin activity and of PF₄ antigen in material released during platelet aggregation by various agents and at various stages of the preparative procedure closely correlated. It has been concluded that PF₄ antigen and antiheparin activity are two properties of the same protein. Comparison of human and pig PF₄ revealed significant biochemical and antigenic differences.

Platelet factor 4 (PF₄), a protein which neutralizes heparin activity, is known to be released during platelet release reaction initiated by agents such as collagen and thrombin. The physiologic role of antiheparin activity released by platelets is not explained, even though it has been described as playing a part in increased coagulability of blood. Increased levels of antiheparin activity have been reported in cases of disseminated intravascular coagulation (DIC) and other thrombotic states. It has been suggested that increased antiheparin activity in plasma may serve as a marker of the intravascular platelet aggregation, since platelet release reaction may occur in vivo as well as in vitro. Most methods currently used for detecting antiheparin activity are clotting assays, depending on the neutralization of heparin. However, it is known that heparin can be neutralized, rather nonspecifically, by many cationic proteins and several other substances. We have attempted to purify and characterize the PF₄ protein from human platelets and have studied its antigenic and immunologic properties in the hope that it might become pos-

*Abbreviations used: PF₄, platelet factor 4; DIC, disseminated intravascular coagulation; ACD, acid citrate dextrose; PRP, platelet-rich plasma; PPP, platelet-poor plasma; BSA, bovine serum albumin; DEAE-C, diethylaminoethyl cellulose; HSA, human serum albumin; AHPF₄, antihuman PF₄ antibody; APPF₄, antipig PF₄ antibody; IE, immunoelectrophoresis; PS, protamine sulfate; SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; SD, standard deviation.
sible to develop a specific and sensitive serologic test for the detection of PF4 antigen in human plasma without interference by other proteins.

MATERIALS AND METHODS

Protein Estimation

Protein estimation was done by the technique of Lowry et al.\textsuperscript{12}

Assay of Antiheparin Activity

Assay of antiheparin activity was conducted using the modified technique of Poplawski and Niewiarowski,\textsuperscript{7} as described by Nath et al.\textsuperscript{13} The antiheparin activity was expressed as units of PF4 or protamine sulfate (PS) equivalents per milliliter. PS is known to neutralize anticlotting activity of heparin. One unit of PF4 is equal to 1 μg of protamine sulfate.

Washed Platelets

Human blood was collected on acid citrate dextrose (ACD). Platelets were separated by differential centrifugation of blood at 120 g and platelet-rich plasma (PRP) at 1100 g. Platelets were washed in Tyrode's solution containing 0.35% bovine serum albumin (BSA), using the technique described by Mustard et al.\textsuperscript{14} However, BSA was omitted from the final suspending medium. All operations were done at 37°C, and washing fluid contained 2-4 U of apyrase per ml (isolated from potatoes by the method of Molnar and Lőránd\textsuperscript{15}). The final suspension usually contained over 10\textsuperscript{7} platelets per ml.

Release and Isolation of Human PF\textsubscript{4}

Washed platelets were aggregated by the addition of bovine thrombin, 1-2 U/ml (Parke Davis & Co., Detroit, Mich.), at 37°C for 15 min with occasional shaking. Aggregated platelets were removed by centrifugation. ZnSO\textsubscript{4} was added to the supernatant (preparation A) containing released PF\textsubscript{4}, and the final concentration was 0.04 M. Precipitation was allowed to develop overnight at 4°C. The precipitated pellet was extracted with a small volume of 1 M NaCl and ultrasonification. The pH of the suspension was adjusted to 2.0, and insoluble material was removed by centrifugation. The supernatant was dialyzed against 1 M NaCl at 4°C for 18-24 hr. This is referred to as crude PF\textsubscript{4} or preparation C.

An aliquot of washed platelet suspension, after the addition of a small amount of Triton X-100, served as an indicator of the total PF\textsubscript{4} contained in the washed platelet preparation.

