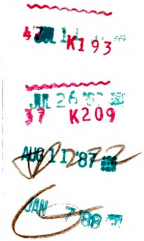

K193
K209

ISOLATION AND PURIFICATION OF HUMAN PLATELET FACTOR 4 BY HEPARIN AFFINITY CHROMATOGRAPHY:

An Enzyme-Linked Immunosorbent Assay

By

Douglas W. Estry

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1978

ABSTRACT

ISOLATION AND PURIFICATION OF HUMAN PLATELET FACTOR 4 BY HEPARIN AFFINITY CHROMATOGRAPHY:

An Enzyme-Linked Immunosorbent Assay

By

Douglas W. Estry

A method is described for the purification of human platelet factor 4 by heparin affinity chromatography. The protein obtained was shown to be pure by both sodium dodecyl sulfate polyacrylamide electrophoresis and disc gel electrophoresis. The protein was obtained at a final yield of 43% and had a specific activity of 271 units of heparin neutralized per milligram. The protein had a subunit molecular weight of 9,300 when compared to known molecular weight standards.

By immunodiffusion a single precipitin arc was observed against rabbit antiserum to human platelet factor 4. Examination of the antiserum by rocket electrophoresis also demonstrated that it was monospecific for heparin neutralizing protein.

Preliminary results of an enzyme-linked immunosorbent assay suggests the feasibility of utilizing specific antiserum labelled with alkaline phosphatase as a substitute for radioimmunoassay in quantitating plasma levels of platelet factor 4.

DEDICATION

To my wife, Sue, for her help and advice and to my parents for their continued support.

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. Joan Mattson, for the outstanding job she has done in providing me with encouragement, advice, and access to her considerable knowledge of hematology and coagulation. I would also like to thank Dr. Garson Tishkoff whose noted expertise in coagulation and biochemical research techniques have provided me with an invaluable source of information. I am also indebted to Dr. Tishkoff for providing laboratory space and adequate funding to allow this project to be completed.

Special thanks to Dr. Thomas Bell for his technical assistance, Martha Thomas, my academic advisor, and Belinda Oxender for her diligent work in preparing this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Current knowledge has established that blood clotting plays an important role in the formation of thrombi that obstruct small and large blood vessels. Platelets are cellular elements that circulate in the blood and constitute an important mechanism in blood clot formation. It is firmly established that platelets may initiate blood clotting by undergoing aggregation. During platelet aggregation certain intracellular metabolities and proteins are released into the circulation, one of which has been called "platelet factor 4". This protein has the unique property of neutralizing the blood clotting chemical inhibitor, heparin.

The aim of this study is to measure circulating blood levels of platelet factor 4 which we believe reflects on-going platelet aggregation, and thus may serve as a sensitive indicator of impending clot formation in acute thrombosis. In order to attain this goal it is the purpose of this thesis to deal with: 1) isolation and purification techniques, 2) antibody formation, and 3) immunologic assay methods including rocketry and enzyme-linked immunosorbent assay (ELISA).

LITERATURE REVIEW

In 1948, Conley et al. demonstrated the *in vivo* presence of a heparin neutralizing factor (1). They found that thrombocytopenic patients had an increased susceptibility to anticoagulation with heparin and that the amount of heparin necessary to prevent clot formation was directly related to the platelet count.

By 1951 the existence of platelet factor 1, prothrombin accelerator, and platelet factor 2, fibrinoplastic factor, had been demonstrated by Seegers and his colleagues. Based on the evidence of Conley, Van Crevald postulated the existence of a third factor which he found had the ability to neutralize heparin (2). Although Van Crevald had demonstrated antiheparin activity in a phosphatide fraction, he had also shown that this fraction had thromboplastic activity which was not disassociated from its antiheparin activity (3). In 1954, Jürgens published work which recognized the heparin neutralizing activity of the platelet as being separate from its procoagulant activity (4). Deutsch et al. were the first investigators to separate the antiheparin activity, which they termed platelet factor 4 (PF4), from other platelet activities (5). They believed that the activity was connected with a "protein principle" and that it was a high molecular weight component.

Until 1960 highly purified preparations of PF4 were unavailable for study. However, in 1961 Deutsch and Kain concentrated the activity of PF4 100-fold by using DEAE chromatography according to

recommendations made by Lüscher (6). Table I lists the chemical and physical properties they demonstrated.

Table I: Chemical and physical properties of platelet factor 4.*

Chemical	:	Protein or high molecular peptide with a carbohydrate component (Deutsch) 13.34% N, 0.34% P, 0.4% glucosamine and 0.08% fucose (Poplawski and Niewiarowski)
Molecular weight	:	Unknown
Heat stability	:	Crude preparation: unstable (activity lost at 50 C/30') Purified preparation: stable (100 C/10')
Storage stability	:	Crude preparations: at 4 C at least 8 days; purified preparations: unlimited; lyophilized platelets: unlimited
Adsorption on	:	Barium sulfate, Kaolin
Ammonium sulfate precipitation	:	Not precipitated at 51% saturation
Sedimentation	:	Not sedimentable (100,000 g/60')

*Deutsch and Kain, 1961.

The assay systems used to determine PF4 levels were, until 1977, based primarily on a classic thrombin clotting time (7, 8, 9, 10, 11). Activity was measured as a function of the quantity of heparin neutralized. A variation of this system was reported in 1974 by Walsh and co-workers (12). Their assay was based on the inactivation of factor Xa by antifactor Xa. This particular system is greatly potentiated by the presence of heparin so again the heparin neutralizing capabilities of PF4 were being utilized. In an attempt to equate the heparin neutralizing activity to known standards, Poplawski and Niewiarowski used protamine sulfate and Levine et al. substituted hexadimithrine bormide (Polybrene ®) rather than protamine (11, 13). Recently, due to the need for more sensitive indicators of platelet release, the assay systems for PF4 have centered around immunologic

techniques including rocketry, radial immunodiffusion, and radioimmunoassay (14, 15, 16, 17).

There have been several attempts to attribute physiological functions, other than heparin neutralization, to PF4. In 1965, Niewiarowski et al. demonstrated the neutralization of fibrinogen breakdown products, antithrombin VI, by PF4 (18). Their PF4 purification procedure involved precipitation with zinc acetate and subsequent DEAE chromatography. During this procedure they reported the simultaneous development of an increased heparin neutralizing activity and antithrombin VI neutralization. The question as to whether this was a true function of PF4 or merely an artifact of the isolation procedure was investigated by Glanzmann and associates (19). Platelet factor 4 was isolated by two techniques; one by gel filtration and the other according to Niewiarowski's zinc acetate procedure. Glanzmann was unable to demonstrate any formation of insoluble fibrin-monomer complexes with the PF4 isolated by gel filtration. When the zinc acetate preparation was treated with EDTA it also failed to precipitate fibrinogen. Thus the removal of divalent cations eliminated fibrinogen precipitation but at the same time failed to inhibit heparin neutralization. In 1978, Harper, Wohl, and Harper found that PF4 inhibits the enzyme collagenase isolated from both human skin and human granulocytes (20). It appears, therefore, that PF4 as well as neutralizing heparin may have a second function involving the inhibition of collagenase. To date a third and final characteristic has been suggested by Fuster et al. (21). Using pigeons known to be susceptible to atherosclerosis they demonstrated increased serum levels of PF4

in the experimental group when compared to a group of controls. In light of recent work by Ross on a platelet mitogenic factor, further work in this area as it relates to endothelial proliferation might clarify the relationship between PF4 and Ross' mitogenic factor (22).

The majority of the knowledge about the PF4 carrier molecule can be attributed to Barber and co-workers (23). Platelet factor 4, released by thrombin aggregation of washed platelets, was removed from its carrier by dissociation at high ionic strengths (I = 0.75). This mixture was chromatographed to separate the active component, PF4, from the aminoglycan carrier. Using cellulose acetate electrophoresis and intrinsic viscosity studies of known standards of glycosaminoglycans it was demonstrated that the PF4 carrier behaves identically to chondroitin 4-sulfate. The multichain proteoglycan carrier has a molecular weight of 59,000 and, after dissociation, a subunit molecular weight of 12,000. Recombinant studies on the carrier and active component led to a binding ratio of four moles of PF4, molecular weight 29,000, per mole of proteoglycan. The final molecular weight of the combined complex is 350,000 suggesting a dimerization of the 175,000 dalton complex.

As well as describing the carrier Barber went on to demonstrate the varying affinities of PF4 for different glycosaminoglycans (22). Although Barber was correct in his characterization and arrangement of these carriers, work by Handin and Cohen in 1976 gave a clearer indication of these relationships (24). Handin's system for determining PF4 affinities was based on the competitive binding of several glycosaminoglycans with [^{3}H] heparin. The quantity of each glycosaminoglycan that displaces 50% of the labelled heparin is shown in

Table II. Heparin clearly had the highest affinity for PF4 while the natural carrier, chondroitin 4-sulfate, had the lowest. The affinities correlated well with the number of sulfated groups on the molecule, heparin having the most, although it also seemed to be due to the arrangment of the carboxyl groups on the uronic acid and the location of the sulfate groups.

Table II: Affinity of PF4 for various glycosaminoglycans.*

Glycosaminoglycan	nmol to displace 50%
Heparin	0.3 nmol/ml
Heparan sulfate	3.6 nmol/ml
Dermatan sulfate	7.0 nmol/ml
Chondroitin sulfates	39.0 nmol/ml**

*Handin and Cohen, 1976.
**Chondroitin 6-sulfate was 10 times as effective as an equal molar concentration of chondroitin 4-sulfate.

A number of techniques have been developed for the isolation of PF4. Only a few, however, have been able to demonstrate a homogeneous preparation by both sodium dodecyl sulfate (SDS) and disc gel polyacrylamide electrophoresis. In 1975, Pepper, Moore, and Cash published work on the isolation of PF4 by gel chromatography (25). Purification was achieved on two large molecular sieve columns utilizing ionic strength differences to separate the carrier from the protein moiety. They were able to show a protein with a molecular weight of 27,000 daltons, a stokes radius of 2.52 nm, and a sedimentation coefficient of 2.4 S. The most recent techniques used to purify PF4 have taken advantage of its intrinsic capabilities to neutralize the inhibitor of coagulation, heparin. The first authors to describe this approach

were Levine and Wohl (26). Using the method of Schmer they prepared an affinity column of heparin epsilon-aminocaproic acid Sepharose. The release products of freeze-thawed platelets were applied to the column and the PF4 eluted by changing the ionic strength of the buffer. They achieved a mean recovery of 40%, a peak fraction specific activity of 375 units/mg, and a purification factor of 855. The product was homogeneous on SDS disc electrophoresis. Using the same technique, but eliminating the epsilon-aminocaproic acid arm, Handin and Cohen obtained a yield of 75% with a specific activity of 235 units/mg (24). The molecular weights of the components from each of these isolation procedures is shown in Table III.

Table III: Molecular weights of PF4 as determined by different isolation techniques.

	Carrier	Protein	PF4 Carrier Complex (Dimer)
Barber, A.J.	59,000	29,000	350,000
Moore, S.		27,100	358,000
(Subunit)		6,900	
Handin, R.I.		9,600	
(Subunit)			
Levine, S.P. (SDS)		11,600	
(Gel Filtration)		40,000	

The development of the heparin-Sepharose affinity column as a tool in the purification of PF4 made available a highly purified protein product. Using these products it has been possible to characterize the PF4 molecule. The amino acid composition shown in Table IV is a composite of work by Handin, Pepper, and Hermodson (24, 25,

27). In all cases acid hydrolysis was carried out for at least 24 and 48 hours and cysteine was measured as cysteic acid. No methionine or trytophan were found. In 1977, Hermodson et al. published the primary amino acid sequence of human PF4 (27). Figure I shows the results of their sequence analysis. They were unable to detect any free cysteine. Thus, two disulfide bonds appear to exist within the molecule. They did not suggest where these bridges might be positioned.

Table IV: Amino acid composition of human PF4.

Amino Acid	Residues/Mole	Amino Acid	Residues/Mole
Alanine	5	Lysine	7
Arginine	3	Methionine	0
Aspartic acid	5	Phenylalanine	0.5
Cysteine	3	Proline	4
Glutamic acid	9	Serine	4
Glycine	3	Threonine	4
Histidine	2	Tryptophan	0
Isoleucine	5	Tyrosine	1
Leucine	9	Valine	3

Figure I: Amino acid sequence of human PF4.

```
1                   5                  10                  15
Glu-Ala-Glu-Glu-Asp-Gly-Asp-Leu-Gln-Cys-Leu-Cys-Val-Lys-Thr-

16                  20                 25                  30
Thr-Ser-Gln-Val-Arg-Pro-Arg-His-Ile-Thr-Ser-Leu-Glu-Val-Ile-

31                  35                 40                  45
Lys-Ala-Gly-Pro-His-Cys-Pro-Thr-Ala-Gln-Leu-Ile-Ala-Thr-Leu-

46                  50                 55                  60
Lys-Asn-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Leu-Gln-Ala-Pro-Leu-Tyr-

61                  65                 70
Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser
```

*Hermodson et al., 1977.

A topic still open for some discussion has been the controversy over the subcellular localization of PF4. In 1972, Barber and co-workers used a glycerol-lysis technique and sucrose density centrifugation to demonstrate the fraction containing the greatest heparin neutralizing activity (22). They isolated a soluble fraction, a granular rich fraction, and a membrane rich fraction. These contained 33%, 55%, and less than 1% of the heparin neutralizing activity respectively. Assays for hexuronic acid gave the same results suggesting that the greatest concentration of PF4 was found in the granules and not associated with the membrane. Using methods similar to those of Barber, Day and co-workers again isolated three major zones from the homogenized platelets (28). They broke down the granular fractions into three sub-zones and showed heparin neutralizing activity in the zone richest in dense granules. This area corresponded to a high level of serotonin and non-metabolic adenine nucleotides. Work by Walsh et al. in 1974 led to two hypotheses (29). First, both ADP and serotonin are stored in dense granules, the contents of which are released quickly, and the heparin neutralizing activity is stored in granules other than the dense bodies or the type of alpha granule in which lysosomal enzymes are stored. Second, ADP, serotonin, and heparin neutralizing activity are all stored in the same granule with PF4 being released more slowly. In 1975, Broekman, Handin, and Cohen using density centrifugation and a nitrogen decompression technique for platelet lysis, separated the constituents of the platelets into 9 fractions (30). The purity of the granular fractions, in particular the alpha granules and mitochondria, was far superior to any yet achieved. In contrast to Day they found a separation of the serotonin

activity and the PF4 activity, with the PF4 activity being localized over the zone of purest alpha granules.

The active site of the PF4 molecule was demonstrated in 1976 by Handin and Cohen (24). They presented evidence that binding to aminoglycans occurs via ionic interactions between lysine residues on the protein and negatively charged groups on the aminoglycan. Using O-methylisourea to convert the lysyl groups to quanidino groups they showed a 72% reduction in the heparin neutralizing activity of PF4. To demonstrate that the effect was not due to conformational changes, the PF4 was also modified at the arginine residues with 1, 2-cyclohexanedione with no resultant change in the heparin neutralizing activity.

To show that the heparin neutralizing protein of the platelet could be successfully used to monitor the platelet release reaction Gjesdal and Pepper measured the half-life in the monkey, Macaca mulatta (31). If the half-life of PF4 were very short, the PF4 concentration would only reflect on-going platelet release. If, on the other hand, the half-life were long, normalization of an increased platelet release would not lead to rapid changes in the plasma PF4 concentrations. Although their studies were restricted to two monkeys the results suggested a plasma half-life of 7-11 hours and an intra-platelet half-life of about 21 hours.

In summary, it would appear that PF4 would make an ideal tool for measuring occult *in vivo* systemic platelet aggregation. The protein and carbohydrate moiety of the PF4 complex have been defined both in terms of their chemical and physical characteristics. It is now only necessary to determine the best biologic and immunologic means of assaying for plasma levels.

MATERIALS AND METHODS

Chromatographic Materials

Bio-Gel P-2, A-5M, and A-15M were purchased from Bio-Rad Laboratories, Richmond, California. Sepharose 4B, DEAE-Sephadex, Sephadex B-200, and all column chromatographic equipment was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey.

Chemicals

Cyanogen bromide and acetonitrile were obtained from Aldrich Chemical Co., Cedar Knoll, New Jersey. Acrylamide, methylenebisacrylamide, N, N, N', N'-tetramethylenediamine, and ammonium persulfate were purchased from Bio-Rad Laboratories and sodium dodecyl sulfate was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, New York. "Sigma 104" phosphatase substrate (p-Nitrophenyl Phosphate, Disodium) was purchased from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were reagent grade.

Proteins

Topical bovine thrombin was obtained from Parke-Davis and Co., Detroit, Michigan. Bovine albumin (molecular weight 67,000), hen egg albumin (molecular weight 45,000), chymotrypsinogen A (molecular weight 25,000), and cytochrome C (molecular weight 12,500) were all obtained from Boehringer Mannhein, Indianapolis, Indiana. Alkaline phosphatase, type VII, from calf intestine was from Sigma Chemicals and had a specific activity of 1035 units/mg. Human thrombin, Fibrindex, was from Ortho Diagnostics, Raritan, New Jersey.

Heparin

Heparin grade 1 from porcine intestinal mucosa was obtained from Sigma Chemical Company. The activity varied between 160 and 170 units/mg. Injectable aqueous heparin, from beef lung, was purchased from Upjohn Company, Kalamazoo, Michigan.

Platelets

Seventy-two hour old bank platelets were donated by the American Red Cross, Lansing, Michigan. The total volume of the platelets was estimated based on an average of 25 ml/unit. Enough EDTA, disodium salt, was added to the pooled platelets to give a final concentration of 0.001 M. The pooled platelets were then acidified to a pH of 6.5 with acid citrate dextrose (NIH formula A).

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was carried out according to the methods of Weber and Osborn (32). The proteins were reduced by heating 1 part protein solution with nine parts of 0.01 M sodium phosphate, pH 7.0, containing 1% SDS and 1% 2-mercaptoethanol to 100 C for two minutes. Ten percent gels were used and the ratio of acrylamide to methylenebisacrylamide was fixed at 37:1. The proteins were applied to the gels in combination with 2-mercaptoethanol, glycerol, and the tracking dye, bromphenol blue, and electrophoresis was carried out at 8 mA/tube. Electrophoresis was continued until the tracking dye had migrated to within 1 to 2 cm of the bottom of the gel. The gels were stained in 0.025% Coomassie brilliant blue in 25% methanol and 9% acetic acid and destained in 5% methanol and 7.5% acetic acid. Molecular weight standards were processed identically and consisted of bovine albumin, hen albumin, chymotrypsinogen A, and cytochrome C.

The standard proteins were aliquoted and frozen at -20 C for use in additional procedures. Molecular weight determinations were made by plotting relative mobility values of the standards on the abscissa and log of the molecular weight on the ordinate.

Protein and Carbohydrate Determination

Protein was measured by the Bio-Rad protein assay using bovine serum albumin as a standard. Protein was also monitored by reading absorbances at 280 nm and 260 nm (33). Calculations of concentration by the absorbance method were according to the formula:

$$1.45(OD_{280}) - 0.74(OD_{260}) = mg/ml$$

Carbohydrate was measured according to the procedure of Dubois et al. (34). Two milliliters of a solution containing carbohydrate was incubated for 20 to 30 minutes at room temperature with 0.05 ml phenol, 80% (w/w), and 5 ml of concentrated sulfuric acid. Known concentrations of heparin were used to establish a standard curve. The absorbance of each sample was read at 595 nm.