DEAE-Cellulose (DEAE-C) Ion-exchange Chromatography

DEAE-C (Whatman, DE-52, preswollen), equilibrated with 0.02 M citrate phosphate buffer, pH 6.0, was packed in glass columns (20 × 1.5 cm). Three milliliters of crude PF\textsubscript{4} (in 1 M NaCl) was applied on each column. Citrate phosphate buffer (0.02 M, pH 6.0) was used for elution. The flow rate of elution was maintained at approximately 45 ml/hr. Fractions were usually collected in 1.5-2-ml volumes and were continuously monitored at 280 nm using a UV monitor (Pharmacia, Piscataway, N.J.). Fractions were dialyzed overnight at 4°C against 0.02-0.05 M Tris-HCl buffer (pH 7.5).

Preparation of Antiserum

Rabbits were inoculated initially in the foot pads, and in subsequent injections subcutaneously, with a 1:1 mixture of DEAE-C-purified PF\textsubscript{4} (fractions having the highest specific antiheparin activity) and Freund's complete adjuvant (Difco). The animals were injected at weekly intervals, and a total of eight to ten injections were given. The total protein injected per animal was about 2.0 mg. Rabbits were bled from the ear vein periodically to monitor the level of antibodies produced. The rabbits' blood was mixed with ACD (1:6 ratio), and platelet-free plasma was obtained by differential centrifugation. Serum was obtained by heating plasma to 56°C for 30 min. Antiserum was absorbed with BSA (2.5 mg/ml) and human serum albumin (HSA) (2 mg/ml) incubated for 30 min at 37°C, and the precipitate was removed by centrifugation. The supernatant
was used as antihuman PF₄ antiserum (AHPF₄). Gamma globulin was obtained from the anti-
serum by ammonium sulfate precipitation.¹⁶

Rabbit antihuman serum albumin was obtained from Miles Laboratories, Kankakee, Ill.
Preparation of purified pig PF₄ and antipig PF₄ was conducted by the method of Nath et al.¹³

**Double-gel Immunodiffusion**

One per cent Noble agar (Difco) in 0.15 M NaCl was used as a diffusion medium (Ouchter-
lony).¹⁷ Three milliliters of molten agar solution was layered on each microscope slide (25 x
75 mm). The diameter of antigen and antiserum wells was about 2 mm, and the distance between
them varied from 3 to 4 mm. The gels were allowed to develop at room temperature overnight in
a humidified chamber.

**Immunoelectrophoresis (IE)**

Immunoelectrophoresis was done following the method described by Ouchterlony and Nilsson.¹⁸
Three milliliters of 0.9% agarose (Sigma) melted in 0.05 M veronal buffer, pH 8.2, was layered
on a microscope slide (25 x 75 mm). Antigen was placed in small wells (1 mm diameter) with a
capillary pipette. An electric potential of 40 V was applied across the gel for 60 min. After the
electrophoretic run, a central trench was cut out in the gel and filled with antiserum. The gel was
allowed to develop overnight in a humidified chamber at room temperature.

Developed slides were washed in 0.15 M NaCl and distilled water, followed by drying and sub-
sequent staining with amido black 10B (Harleco, Philadelphia, Pa.). Photo prints were made from
the stained slides by using the slides directly as negatives.

**Sodium Dodecyl Sulfate (SDS)-polyacrylamide Gel Electrophoresis**

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to
the method described by Weber and Osborn.¹⁹ Ten per cent gels were electrophoresed at 1.5 mA
per gel for 18 hr. In some experiments, protein samples were reduced by the addition of β-
mercaptoethanol before layering on the top of gels. Protein samples were mixed with SDS buffer
to have at least 100 μg/ml of protein. The gels were stained with Coomassie brilliant blue. Hu-
man serum albumin (mol wt, 68,000), soybean trypsin inhibitor (SBTI) (mol wt, 20,095), whale
sperm myoglobin (mol wt, 17,500), egg white lysozyme (mol wt, 14,300), ribonuclease (mol wt,
13,700), and bovine pancreatic trypsin inhibitor (mol wt, 6513)²⁰ were used as the standard
proteins of known molecular weights. In addition, cyanogen bromide derivatives of lysozyme (mol
wt, 10,200) and of myoglobin (mol wt, 8270 and 6420) were prepared by the method described by
Budzynski et al.²¹ Ribonuclease was from Pharmacia (Sweden); lysozyme from Worthington Bio-
chemical Corporation, N.J.; other standards were from Sigma.

**Amino Acid Analysis**

Amino acid analysis was performed by Worthington Biochemical Corp., Freehold, N.J. Sam-
ples were hydrolyzed at 110°C for 24, 48, and 72 hr, and then analyzed on an amino acid ana-
lyzer. Corrections have been made for hydrolytic losses. Values were expressed as nanomoles of
amino acid per milligram of protein.

**Effect of Trypsin on PF₄**

Preparation A and DEAE-C PF₄ were tested for their sensitivity to trypsin treatment. To 0.4 ml
of PF₄, preparation, 0.05 ml of trypsin (0.001%) was added, and the mixture was incubated at
37°C for 30 min. Addition of 0.05 ml soybean trypsin inhibitor (SBTI) (0.05%) was used to stop
the action of trypsin.

In the control experiment, 0.05 ml of trypsin and SBTI each were mixed and allowed to react
for 3 min at room temperature before the addition of the PF₄ sample. Residual antiheparin ac-
tivity and antigenicity were determined by clotting assay and agar-gel diffusion techniques,
respectively.
Table 1. Purification and Recovery of PF₄ From Washed Human Platelets

<table>
<thead>
<tr>
<th></th>
<th>Mean Values From Six Experiments</th>
<th>Values of One of the Best Samples</th>
<th>Mean Recovery of Antiheparin Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS U/mg Protein</td>
<td>Purification Index</td>
<td>PS U/mg Protein</td>
</tr>
<tr>
<td>Tritonized platelets</td>
<td>7.0</td>
<td>1</td>
<td>9.0</td>
</tr>
<tr>
<td>Supernatant of thrombin-treated platelets</td>
<td>26.6</td>
<td>3.8</td>
<td>35.0</td>
</tr>
<tr>
<td>Crude PF₄</td>
<td>58.4</td>
<td>8.3</td>
<td>124.0</td>
</tr>
<tr>
<td>DEAE-C fractions</td>
<td>87.8</td>
<td>12.5</td>
<td>190.0</td>
</tr>
</tbody>
</table>

Aggregation of Platelets and Release of PF₄

Washed platelets (10⁹/ml) were studied in a Payton aggregometer (Payton Industries, Scarborough, Ontario, Canada) for aggregation. To 0.8 ml of platelets in a cuvette was added 0.1 ml saline (0.15 M NaCl) and 0.1 ml of thrombin (0.2 U/ml), or 0.1 ml of human fibrinogen (0.1%) and 0.1 ml of ADP (50 μmol), and the reaction was allowed to proceed for 1, 2, and 5 min. At the end of the appropriate time, the contents of the cuvette were spun for 30 sec in an Eppendorf Centrifuge (Brinkmann Instrs., New York, N.Y.). The supernatant was studied for antiheparin activity by clotting assay and for PF₄ antigen using Ouchterlony’s test.

RESULTS

PF₄ antiheparin activity was released from human platelets by the action of thrombin and subsequently purified by ZnSO₄ precipitation and DEAE-C chromatography. Table 1 shows the results of six experiments. The antiheparin activity detected in platelet suspension lysed by Triton X-100 was considered as the total antiheparin activity contained in the platelet suspension. Other values are compared with this basal value of tritonized platelets. The PF₄ protein obtained by ZnSO₄ precipitation and extraction, called crude PF₄, revealed an average specific activity about 12 times the basal value. Purification of crude PF₄ by DEAE-C chromatography resulted in a further gain in specific activity which reached a value of 17 times the basal value. The recovery of PF₄ precipitated by ZnSO₄ was about 45% as compared with starting material; the recovery of PF₄ eluted from DEAE-C column was about 18%.

Protein recovered in the supernatant after platelet aggregation accounted for about 10% of the total protein present in the solution of tritonized platelets. ZnSO₄ precipitated about 50% of protein releasable during platelet aggregation by thrombin. From this, about 40% was recovered in the PF₄ containing material eluted from the DEAE-C column.

In order to further purify crude PF₄, it was applied to the DEAE-C column. Fractions obtained were dialyzed against 0.02-0.05 M Tris-HCl, pH 7.5, and each fraction was tested for its antiheparin activity using the clotting assay, and for PF₄ antigen using the gel diffusion (Ouchterlony) technique. Ten different batches of crude PF₄ were used for chromatography on 15 separate columns. There was an average of 166% gain in the specific activity of the fraction having peak antiheparin activity as compared to the specific activity of the crude PF₄ charged on the column.