Heparin Neutralizing Activity

Platelet poor plasma was obtained from blood treated with 3.8% trisodium citrate at a ratio of 9:1. The blood was centrifuged at 3000 rpm for 15 minutes at room temperature in a Sorvall GLC-1, Norwalk, Connecticut. The assay system used was Dana's modification of Harada and Zucker's original procedure (7). Prior to testing, dilutions of aqueous heparin (Upjohn) were prepared in 0.15 M NaCl varying from 0.1 to 2.0 units/ml. The test system was a mixture of 0.10 ml of a test sample or blank, 0.10 ml of substrate plasma, and 0.05 ml of the desired heparin dilution. This mixture was incubated at 37 C for one minute and 0.05 ml of a thrombin solution was then

added and the clotting time determined. Human thrombin, diluted with imidazole buffer, was used at a final concentration of 16.5 units/ml (Ortho). Progressively higher concentrations of heparin were added to determine the minimal concentration of heparin necessary to give a clotting time greater than 20 seconds. A blank curve was constructed by substituting buffer for the test plasma. The curve was drawn with the heparin values on the abscissa and the clotting times on the ordinate. The concentration of heparin at which the curve crossed the 20 second mark was taken as the heparin neutralizing activity and the test value was obtained by subtracting the value of the blank from the value obtained from the unknown at 20 seconds. All assays were performed on a Fibrometer (Baltimore Biologic Laboratories).

Conductivity Measurements

Conductivity was measured on the Radiometer type CDM2e conductivity meter and the values calculated by comparison to buffers of known composition.

Preparation of Heparin-Agarose

Heparin was covalently linked to Bio-Gel A15M according to the method of March et al. (35). The acetonitrile was redistilled prior to use. The acetonitrile-CNBr mixture was prepared by adding 50 ml of redistilled acetonitrile to a 100 g bottle of CNBr. This solution could be stored at -20 C when not in use. One hundred milliliter portions of agarose were washed extensively with double distilled water and then mixed as a 1:1 slurry with 2 M sodium carbonate. Ten milliliters, or 0.05 volumes, of the acetonitrile-CNBr solution was added with rapid stirring. The reaction was allowed to continue

for one and one-half to two minutes, or until all the CNBr had redissolved. The agarose was then washed alternately with 0.1 M sodium bicarbonate, pH 9.5 and double distilled water. The final wash was in the buffer in which the heparin was to be dissolved. The washing procedure should not take more than 90 seconds. The washed gel was incubated at 4 C with constant stirring in 100 ml of 0.1 M sodium bicarbonate, pH 9.5 containing 600 mg of heparin (Sigma). The incubation was continued for 18 to 20 hours and unreacted groups were then masked by adding 1 M glycine and mixing for an additional 4 hours at room temperature. The coupled agarose was washed alternatively with 2 liters of 0.5 M NaCl/ 0.1 M sodium acetate, pH 4.0, 2 liters of 0.5 M NaCl/ 2 M urea, and 2 liters of 0.5 M NaCl/ 0.1 M sodium bicarbonate, pH 10.0. After washing the heparin gel was equilibrated with 0.15 M NaCl/ 0.01 M Tris, pH 8.6.

Regeneration of Heparin-Agarose

In most cases the heparin-agarose can be reused after alternate washing in high and low ionic strength buffers and a final equilibration with starting buffer. After several preparations it may be necessary to wash more extensively. The heparin-agarose can be suspended in 5 volumes of 1 M sodium chloride containing 0.5% SDS with gentle stirring at room temperature for 1 hour. The gel is then allowed to settle. The supernatant is aspirated off and the gel washed twice with 5 volumes of distilled water for 60 minutes at room temperature with constant stirring. The supernatant is removed and the column repacked. The gel is then washed with a 30-fold volume of distilled water, 5 volumes of 2 M NaCl, 1 M NaCl/ 0.01 M Tris, pH 8.6 and finally again with distilled water. The washed gel is then equilibrated with starting buffer.

Purification of PF4

Fifty units of platelets pooled as previously described were centrifuged at 120 x g for 15 minutes at 4 C to remove red blood cells. The platelets were then pelleted by centrifugation at 2800 x g for 15 minutes at 4 C. The platelet poor plasma was removed by aspiration and the platelets were washed twice with a solution containing 0.15 M NaCl, 0.01 M trisodium citrate, and 0.001 M EDTA, disodium salt. The washed platelets were resuspended in 0.15 M NaCl/ 0.01 M Tris, pH 8.6 containing 0.025 M $CaCl_2$ and warmed to 37 C. Topical bovine thrombin was added to a final concentration of 1 unit/ml and visible aggregation occurred within 1 minute. After an additional 3 minutes of incubation the platelets were cooled in an ice bath and then centrifuged at 12100 x g for 30 minutes at 4 C. The supernatant was stored at -20 C prior to application to the column. A precipitate will develop with storage and should be removed by centrifugation at 12100 x g for 30 minutes at 4 C.

The supernatant from the platelet aggregation can be either applied directly to a packed column of heparin-agarose or mixed with 40 ml of the gel in starting buffer for one hour at 4 C. In the latter case the agarose was then poured into a 1.6 by 20 cm column and allowed to pack by hydrostatic pressure. The column was washed with starting buffer until the O.D. at 280 nm returned to baseline. The elution was performed in two steps. First, 0.5 M NaCl/ 0.01 M Tris, pH 8.6 was applied and washing continued until no protein could be detected by optical density determinations. Finally 1.0 M NaCl/ 0.01 M Tris, pH 8.6 was run onto the column and the PF4 eluted in

one peak. The column could also be washed by application of a salt gradient having ionic strengths ranging from 0.5 M NaCl to 3.0 M NaCl in 0.01 M Tris, pH 8.6. The tubes containing the highest activity were concentrated by desalting on a P-2 column equilibrated with 0.5 M NH_4HCO_3/ 0.5 M NaCl. The protein in the void volume was then pooled and lyophilized. Alternately, the protein was concentrated by ultrafiltration on an Amicon UM-2 membrane, Amicon Corporation, Lexington, Massachusetts.

Preparation of Antibody and Immunoassay

Antibody to purified protein was prepared by immunizing rabbits with 20 ug of PF4 in Freunds complete adjuvant. Injections were made intradermally in 10 to 15 sites on the rabbits back. A second injection of 20 ug in Freunds complete adjuvant was made 6 weeks later and the rabbits were bled two weeks after the last injection. Blood was drawn into 3.8% trisodium citrate at a ratio of 9:1. Red cells and platelets were removed by centrifugation at 5000 x g and clotting of the plasma was initiated by addition of topical bovine thrombin at a final concentration of 1 unit/ml.

a. Ammonium Sulfate Precipitation

The gamma globulin portion of the serum was precipitated by addition of ammonium sulfate. A saturated solution of $(NH_4)_2SO_4$ was adjusted to pH 7.8 by the addition of 2 M NaOH. This was done just prior to precipitation of the IgG fraction since ammonia is released upon prolonged standing. With constant stirring 25 ml of the $(NH_4)_2SO_4$ solution was added dropwise to 50 ml of serum resulting in one-third saturation. Stirring was continued at room temperature

for 2 hours after addition in order to avoid mechanical trapping of serum components other than IgG in the precipitate. The suspension was then centrifuged at room temperature for 30 minutes at 1400 x g. The precipitate was redissolved in an amount of saline equal to the original volume and a second and third precipitation were performed as above. After the final precipitation the protein pellet was dissolved in a volume of phosphate buffered saline (PBS), pH 7.2, equal to one-half of the original volume. The solution was then extensively dialyzed against several changes of PBS at 4 C. Any precipitate remaining after dialysis can be removed by centrifugation at 1400 x g for 30 minutes at 4 C.

b. Immunoadsorption

To 12 ml of antigen, in PBS, containing 20 to 50 mg/ml of protein 3 ml of 2.5% glutaraldehyde was added with vigorous stirring (33). If the protein concentration is not sufficient bovine serum albumin (BSA) can be added. The solution was stirred for 2 to 3 hours, care being taken to avoid foaming. A protein gel should form. If not a few more drops of glutaraldehyde may be added and stirring continued for an additional hour. The gel was then washed by addition of a 5-fold volume of PBS. Washing was continued until the absorbance of the wash at 280 nm fell below 0.04. The buffer was then changed to 0.1 M glycine pH 2.8 and the gel mixed for 5 to 10 minutes. Following the glycine wash the gel was then reequilibrated with PBS. Five milliliters of antiserum to be adsorbed were added to the washed gel and mixed vigorously for 15 minutes without foaming. The solution was then centrifuged and the supernatant removed. The adsorbed antibody was removed by addition of 0.1 M glycine, pH 2.8, after the gel had

been washed in PBS. The gel, which can be reused, should be stored at 4 C in PBS containing 0.02% sodium azide.

c. Immunodiffusion and Immunoelectrophoresis

Immunodiffusion and immunoelectrophoresis were carried out in 1% agarose (Bio-Rad) in 0.075 M barbital buffer containing 2 mM calcium lactate. The Ouchterlony's were run on 7.1 x 7.1 cm glass slides according to standard techniques using 4 mm wells (33). The slides were incubated in a humidified chamber at 4 C for 24 hours. Prior to washing the slides were compressed under several layers of filter paper for 3 minutes, taking care to avoid trapping air bubbles. The slides were washed for 24 hours in 0.3 M NaCl and the salt was then removed by an additional 3 to 4 hour wash in distilled water. The slides were compressed under filter paper for 10 minutes using a 2 kg weight and then allowed to dry. The slides were stained in 0.25% Coomassie brilliant blue in methanol, acetic acid, and water (5:1:4) for 3 to 5 minutes and then destained in a solution containing 10% methanol and 10% acetic acid.