Figure 1 compares the ability of tritonized washed human platelets (T), the released material from platelets (A), crude PF₄ (C), and supernatant after ZnSO₄ precipitation (Z) to form a precipitation band in gel diffusion. A pre-
Fig. 1. Comparison of tritonized platelets (T), thrombin-induced released material (A), crude PF₄ (C), and supernatant after ZnSO₄ precipitation (Z) for the presence of PF₄ antigen. Central well(s) contained anti-PF₄ serum absorbed with BSA and human albumin.

cipitation band appeared with (T), (A), and (C), but no band appeared with (Z). In this, as in most experiments, anti-PF₄ serum was absorbed with BSA (2.5 mg/ml) and HSA (2 mg/ml). Nonabsorbed serum formed a second precipitation band, apparently due to albumin. Usually, the early fractions from DEAE-C column gave one precipitation band in immunodiffusion with either absorbed or nonabsorbed anti-PF₄ serum, suggesting elimination of albumin contaminants.

Figure 2 shows that antihuman PF₄ (AHPF₄) gamma globulin gave a precipitation line with human tritonized platelets and with DEAE-C-purified human PF₄. On the other hand, it did not react with pig tritonized platelets and with purified pig PF₄. Antipig PF₄ (APPF₄) gamma globulin gave precipitation

Fig. 2. Immunodiffusion of human and pig PF₄. Left central well contains antihuman PF₄ gamma globulin (AHPF₄) prepared from serum absorbed with BSA and human albumin, and the right central well contains antipig PF₄ gamma globulin (APPF₄) prepared from serum absorbed with BSA and pig platelet-poor plasma. Peripheral wells contain human or pig platelets (tritonized), human PF₄ (HPF₄), and pig PF₄ (PPF₄).
lines with pig platelets and purified pig PF₄, but it did not react with human platelets and human PF₄.

Each fraction from the DEAE-C column was tested for the presence of PF₄ antigen by the gel diffusion test using antiserum to human PF₄ (AHPF₄). The results of a representative column showing the amount of protein, antiheparin activity, PF₄ antigen, and HSA antigen are shown in Fig. 3. In this experiment, antiheparin activity was eluted in fractions 14–19. The presence of a PF₄ antigen in the gel diffusion test correlated well with the presence of antiheparin activity in the fractions. The second protein peak appearing in fraction 17 was found to have human serum albumin when tested with anti-HSA serum. Fractions 14 and 15 appeared to be free of albumin.

In order to test the extent of purity of PF₄ antigen, immunoelectrophoresis (IE) and SDS-acrylamide gel electrophoresis were performed on various fractions from the DEAE-C column as well as some other preparations. The IE of PF₄ using unabsorbed AHPF₄, indicated the presence of BSA as well as human albumin antigens, along with PF₄ antigen in crude PF₄ and some fractions from the DEAE-C column. Absorption of antiserum with BSA and HSA resulted in antisera which were monospecific, i.e., which gave a single precipitation arc in IE (Figs. 4 and 5) when tested against human PF₄.

The effect of heparin on the immunoelectrophoretic behavior of PF₄ antigen and AHPF₄ antibody binding is shown in Fig. 3. PF₄ antigen normally formed a precipitation arc slightly towards the cathode; however, addition of heparin to PF₄ antigen resulted in the formation of a long streaking precipitation band moving into the anode (Fig. 4). This phenomenon was observed with preparation A as well as with DEAE-C-purified PF₄. It is noteworthy that even during a longer electrophoresis run prior to immunoprecipitation (up to 3 hr), no separation of PF₄ antigen into different components was observed.

The PF₄ antigen mixed in human plasma could easily be detected by IE or immunodiffusion, indicating the possibility that this technique might be useful in detecting PF₄ in plasma.

Figure 5 compared the mobility of DEAE-C-purified human and pig PF₄ antigens using IE. Human PF₄ moved towards the cathode, while pig PF₄ moved distinctly towards the anode. The two antigens did not cross react in IE.
Fig. 4. The effect of heparin on the immuno-electrophoretic mobility of DEAC-C-purified PF₄. The upper well was filled with 5 µl of PF₄ (480 µg/ml) diluted 1:2 with 0.15 M NaCl, and the lower well with 5 µl of PF₄ diluted 1:2 with heparin (100 µ/ml). Cathode on the left, anode on the right.

Fig. 5. Comparison of PF₄ antigen isolated from washed platelets of human and pig using immunoelectrophoresis. AHPF₄ was absorbed with BSA and human albumin, while APPF₄ was only absorbed with BSA. 0.9% agarose in 0.05 M veronal buffer was used as the diffusing medium, and an electric potential of 40 V was applied across the slide for 60 min. The volume of PF₄ solution applied was 5 µl.
Tritonized platelets gave at least 14 stainable bands (gel 1) on SDS-acrylamide gel in the presence of β-mercaptoethanol (Fig. 6A). However, the number of bands was reduced to only four in the case of preparation A (gel 2) and only three when crude PF₄ was applied on the gel (gel 3). The two bands representing peptides X and Y are more dense in gel 3 than in gel 1. Figure 6B compares various fractions from a DEAE-C column when applied on SDS-acrylamide gel. Gel 4, representing the DEAE-C fraction 15, showed only band Y which, in subsequent gels representing DEAE-C fractions 16, 17, and 18, became gradually less dense. On the other hand, band X appeared on gels 5, 6, and 7. The faint protein band, observed occasionally in the proximity of band Y on gels 6 and 7, never appeared on gels 4 and 5. A comparison with the standard proteins revealed the molecular weight of the band X peptide to be about 68,000 and the band Y peptide to be 7,100.

Table 2 and Fig. 3 compare the protein content and antiheparin activity of the various DEAE-C fractions which were applied on SDS-acrylamide gels shown in Figs. 6A and 6B. The high antiheparin specific activity of fraction 15 shown in Table 2 corresponds with the gel 4 in Fig. 5B showing only band Y. Eluates from various DEAE-C columns often revealed a pattern of fractions similar to the one shown in Fig. 5B. Fractions similar to fraction 15 were always found in earlier fractions and were low in their protein content, as shown by absorbance at 280 nm as well as by direct protein determination; however, in some column runs, fractions with a single peptide failed to appear altogether. Immunodiffusion studies indicate that peptide X corresponds to albumin and peptide Y to PF₄ antigen (Fig. 3).
Table 2. Specific Activity of PF₄ at Various Stages of Purification

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Purification Step</th>
<th>Protein (mg/ml)</th>
<th>Antiheparin Activity (PS U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tritonized platelets (185H)</td>
<td>2.38</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Preparation A (185A)</td>
<td>0.18</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Crude PF₄ (185C)</td>
<td>1.08</td>
<td>72</td>
</tr>
<tr>
<td>4</td>
<td>DEAE-C fractions (185C/1/15/74)</td>
<td>0.07</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>0.09</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>0.21</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>0.13</td>
<td>29</td>
</tr>
</tbody>
</table>

These fractions were used for SDS-acrylamide gel electrophoresis shown in Figs. 6A and 6B. Gels 1–7 correspond to the samples 1–7 in this table.

Fractions from DEAE-C column dialyzed against 0.02–0.05 M Tris-HCl buffer, pH 7.5, could be stored for months if frozen, or several days if left at 4°C. However, if several fractions from a column or different columns all sharing high antiheparin specific activity were pooled, precipitate appeared within a few hours. The precipitate consisted of filamentous particles of various length. Removal of this precipitate by centrifugation or filtration resulted in almost complete loss of antiheparin activity. The precipitate failed to go into solution even when the molarity of the solution was raised to 1 M by the addition of sodium chloride or by varying the pH.

DEAE-C-purified PF₄ with only one protein band (Y) revealed the amino acid composition as listed in Table 3. Assuming that there is only one residue