Rocketry was performed according to Laurell (36). The gels were 1 mm thick. Four millimeter wells were cut to which 10 ul of sample was applied. The gels contained a 1 to 100 dilution of antisera and electrophoresis was run at 20 V/cm for 4 hours. Staining was performed as previously described for Ouchterlony's.

Enzyme-Linked Immunosorbent Assay

a. Alkaline Phosphatase Labeling

Glutaraldehyde coupling of enzyme to antibody was done according to modifications of Avrameas (37, 38). The procedure of conjugation was as follows: 0.3 ml of alkaline phosphatase solution

Sigma Type VII was centrifuged at 2000 rpm for 10 minutes at 4 C and 0.2 ml of the clear supernatant was removed. To this solution 0.1 ml of an $(NH_4)_2SO_4$ cut of immune serum containing 0.54 mg/ml of protein was added. The final ratio of enzyme to antibody was 3:1. This solution was mixed and dialyzed for 8 hours at 4 C against 3 changes, 2 liters each, of phosphate buffered saline, pH 7.2. Just prior to use 4.2% glutaraldehyde in PBS was prepared from a 50% stock solution. The enzyme-antibody mixture was removed from dialysis and enough of the 4.2% glutaraldehyde was added to give a final concentration of 0.2%. The reaction was allowed to take place for 2 hours at room temperature. The solution was then diluted to 1 ml with PBS and dialyzed overnight at 4 C against 2 changes of PBS. The protein was then chromatographed on a 2.5 x 100 cm Sephadex G-200 column equilibrated with 0.05 M Tris/HCl, pH 8.0, containing 0.02% sodium azide. The protein fractions eluted in the void volume were pooled and enough BSA (Sigma RIA Grade) added to give a final concentration of 4g%. This solution is stored at 4 C in a dark container and used at a 1:10 dilution. The void volume of the G-200 column was determined by the use of Blue Dextran 2000 (Pharmacia) at a concentration of 2 mg/ml.

b. Glutaraldehyde Treatment of Polypropylene Tubes

Polypropylene tubes, 12 mm x 75 mm, were obtained from Curtin Matheson, Detroit, Michigan. One milliliter of freshly prepared 0.1% glutaraldehyde in 0.1 M carbonate/HCl, pH 9.0, was added to each tube. The tubes were capped and incubated for 3 hours at 56 C. After incubation each tube was washed three times with double distilled water and used immediately for coating with antibody (39).

c. Antibody Coating of Plastic Tubes

An appropriate dilution of antibody in 0.1 M carbonate/HCl, pH 9.0, as determined by titration experiments, was prepared. One milliliter was added to each tube and then incubated for at least 20 hours at 4 C. The tubes can be stored with the immunoglobulin fraction in place. Before use the tubes were washed 5 times with 0.15 M NaCl containing 0.05% Tween 20.

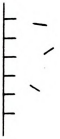
d. Antigen Standards

Platelet factor 4 prepared as previously described was assayed for activity and protein content according to the procedures outlined. Dilutions of the purified protein ranging from 2 to 87 ng/ml were prepared in PBS containing 1g% BSA.

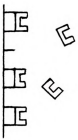
e. Enzymeimmunoassay System

One-half milliliter of standard or an appropriate dilution of test plasma was incubated in the glutaraldehyde-treated and antibody coated tubes for 5 hours at room temperature on a 45^{o} angle roller drum. One percent BSA in PBS was used to make all dilutions. The incubation mixture from the first reaction was discarded and the tubes were washed 5 times with an excess of 0.15 M NaCl containing 0.05% Tween 20. To each tube 0.5 ml of a 1:10 dilution of ELISA in PBS, BSA buffer was added. The tubes were incubated at room temperature on the roller drum for 16 hours or overnight. The 0.5 ml aliquots of ELISA were then removed and the tubes again washed 5 times with the Tween-NaCl solution. The tubes were then incubated for 30 minutes at room temperature with a 0.1% solution of freshly prepared p-nitrophenylphosphate substrate in 0.05 M sodium carbonate/HCl, pH 9.8. Color development was stopped by addition of 0.2 ml of

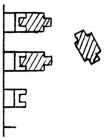
0.5 M NaOH. The absorptivity was measured at 400 nm and unknowns were calculated from standard curves plotted with log of the PF4 concentration versus absorbance (Figure II).



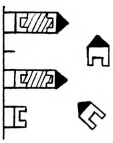
Glutaraldehyde (GDA) heat fixed to polyprolylene tubes at 56 C.



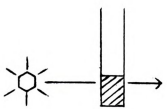
Nonspecific coupling of antiserum to GDA-treated polypropylene tubes.



Antigen interaction with immobilized antiserum.



Enzyme-linked antiserum attached to bound antigen.



Measure absorbance at 400 nm after the development of a yellow color with the p-nitrophenylphosphate (enzyme substrate). The intensity of the color is proportional to the amount of antigen bound.

Figure II

RESULTS

The heparin neutralizing protein was purified from outdated human platelets. The procedure involved affinity chromatography of crude platelet extracts on a heparin-agarose affinity column. To determine percent yield, the amount of PF4 in the starting material was based on the heparin neutralizing activity of the pooled platelet rich plasma which had been treated to release PF4. Because of the platelet damage due to prolonged storage at 4 C, 80 to 90% of the heparin neutralizing activity was lost by the initial washing to remove plasma proteins. Table V outlines the specific activities and yields based on results obtained from three different preparations.

Table V: Purification of heparin neutralizing protein.

	Total Protein mg	Hep. Neut. Total Units	Specific Act. U/mg	Yield %
Pooled Platelet Rich Plasma*	92,682.5	9,333.8	0.10	100
Thrombin Extract**	211.5	1,395	6.6	15(100)***
Heparin-Agarose	2.2	595.5	270.9	6(43)

*Represents the PF4 activity in platelets plus the PF4 activity in plasma.
**Represents the PF4 thrombin aggregated extract of washed platelets.
***Yield data in parenthesis is based on the PF4 activity of the thrombin aggregated extract of washed platelets.

The release products of thrombin aggregated washed platelets had a specific activity of 6.6 U/mg. This represented a 66-fold initial purification of the heparin neutralizing activity based on a specific activity of 0.10 U/mg for the platelet rich plasma. Application of the thrombin extract to the heparin-agarose column resulted in a 41-fold further purification. The additional figures listed in parenthesis are values obtained if the thrombin extract is considered the starting point for purification and represent the efficiency of the heparin affinity column (Table V).

Measurements of heparin covalently linked to agarose were done using Dubois' carbohydrate assay (34). Starting material and washes from the heparin-agarose preparation were assayed. The difference between these values was taken as the quantity of heparin on the agarose gel. The values obtained varied from 50 to 100 U/ml of gel.

Several methods were used to concentrate the PF4. Dialysis against low ionic strength buffers precipitated the protein and the heparin neutralizing activity was lost. It was found to be more efficient to exchange buffers on a P-2 column equilibrated with 0.5 M NaCl/ 0.5 M NH_4HCO_3. Lyophilization after chromatography resulted in negligable loss of heparin neutralizing activity. Once lyophilized the protein was stored in a desiccator at -20 C without loss of activity. A UM-2 membrane could also be used to concentrate the heparin neutralizing protein.

Figure III illustrates a stepwise elution pattern from the heparin affinity column. The peak of protein eluting in the starting buffer is not shown. A gradient obtained with NaCl at a starting concentration of 0.5 M NaCl and a limit concentration of 3 M was also used to elute

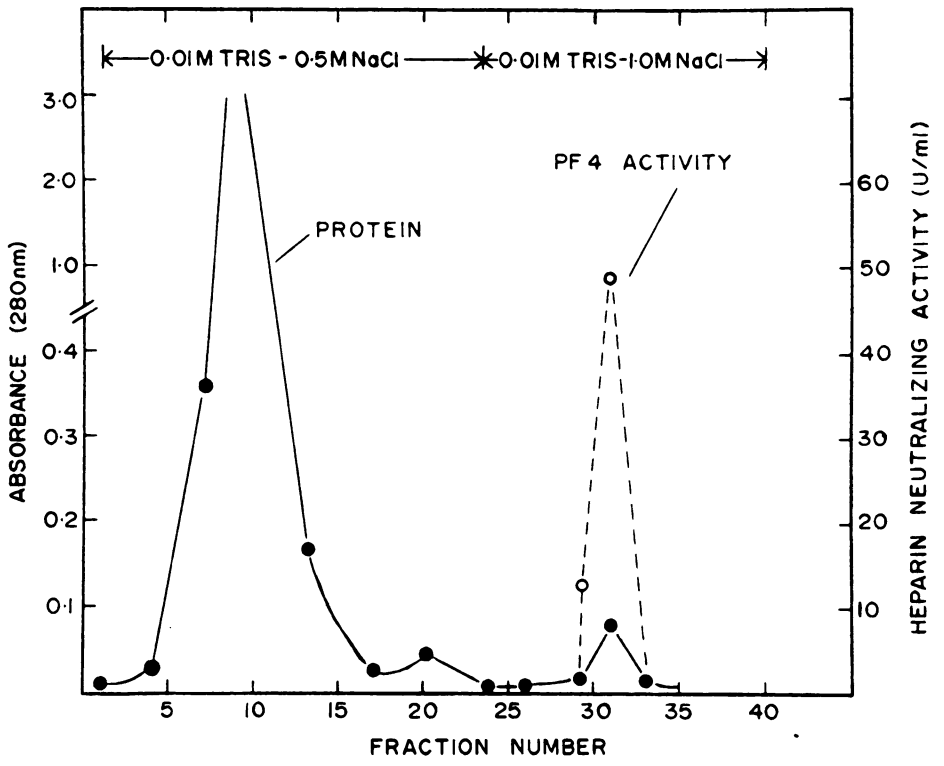


Figure III. Elution pattern of heparin neutralizing protein from heparin-agarose. The protein eluting with the starting buffer is not shown. Starting material was applied in 0.15 M NaCl/ 0.01 M Tris, pH 8.6. When the absorbance had fallen to zero, 0.5 M NaCl/ 0.01 M Tris, pH 8.6 was applied, followed by 1.0 M NaCl/ 0.01 M Tris, pH 8.6.

the column. In these experiments the gradient was not applied until the first peak had been washed from the column with 0.5 M NaCl/ 0.01 M Tris, pH 8.6. Because the two eluting peaks have identical mobility on SDS-PAGE it was difficult to determine if one was contaminated with the other. Care must be taken to assure that the absorbance at 280 nm returns to baseline prior to application of the next buffer. A typical run consisted of approximately 100 units of pooled platelets with 2 to 3 mg of purified protein isolated per experiment. In all cases the heparin neutralizing activity eluted with a salt concentration of 0.9 to 1.0 M NaCl, and an additional peak eluted with 0.5 M NaCl which did not have heparin neutralizing activity as determined by the biological assay.