Table 3. Amino Acid Composition of PF₄*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mol. Ratio</th>
<th>No. of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYS</td>
<td>9.2</td>
<td>9</td>
</tr>
<tr>
<td>HIS</td>
<td>1.4</td>
<td>1</td>
</tr>
<tr>
<td>ARG</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>ASP</td>
<td>9.0</td>
<td>9</td>
</tr>
<tr>
<td>THR</td>
<td>4.2</td>
<td>4</td>
</tr>
<tr>
<td>SER</td>
<td>5.6</td>
<td>6</td>
</tr>
<tr>
<td>GLU</td>
<td>9.3</td>
<td>9</td>
</tr>
<tr>
<td>PRO</td>
<td>2.8</td>
<td>3</td>
</tr>
<tr>
<td>GLY</td>
<td>6.3</td>
<td>6</td>
</tr>
<tr>
<td>ALA</td>
<td>6.0</td>
<td>6</td>
</tr>
<tr>
<td>CYS 1/2</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>VAL</td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>ILE</td>
<td>4.9</td>
<td>5</td>
</tr>
<tr>
<td>LEU</td>
<td>6.8</td>
<td>7</td>
</tr>
<tr>
<td>TYR</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>MET</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>PHE</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>TRP†</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Amino sugars, trace.
Basic residues, 12; acidic residues, 18; hydrophobic residues, 19; other, 35; total, 74.
*Average of two determinations.
†Not determined.
The regression line of molecular weights and relative electrophoretic mobilities of various protein standards calculated by the least square method. 1, SBTI; 2, myoglobin; 3, lysozyme; 4, ribonuclease, 5, CNBr derivative I of lysozyme; 6, CNBr derivative I of myoglobin; 7, CNBr derivative II of myoglobin; 8, pancreatic trypsin inhibitor. All values of the electrophoretic mobility of protein standards are means of at least five determinations. The mean electrophoretic mobility of reduced PF₄ was 0.941 ± 0.017 (48 determinations) and of nonreduced, 0.837 ± 0.049 (13 determinations). All standards were tested in a reduced system.

of tyrosine per PF₄ molecule, a total of 74 amino acid residues (12 basic, 18 acidic, and 19 nonpolar) was calculated. The minimal molecular weight on the basis of amino acid residues after subtraction of water of hydration was calculated to be approximately 8000 daltons. On the basis of SDS-acrylamide gel electrophoresis, it has been calculated that the molecular weight of nonreduced PF₄ corresponds to 11,700 daltons and that of β-mercaptoethanol-reduced PF₄ to 7100 daltons (Fig. 7).

Incubation of DEAE-C-purified PF₄ (9.7 PS U/ml) with its antibody (gamma globulin fraction, titer 1:16 in immunodiffusion) for 30 min resulted in antiheparin activity 54% of the initial, a loss of 46% activity. Longer preincubation, up to 4 hr, resulted in almost complete loss of antiheparin activity. Preimmune serum, however, had no significant effect on the antiheparin activity of PF₄.

PF₄ antigen and its antiheparin activity showed similar heat stability. Heating at 80°C for 10 min caused a drop to 71.1% of the initial antiheparin activity. Even boiling for 3 min at 100°C caused the retention of 54.4% of the initial activity. PF₄ antigenicity was not lost at any stage. In another experiment, we incubated 0.001% trypsin with preparation A or DEAE-C-purified PF₄ for 30 min at 37°C. Trypsin eliminated antiheparin activity, as well as the antigenicity of PF₄ protein, completely.

Table 4 shows that washed human platelets, when aggregated with thrombin, release antiheparin activity as well as PF₄ antigen. The release of the two occurred in parallel. On the other hand, washed human platelets, when treated with ADP in the presence of fibrinogen, do not cause any significant release of antiheparin or PF₄ antigen activity even though ADP caused platelet aggregation. Similarly, immunodiffusion detected the presence of PF₄ antigen after platelets were aggregated in PRP with thrombin. On the other hand, human PPP did not give a precipitation line with anti-PF₄ serum.

**DISCUSSION**

Purification of antiheparin activity (PF₄) from human platelets was achieved following basically the same technique as described for pig platelets. However, we resorted to washing human platelets at 37°C in the presence of calcium and apyrase, using the improved procedure of Mustard et al. This procedure enabled us to recover better yields of PF₄. Bovine serum albumin was used, as recommended. Later during the study, however, it was discovered that the pres-
Table 4. Comparison of the Release of Antiheparin Activity and PF₄ Antigen From Washed Human Platelets