Polyacrylamide Gel Electrophoresis

The peak fractions eluting from the column were applied to SDS-PAGE in quantities of 10 to 20 ug/gel of purified protein or standard. There was a single protein band observed in all cases. Both the first peak eluting from the column and the peak containing heparin neutralizing activity had identical mobilities (Figure IV). Figure V illustrates the migration of peaks in comparison with known standards on SDS-PAGE. A single band of protein from the tubes containing the highest heparin neutralizing activity was also found on native disc gels.

Molecular Weight

The protein migrated as a single band and had a subunit molecular weight of 9,300 daltons in comparison to standards of known molecular weight (Figure VI). The protein eluting at an ionic strength of 0.5 M NaCl had a molecular weight identical to the heparin neutralizing protein.

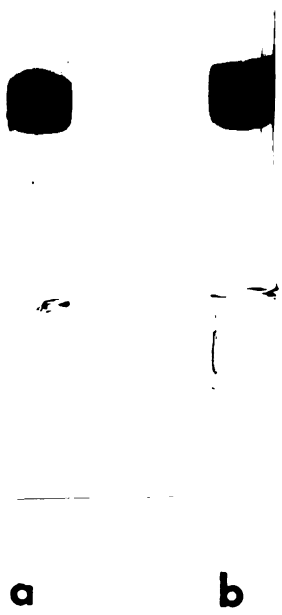


Figure IV. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of highly purified human PF4. a, protein eluting at a salt concentration of 0.5 M NaCl. b, heparin neutralizing protein eluting in 1.0 M NaCl.

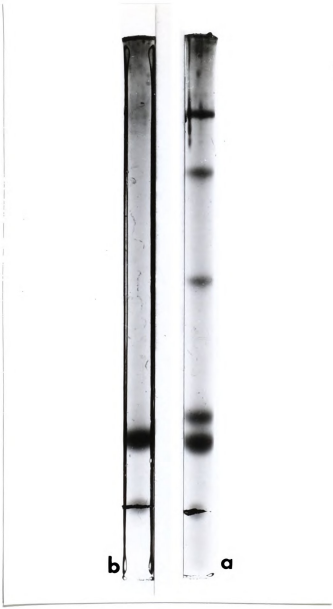


Figure V. Ten percent sodium dodecyl sulfate polyacrylamide gels illustrating the migration of PF4 relative to proteins of known molecular weight. a, mixing experiment using bovine albumin, 67,000, egg albumin, 45,000, chymotrypsinogen A, 25,000, and cytochrome C, 12,500 with human PF4. b, purified heparin neutralizing protein.

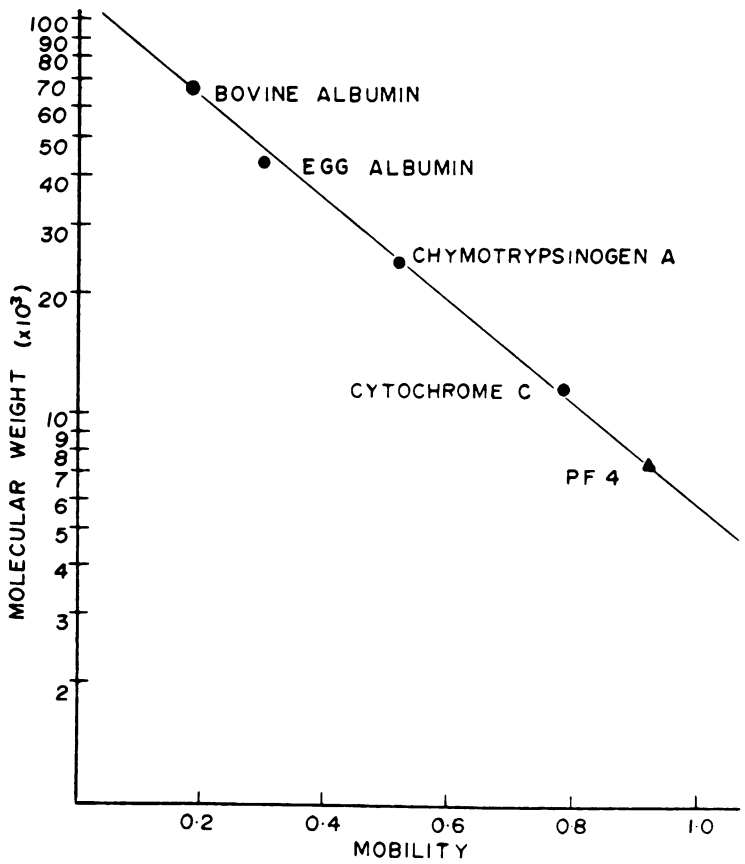


Figure VI. Determination of the molecular weight of human PF4. The proteins were run on duplicate gels and the relative mobility of the PF4 was 0.90 and 0.93. The extrapolated value for the molecular weight of PF4 was 9,300 daltons.

Antibody Production, Immunodiffusion, and Immunoelectrophoresis

Two different injection schedules were followed. The one found to be most successful consisted of a low-dose intradermal injection with booster shots at six weeks and bleedings at eight weeks. It was not necessary to couple the protein to a carrier in order to elicit an antibody response.

By immunodiffusion a single precipitin arc was observed against rabbit antiserum to human PF4. In three of the rabbits immunized a second precipitin band was observed which appeared to be against the protein eluting from the column at a lower ionic strength. Immunoadsorption of the antisera with this protein peak resulted in a single precipitin arc (Figure VII a, b). Examination of the antiserum by rocket electrophoresis also demonstrated a monospecific antiserum to PF4 (Figure VIII).

Several attempts were made to assay for plasma levels of PF4 by rocket immunoelectrophoresis. Because of the suspected low levels in plasma, nanogram quantities, it was not possible to detect any PF4 by standard techniques. In one series of experiments the heparin neutralizing protein was concentrated from 5 ml of citrated plasma by mixing with 0.5 ml of heparin-agarose. Minimal success was obtained in this experiment but results could not be used to accurately quantitate PF4 levels. PF4 levles were detected when assaying serum samples.

Enzyme-Linked Immunosorbent Assay

Initial tests on the Enzyme-Linked Immunosorbent Assay (ELISA) gave results indicating its potential use as a very sensitive marker of PF4 levels.

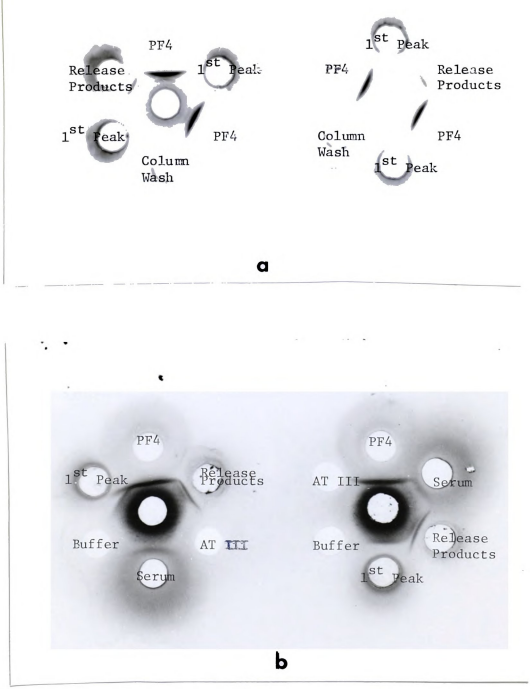


Figure VII. Immunodiffusion of various serum and platelet components against antiserum to human PF4. a, precipitation pattern demonstrating the monospecificity of the antiserum after absorption. b, heterogeneous antiserum illustrating precipitation against protein eluting in 0.5 M NaCl.

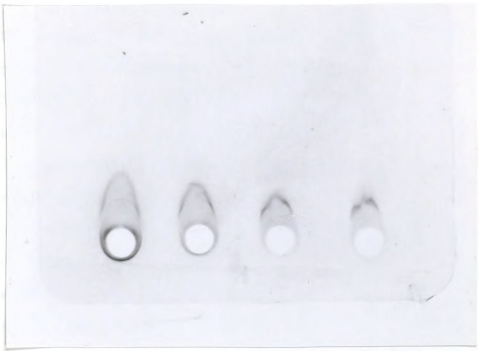


Figure VIII. Rocket electrophoresis of serial dilution of human serum against anithuman PF4 demonstrating the monospecificity of the immune serum. Antiserum was diluted 1:100 in 1% agarose and dilutions of serum from 1:1 to 1:8 were run into the agarose. The electrophoresis was carried out at 10 C for 4 hours and the voltage was maintained at 20 V/cm.