<table>
<thead>
<tr>
<th>Reagents Added to Platelets</th>
<th>Incubation Time (min)</th>
<th>Antiheparin Activity (PS U/ml)</th>
<th>Immunodiffusion Precipitation Band</th>
<th>Platelet Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1</td>
<td>5.5</td>
<td>+</td>
<td>85</td>
</tr>
<tr>
<td>Thrombin</td>
<td>2</td>
<td>5.5</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Thrombin</td>
<td>5</td>
<td>6.9</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>Fibrinogen + ADP</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>Fibrinogen + ADP</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>Fibrinogen + ADP</td>
<td>5</td>
<td>0</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>Sonicated platelets†</td>
<td>0</td>
<td>7</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
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*Values represent the mean of four identical experiments. Platelet aggregation was directly recorded on the chart paper as the reciprocal of light transmission through the platelet suspension. A 100% aggregation means that most platelets were removed from the suspension. Assay was performed by transferring 0.8 ml of washed platelets (10⁷/ml) into aggregometer cuvette; to this, 0.1 ml saline (0.15 M NaCl) and 0.1 y 1 thrombin (2 U/ml) was added; or to 0.8 ml platelet suspension, 0.1 ml of human fibrinogen (1%) and 0.1 ml ADP (5 x 10⁻⁴ M) were added. Reactants were centrifuged in an Eppendorf centrifuge at the end of desired incubation period. Supernatant was removed and assayed for the antiheparin activity and the presence of PF₄ antigen.

†Platelet suspension sonicated for 1 min. The sonicator used was model 1000, Ultrasonic System, Inc., Farmingdale, N.Y.

ence of BSA in PF₄ was not desirable in the immunologic experiments; therefore, BSA was omitted from the medium in which platelets were finally suspended. Nevertheless, the washing medium continued to have BSA. Higher concentrations of ZnSO₄ were required to precipitate human PF₄ as compared with pig material. We succeeded in isolating homogenous PF₄ protein as confirmed by means of immunodiffusion (Fig. 1), immunoelectrophoresis (Figs. 4 and 5), and SDS-acrylamide gel electrophoresis (Fig. 6B). The recovery of albumin-free PF₄ was low. Antibodies to both BSA and HSA were found to be present in AHPF₄. However, AHPF₄ could be made monospecific by absorption with BSA, HSA, or normal human PPP. Monospecificity was tested in immunodiffusion and in IE. Contamination of PF₄ antigen with BSA and HSA might have occurred because fractions from DEAE-C were chosen for injection in rabbits on the basis of high specific activity, without considering their purity on SDS gel.

In agreement with other investigators,²³,²⁴ we found that PF₄ is a low-molecular-weight, heat-stable protein. Experiments were performed on DEAE-C-purified PF₄ fractions which gave only one band on SDS-acrylamide gel and were free of albumin. The minimal molecular weight of PF₄ calculated from amino acid analysis was about 8000. The molecular weight of PF₄ by SDS-polyacrylamide gel electrophoresis in the nonreduced system was 11,700 daltons. Treatment of PF₄ with β-mercaptoethanol resulted in a decrease of molecular weight to 7100 daltons. As it can be seen from Fig. 7, there was no straight-line correlation between relative electrophoretic mobility and molecular weights of proteins below 15,000. The electrophoretic mobility of non-reduced PF₄ was close to that of lysozyme, another cationic protein which has a molecular weight of 14,300.²² Therefore, it is possible that the molecular weight of PF₄ is also in this approximate vicinity, and that β-mercaptoethanol splits PF₄ into two subunits which have identical molecular weights. Kaser-
Glanzmann et al. calculated the molecular weight of human PF₄ as 29,000 by means of sedimentation velocity measurement. Moore et al. suggested that the PF₄ molecule may be composed of four subunits, each of 7000 daltons. Direct evidence of polymerization in our experiments was encountered when various DEAE-C fractions were mixed, which resulted in the appearance of filamentous precipitate.

Although PF₄ is released from platelets in a complex with proteoglycan carrier, the purified material was free from amino sugars. Therefore, the heat stability of PF₄ is not likely to be related to the protective action of carbohydrate.

The low solubility of purified PF₄ may be due to the high content of non-polar residues. This finding is in agreement with the observation of Moore et al.

The content of acidic residues in purified PF₄ was slightly higher than that of basic residues. However, the mobility of PF₄ in electrophoresis indicated that it might be a cationic protein. It is possible that carboxylic residues of aspartic and glutaminic acid may occur in PF₄ molecules as amides (asparagine, glutamine); thus, the negative charge may be neutralized.