A solid-phase, non-competitive enzymeimmunoassay was used. Glutaraldehyde was heat fixed to polypropylene tubes and antisera was then linked to the glutaraldehyde by the supposed formation of Schiff bases between the aldehyde residue on the glutaraldehyde-polymer and the amine residue of the protein. Antigen was then incubated for five hours with the coated tubes followed by a 16 hour incubation with the alkaline phosphatase coupled IgG. The alkaline phosphatase-IgG coupling was done according to modification of Avrameas. Conjugated protein eluting in the void volume of a G-200 column was used. Color was developed in the reaction tubes with p-nitrophenylphosphate and antigen levels determined by absorbance at 400 nm.

Eighty to 90% of the conjugated antiserum could be recovered in a high molecular weight fraction when applied to a G-200 column. A total volume of 1 ml, containing 2.04 mg of protein, was applied to the column. Fractions eluting in the void volume and having an absorbance at 280 nm of 0.1 or greater were pooled. The final volume of the conjugate in the first experiment was 13.5 ml. In order to avoid unreacted IgG, the trailing edge of the elution peak was discarded.

The results presented will deal with the following variables:

1. The buffering system for coupling IgG to glutaraldehyde treated tubes.

2. The concentration of antiserum used to coat the tubes.

3. The concentration of conjugated enzyme to give optimum absorbance readings over a specific range of protein standards.

4. The optimum temperature for the antigen-antibody reaction.

Other variables to be considered, but which are not evaluated here, include the effects of antibody titer on the assay system and the optimum time of reaction for each step in the assay system.

In the initial experiments the concentration of enzyme conjugated antiserum was kept constant at a dilution of 1:10. In order to determine the effects of antibody concentration and the type of buffering system to give maximum coupling of antibody to polypropylene tubes, four series of tubes were prepared. The first set contained antiserum at a dilution of 1:250 and 1:500 in PBS, pH 7.2. These concentrations corresponded to 20 µg/ml and 10 µg/ml of protein respectively. The second set of tubes contained the same concentration of antiserum but diluted in carbonate/ HCl, pH 9.0. The assay was run at room temperature with standards of purified PF4 varying from 5 to 45 ng/ml. Table VI lists the O.D. values at 400 nm obtained from this experiment. Data plotted on semi-log paper suggests that the most efficient coupling was obtained at an antisera concentration of 10 µg/ml in carbonate/ HCl, pH 9.0 (Figure IX).

Table VI: The effects of antiserum and the buffer system on the coupling of IgG to glutaraldehyde treated polypropylene tubes.

ng/ml PF4	Buffer system and dilution of antisera			
	Carb. 1:500	Carb. 1:250	PBS 1:500	PBS 1:250
43.8	0.450	0.261	0.082	0.126
21.9	0.304	0.128	0.023	0
10.9	0.139	-----	0	0
5.5	0.063	0.077	0	0

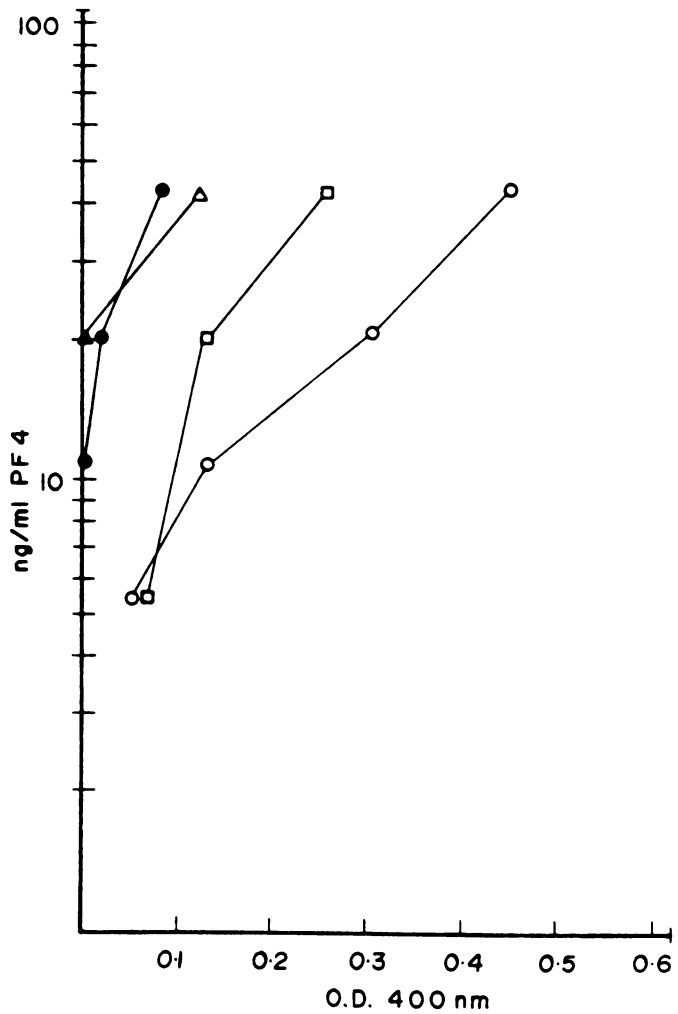


Figure IX. Results of varying the buffering system and the dilution of antiserum utilized to coat the glutaraldehyde treated polypropylene tubes. ○, 10 µg/ml of anti-PF4 in carbonate/ HCl, pH 9.0. □, 20 µg/ml in carbonate buffer. ●, 10 µg/ml in PBS, pH 7.2. △, 20 µg/ml in PBS.

The concentration of protein chosen for these experiments was based on the results of work by Boenisch (40). He found that with a more dense antibody coat on polypropylene tubes more antigenic sites could be expected to participate in the binding of antigen to immobilized antibodies. As a result of this, fewer antigenic sites would be available to participate in the subsequent reaction with the antibody conjugated alkaline phosphatase. Thus, too high a concentration of antibody coating the tubes could be as detrimental to the assay as too low a concentration of antibody.

Although this particular study has not evaluated the long-term stability of the enzyme conjugated antiserum, storage at 4 C in the presence of albumin for up to a month resulted in no loss of activity.

In order to determine the optimum dilution of conjugated enzyme to give maximum absorbance values over a suitable range of antigen levels, several concentrations were run in a simultaneous experiment. The parameters of the test were those established in the first experiment. Antibody tubes coated with a 1:500 dilution of antiserum in carbonate buffer were used and the reaction took place at room temperature. The dilutions of conjugated antiserum ranged from 1:10 to 1:40. The results obtained in these tests are given in Table VII, and illustrated in Figure X. With increasing concentrations of conjugated antiserum the sensitivity of the assay can be increased. However, lowering the concentration resulted in a decrease in the slope of the curve with the results eventually falling to zero. A 1:10 dilution was choosen as the optimum dilution for the range of antigen concentrations used. Statistical analysis of the optimum curve resulted in a coefficient of determination of 0.8.

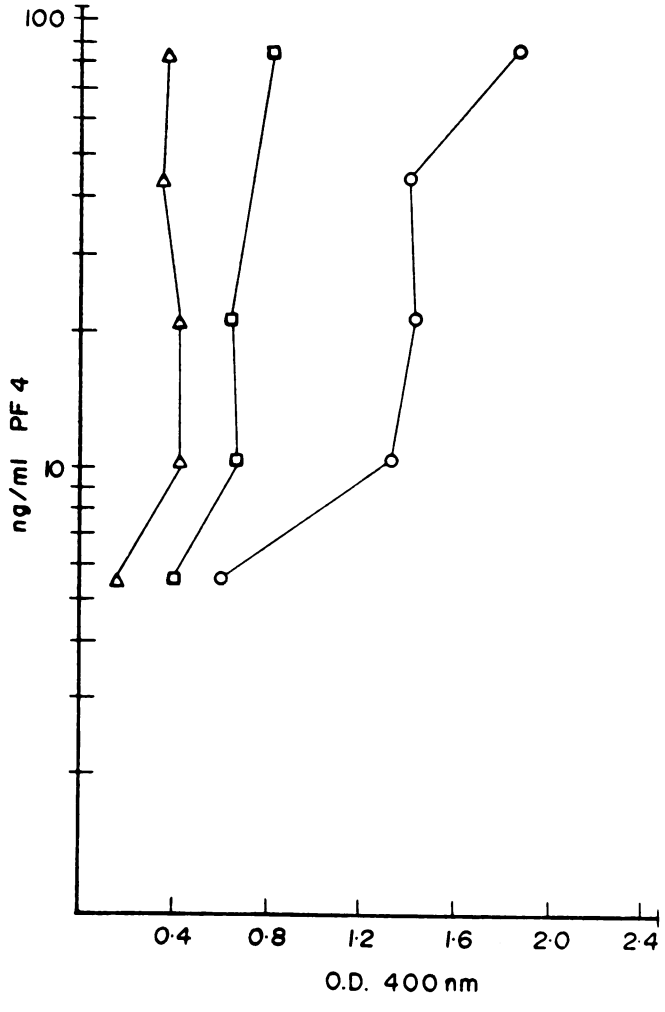


Figure X. Effects of increasing the dilution of alkaline phosphatase conjugated anti-PF4 on the enzyme-linked immunosorbent assay. ○, 1:10 dilution, □, 1:20 dilution, △, 1:40 dilution.

Table VII: Effects on absorbance values as a result of changing the concentration of enzyme conjugated antiserum.

ng/ml PF4	Dilutions of conjugated antiserum 1:10	1:20	1:40
87.5	1.872	0.834	0.358
43.8	1.407	-----	0.353
21.9	1.441	0.624	0.409
10.9	1.347	0.652	0.416
5.5	0.604	0.401	0.173

The final parameter to be evaluated was the effect of temperature on the assay system. As previously indicated, a 1:500 dilution of antiserum was used to coat the glutaraldehyde treated tubes, and the conjugated antiserum was used at a final dilution of 1:10. The tubes were incubated at 4 C for both the 5-hour incubation with the antigen and the 16-hour incubation with the alkaline phosphatase conjugated antiserum. Results in Table VIII indicate the increased variability as a result of the change in temperature. Duplicate determinations showed a wide variation in absorbance readings. The coefficient of determination for the averaged data was 0.78. A comparison of the curves obtained at room temperature and 4 C shows very little statistical difference when the r^2 values are compared. Without further testing of samples at both temperatures no firm conclusion can be drawn on the optimum temperature at which the assay should be run. Results of the data are illustrated in Figure XI.

Table VIII: Results of testing the ELISA system at 4 C.

ng/ml PF4	Absorbance (400 nm) on duplicate determinations 1	2	$\bar{X}$
87.5	1.431	1.417	1.424
43.8	0.815	1.583	1.177
21.9	1.221	1.521	1.371
10.9	0.783	0.562	0.673
5.5	1.069	0.702	0.886
2.7	0.269	0.309	0.289

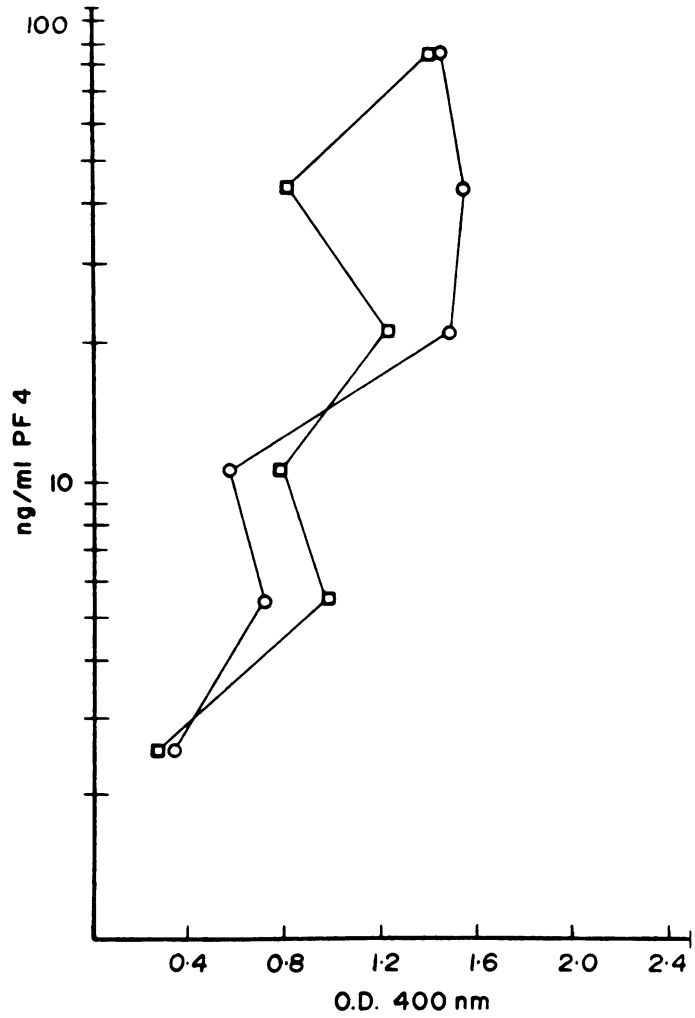


Figure XI. Variability of ELISA as a result of running the assay at 4 C. The two plots represent duplicate determinations.

DISCUSSION

Platelet factor four is a low molecular weight protein having heparin neutralizing activity. We have used affinity chromatography to isolate this heparin neutralizing factor and to demonstrate a highly purified protein on SDS-PAGE. Handin has demonstrated the affinity sequence of this protein for various glycosaminoglycans and has shown by the use of O-methylisourea that the most probable binding site is the lysine residue on the PF4 molecule (24). The ability of PF4 to bind to and release from the heparin affinity column is further evidence of the noncovalent linkage of PF4 to heparin.

Two specific proteins were isolated from the column. Niewiarwoski has termed these a "low" and "high" affinity protein (41). We have not determined the significance of the "high" and "low" affinity species. The possibility exists that the "low affinity" character is an artifact of specimen preparation or an analogue to the heavy and light chains of gamma globulin. It has been suggested that the mitogenic factor of platelets is a low molecular weight, heat stable protein. Further investigation of the "low affinity" peak might suggest a function not related to the heparin neutralizing protein (22).

As is evident from the literature presently available the most successful and the most efficient means of isolating PF4 appears to be affinity chromatography (24, 26). In this particular system outdated human platelets are the source of PF4. Because of the initial

loss in the heparin neutralizing protein, as a result of removing the plasma, the efficiency of the purification is greatly reduced. Several attempts were made at isolating the PF4 from plasma but one of two problems generally resulted. If a 50% ammonium sulfate cut were first performed on the plasma, extensive dialysis on very large quantities of plasma was necessary. The final yield of purified protein was so small that the use of this procedure was not warranted. If, on the other hand, the plasma were applied directly to the affinity column the PF4 could be isolated in fairly good yield but it was generally contaminated with a higher molecular weight protein suspected to be heparin cofactor (antithrombin III). Levine and Wohl's success at isolating PF4 from plasma was based on the use of an Amicon ultrafiltration device for dialyzing and concentrating the ammonium sulfate cut prior to application to the affinity column (26).

The molecular weight of 9,300 obtained for PF4 by the purification procedure used correlated well with the results obtained by both Handin and Levine (24, 26). The range of subunit molecular weights for PF4 are shown in Table III.

Several groups have investigated the possibility of a homologous antigen existing in a variety of different species (31, 42). If this were the case the development of a monospecific antibody would be easiest in an animal demonstrating the greatest amount of intraspecies variation. Studies performed to date indicate the existence of a homologous protein in the monkey, Macca mulatta, and in rabbits. According to Ginsberg the rabbit protein demonstrated the same heparin neutralizing activity as the human protein, had a similar amino acid composition, and a similar subunit molecular weight (42). The protein

was also immunologically cross-reactive with human PF4. Although the rabbit appears to be immunologically responsive to human PF4, the use of either a goat or sheep in an attempt to develop a stronger immunologic response might be suggested.

Initial attempts at the production of antibody in rabbits were only minimally successful. This was attributed to the quantity of protein and the type of injection schedule. It was found that the use of smaller amounts of protein resulted in a better immunologic response. The development of a second antibody in one group of rabbits was thought to be due to contamination of the PF4 by the protein eluting in the "low affinity" peak. Adsorption of the antiserum with this peak resulted in a monospecific antiserum which could then be used in specific immunologic assays.

The clinical applicability of measuring PF4 was demonstrated by Handin (43). Plasma PF4 was measured in a group of patients admitted to the Coronary Care Unit with acute chest pain, and in patients who had undergone cardiac valvular surgery. Mean levels of PF4 were elevated in both cases. He showed that administration of antiplatelet agents such as dipyridamole to the patients with prosthetic valves lowered their PF4 level to within normal range.

Although several investigators have developed a radioimmunoassay for PF4, an enzyme-linked immunosorbent assay was choosen as a viable alternative to RIA. Radioimmunoassay, although it is a very sensitive system, is expensive, carries risks from hazardous substances, utilizes substances with short half-life and requires expensive equipment which is not readily available to all laboratories. The use of enzyme assays overcomes some of these disadvantages. The enzyme-linked antiserum has

been shown to have a long shelf-life, utilize equipment that is generally available in all laboratories, and does not require any special handling precautions. The assay is based on the assumptions that antigen or antibody can be coupled to a solid phase and retain immunologic activity and that enzyme can be conjugated to either antibody or antigen and retain both enzymatic as well as immunologic activity. It has been shown by experience that antigen or antibody can be attached to plastic surfaces, polypropylene or polystyrene, either chemically or nonspecifically. It has also been shown by Nekane and Avrameas that enzymes can be linked to immunoglobulins by one of several techniques (44, 45).

According to Avrameas, glutaraldehyde is an efficient cross-linker and meets the criteria of maintaining both immunologic and enzymatic activity of the components involved in the reaction. His evidence is based on the assumption that the glutaraldehyde linking is almost exclusively by free amino groups of the protein. Most often these free amino groups do not actively participate in the catalytic activity of an enzyme molecule, and this seems to hold true for the antibody. He suggests that the average ratio of enzyme to IgG varies between one and two.

In order for an ELISA assay to be a functional replacement for the RIA it must provide a sensitivity in the range of nanograms of protein per ml. It has been shown that the level of plasma PF4 is partially dependent on the type of collection and the conditions under which the plasma is separated from the cells (15). Using citrated plasma, the expected values fall somewhere between 48 and 128 ng/ml depending on the speed of centrifugation. Citrated plasma

was chosen as the anticoagulant because it is readily available in all clinical situations.

Initial testing of the enzymeimmunoassay resulted in irregular curves with very little reproducibility between samples. The slopes of the curves were also too steep to be able to detect small differences in levels of PF4 between test samples. In order to determine if this were due to problems in the assay system or to the instability of the purified PF4, as it exists in its subunit molecular form, serial dilutions of citrated plasma were assayed for PF4. Although a limited number of results are available, data from the plasma assay showed a greatly improved linearity between samples. Coefficient of determinations (r^2) for these curves were in the area of 0.98.

To date the data collected is not sufficient for any statistical conclusions to be drawn. However, the results so far suggest a sensitivity in the range necessary to make the immunoassay feasible. It is believed that, with the increased stability in plasma, the assay can now be developed into a workable system.

CONCLUSION

A method was described for the purification of human platelet factor 4 by heparin affinity chromatography. The protein obtained was shown to be pure by both sodium dodecyl sulfate polyacrylamide electrophoresis and disc gel electrophoresis. The protein was obtained at a final yield of 43% and had a specific activity of 271 units of heparin neutralized per milligram. The protein had a subunit molecular weight of 9,300 daltons when compared to known molecular weight standards.

By immunodiffusion a single precipitin arc was observed against rabbit antiserum to human platelet factor 4. Examination of the antiserum by rocket electrophoresis also demonstrated that it was monospecific for heparin neutralizing protein.