Our method of PF₄ purification differs from those used by Kaser-Glanzmann et al. and Moore et al. These authors utilized filtration on Biogel A at high ionic strength to separate PF₄ from its proteoglycan carrier. Our trials with this method were not very successful and resulted in much lower recovery of the final product. However, it seems that our DEAE-C-purified PF₄ resembles preparations obtained by means of Biogel A filtration. Amino acid composition of purified PF₄, recently reported by Kaser-Glanzmann et al. and by Moore et al., is similar to that of our preparation, except that our PF₄ contains slightly higher percentages of serine and aspartic acid. Kaser-Glanzmann et al. reported that 1 mg of their PF₄ neutralizes 34 U of heparin, which, according to our calculation, corresponds to approximately 190 PS U/mg protein. This value has been found for some of our preparations. Recently we had a chance to examine a sample of PF₄ preparation kindly supplied by Dr. Moore and Dr. Pepper, and we found that this product gives a distinct precipitation line with our antihuman PF₄ serum.

It is interesting to note the differences in the immunoelectrophoretic migration of PF₄ of human and pig origin. Pig PF₄ was found to migrate towards the anode, while human PF₄ formed a precipitation band slightly but definitely towards the cathode. Specific antiheparin activity of pig PF₄ was five to ten times higher than that of human PF₄. It is surprising that a protein like human PF₄ with more cationic charge, has lesser antiheparin activity as compared to anionic, or less cationic, protein such as pig PF₄. However, this does suggest that antiheparin activity of PF₄ is not simply neutralization of the anionic charge of the heparin molecule, but is rather a specific binding activity. It seems that significant differences exist between human and pig PF₄. Pig PF₄ had a higher molecular weight (21,000 daltons), and no cross-reaction was seen between antigens of human and pig PF₄ by immunodiffusion test and only 5%–10% cross-reaction in a more sensitive system of platelet immune aggregation.

The precipitation pattern in IE of preparation A and DEAE-C-purified PF₄...
was quite identical, but addition of heparin to these PF₄ preparations resulted in very significant changes in their precipitation pattern (Fig. 4). This test emphasizes the fact that the precipitation arc in IE is indeed formed by biologically active PF₄ molecules retaining the capacity to bind with heparin. It has been established that pig PF₄ and human PF₄ form complexes with heparin. Our experiment suggests that the interaction of PF₄ with heparin results in the formation of a complex which changes its electrophoretic mobility.

Incubation of purified PF₄ with its antibody at 37°C resulted in loss of antiheparin activity. This fact, in conjunction with the IE (Fig. 4), suggests that PF₄ antigen and heparin-neutralizing activity is a property of the same protein. The heat resistance of PF₄ antigen and PF₄ antiheparin activity, as well as the destruction of both activities by trypsin, are also consistent with the above suggestion.

In order to further establish the relationship of antiheparin activity and PF₄ antigen, the effect of thrombin and ADP on the suspensions of washed human platelets was compared. Thrombin caused the aggregation of platelets and released antiheparin activity as tested by the clotting assay. It was also found to cause the release of PF₄ antigen as tested by immunodiffusion test. On the other hand, ADP-induced aggregation did not result in the release of antiheparin activity, and the same was true of PF₄ antigen. Thrombin causes release of serotonin, ADP, antiheparin activity in the suspension of washed human platelets prepared according to the method of Mustard et al., while ADP does not cause release in this system. The same close relationship of antiheparin activity and PF₄ antigen was found when fractions from a DEAE-C column were tested. Preliminary experiments performed in cooperation with J. Hawiger indicate that leukocytes do not reveal PF₄ antigen, although they contain a potent antiheparin activity. As established in experiments with the immunodiffusion and immunoelectrophoresis, plasma and heparin do not interfere with the detection of PF₄ antigen, although its mobility and precipitation pattern change in the presence of heparin. It is believed that establishment of the specificity of the PF₄ antigen and preparation of monospecific AHPF₄ antisera will enable the development of a sensitive serologic test free from nonspecific reactions encountered with the clotting assay. Since the PF₄ molecule contains one molecule of tyrosine, elaboration of the radioimmunoassay of this protein by labeling with radioactive iodine could be considered.

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NOTE ADDED IN PROOF

K. Gjesdal (Scand J Haemat 13:232, 1974) obtained recently antibody to human PF₄ in rabbits and reported an immunologic method for the quantitation of PF₄ in human plasma and serum.

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Antigenic and antiheparin properties of human platelet factor 4 (PF4)

N Nath, CT Lowery and S Niewiarowski

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