Preliminary results of an enzyme-linked immunosorbent assay suggest the feasibility of utilizing specific antisera labelled with alkaline phosphatase as a substitute for radioimmunoassay in quantitating plasma levels of platelet factor 4.

LITERATURE CITED

LITERATURE CITED

1. Conley, C.L., Hartmann, R.C., Lalley, J.S. The Relationship of Heparin Activity to Platelet Concentration. Proc. Soc. Exp. Biol. Med. 69: 284, 1948.

2. Van Crevald, S., Paulssen, M.M.P. Significance of Clotting Factors in Blood-Platelets, in Normal and Pathological Conditions. Lancet ii: 242, 1951.

3. Van Crevald, S., Paulssen, M.M.P. Isolation and Properties of the Third Clotting Factor in Blood-Platelets. Lancet 1: 23, 1952.

4. Jürgens, R. Funktion der Thrombocyten bei Blutung und Thrombose. Wiener klinische Wochenschrift 66: 850, 1954.

5. Deutsch, E., Wawersich, E., Franke, G. Über das Vorkommen eines Antiheparinfaktors in Thrombozyten und Gewcben. Thromb. Diath. Haemorrh. 1: 397, 1957.

6. Deutsch, E., Kain, W. Studies on Platelet Factor 4. In: Blood Platelets: Henry Ford Symposium. Johnson, Monto, Rebuck, Horn (eds.), Little, Brown and Co., Boston, MA. pp. 337, 1961.

7. Dana, B., Carvalho, A., Ellman, L. Plasma Heparin Neutralizing Activity: Its Use in the Evaluation of Thrombocytopenia and Thrombosis. Amer. J. Clin. Path. 65: 964, 1976.

8. Harada, K., Zucker, M.B. Simultaneous Development of Platelet Factor 4 Activity and Release of ^{14}C-Serotonin. Thromb. Diath. Haemorrh. 25: 41, 1972.

9. O'Brien, J.R., Etherington, M., Jamieson, S., Lawford, P. Heparin Thrombin Clotting-Time and Platelet Factor 4. Lancet 2: 656, 1974.

10. Fuster, V., Kazmier, F.J., Cash, J.D., Bowie, E.J.W., Owen, C.A. Assay of Platelet Factor 4 in Plasma. Mayo Clin. Proc. 48: 103, 1973.

11. Poplawski, A., Niewiarowski, S. Method for Determining Antiheparin Activity of Platelets and Erythrocytes. Thromb. Diath. Haemorrh. 13: 149, 1965.

12. Walsh, P.N., Biggs, R., Gagnatelli, G. Platelet Antiheparin Activity: Assay Based on Factor-Xa Inactivation by Heparin and Antifactor-Xa. Brit. J. Haematol. 26: 405, 1974.

13. Levine, S.P., Wohl, H., Marzec, U., Bernstein, E., Kroener, J. Release of Platelet Factor 4 (PF4) Measured by a Polybrene (R) Assay in Response to In Vitro Platelet Damage. Thromb. Res. 10: 1, 1977.

14. Niewiarowski, S., Lowery, L.T., Howiger, J., Millman, M., Timmons, S. Immunoassay of Platelet Factor 4 (PF-4 Antiheparin Factor) by Radial Immunodiffusion. J. Lab. Clin. Med. 87: 720, 1976.

15. Levine, S.P., Krentz, L.S. Development of a Radioimmunoassay for Human Platelet Factor 4. Thromb. Res. 11: 673, 1977.

16. Bolton, A.E., Ludlam, C.A., Pepper, D.S., Moore, S., Cash, J.D. A Radioimmunoassay for Platelet Factor 4. Thromb. Res. 8: 51, 1976.

17. Handin, R.I., McDonough, M., Lesch, M. Clinical Application of a Radioimmunoassay for Platelet Factor Four. Thromb. Res., in press.

18. Niewiarowski, S., Farbiszewski, R., Poplawski, A. Neutralization of Antithrombin VI (Fibrinogen Breakdown Products) with Platelet Antiheparin Factor (Platelet Factor 4). Thromb. Diath. Haemorrh. 14: 490, 1965.

19. Käser-Glanzmann, R., Jakábová, M., Lüscher, E.F. Heparin-Neutralizing Factor (Platelet Factor 4) from Human Blood Platelets and its Reactivity with Fibrinogen and Soluble Fibrin-Monomer Complexes. Haemosta. 1: 136, 1972/1973.

20. Hiti-Harper, J., Wohl, H., Harper, E. Platelet Factor 4: An Inhibitor of Collagenase. Sci. 199: 991, 1978.

21. Fuster, V., Lewis, J.C., Kottke, B.A., Ruiz, C.E., Bowie, E.J.W. Platelet Factor 4-Like Activity in the Initial Stages of Atherosclerosis in Pigeons. Thromb. Res. 10: 169, 1977.

22. Rutherford, R.B., Ross, R. Platelet Factors Stimulate Fibroblasts and Smooth Muscle Cells Quicscent in Plasma Serum to Proliferate. J. Cell Biol. 69: 196, 1976.

23. Barber, A.J., Käser-Glanzmann, R., Jakábová, M., Lüscher, E.F. Characterization of a Chondroitin 4-Sulfate Proteoglycan Carrier for Heparin Neutralizing Activity (Platelet Factor 4) Released from Human Blood Platelets. Biochim. Biophys. Acta 286: 312, 1972.

24. Handin, R.I., Cohen, H.J. Purification and Binding Properties of Human Platelet Factor Four. J. Biol. Chem. 251: 4273, 1976.

25. Moore, S., Pepper, D.S., Cash, J.D. Platelet Antiheparin Activity: The Isolation and Characterization of Human Platelet Factor 4 Released from Thrombin-Aggregated Washed Human Platelets and its Dissociation into Subunits and the Isolation of Membrane-Bound Antiheparin Activity. Biochim. Biophy. Acta 379: 370, 1975.

26. Levine, S.P., Wohl, H. Human Platelet Factor 4: Purification and Characterization by Affinity Chromatography. J. Biol. Chem. 251: 324, 1976.

27. Hermodson, M., Schmer, G., Kurachi, K. Isolation, Crystallization, and Primary Amino Acid Sequence of Human Platelet Factor 4. J. Biol. Chem. 252: 6276, 1977.

28. Day, H.J., Stormorken, H., Holmson, H. Subcellular Localization of Platelet Factor 3 and Platelet Factor 4. Scand. J. Haematol. 10: 254, 1973.

29. Walsh, P.N., Gagnatelli, G. Platelet Antiheparin Activity: Storage Site and Release Mechanism. Blood 44: 157, 1974.

30. Broekman, M.J., Handin, R.I., Cohen, P. Distribution of Fibrinogen, and Platelet Factor 4 and XIII in Subcellular Fractions of Human Platelets. Brit. J. Haematol. 31: 51, 1975.

31. Gjesdal, K., Pepper, D.S. Half-Life of Platelet Factor 4 (PF-4) in Plasma and Platelets from Macaca mulatta. Thromb. Haemosta. 37: 73, 1977.

32. Weber, K., Osborn, M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J. Biol. Chem. 244: 4406, 1969.

33. Brewer, J.M., Pesce, A.J., Ashworth, R.B. Experimental Techniques in Biochemistry. Prentice Hall, Inc., New Jersey, pp. 328, 1974.

34. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28: 350, 1956.

35. March, S.C., Parikh, I., Cuatrecasas, P. A Simplified Method for Cyanogen Bromide Activation of Agarose for Affinity Chromatography. Anal. Biochem. 60: 149, 1974.

36. Laurell, C.B. Electroimmuno Assay. Scand. J. Clin. Lab. Invest. 29(Suppl. 124): 21, 1972.

37. Engvall, E., Perlmann, P. Enzyme-Linked Immunosorbent Assay (ELISA) Quantitative Assay of Immunoglobulin G. Immunochem. 8: 871, 1971.

38. Engvall, E., Jonsson, K., Perlmann, P. Enzyme-Linked Immunosorbent Assay. II. Quantitative Assay of Protein Antigen, Immunoglobulin G, by Means of Enzyme-Labelled Antigen and Antibody-Coated Tubes. Biochim. Biophys. Acta 251: 427, 1971.

39. Barrett, M.J., LeMat, B., Chow, S. Antibodies Coupled to the Inside of Plastic Test Tubes and Their Use in a Radioimmunoassay. Clin. Chem. 21: 1011, 1975.

40. Boenisch, T. Improved Enzymeimmunoassay for Trace Proteins. ELISA - Enzyme-Labeled Immunosorbant Assay: ASCP Educational Center Program, Sept. 7-9, 1977.

41. Niewiarowski, S. (Communication)

42. Ginsberg, M.H., Haskin, R., Sigrist, P. Purification and Properties of a Heparin Neutralizing Protein from Rabbit Platelets and its Homology with Human Platelet Factor 4. Fed. Proc. 37-6: 1444, 1978. (abst.)

43. Handin, R.I. Workshop on Platelets. Philadelphia, PA. pp. 41, 1976.

44. Nekane, P.K., Kawaoi, A. Peroxidase-Labeled Antibody a New Method of Conjugation. J. Histochem. Cytochem. 22: 1084, 1974.

45. Avrameas, S. Coupling of Enzymes to Proteins with Glutaraldehyde: Use of the Conjugates for the Detection of Antigens and Antibodies. Immunochem. 6: 43, 1969.

VITA

VITA

The author was born in Jackson, Michigan, on April 8, 1949. He attended Jackson Community College and Western Michigan University where he obtained his Bachelor of Science degree in Biology. His medical technology internship was completed in 1974 at Foote Memorial Hospital, Jackson, Michigan. The author worked two years at Mercy Hospital as a staff technologist. In January 1976, he was admitted to the graduate program in Clinical Laboratory Science at Michigan State University.

He was married on September 18, 1977 to Suzanne Catherine Fabing.