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thesis entitled

THE DEVELOPMENT OF A BIOLOGICAL PLATELET FACTOR 4

PURIFIED SUBSTRATE ASSAY

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Pathology

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Date August 15, 1978

THE DEVELOPMENT OF A BIOLOGICAL PLATELET FACTOR 4 PURIFIED SUBSTRATE ASSAY

By

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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

THE DEVELOPMENT OF A BIOLOGICAL PLATELET FACTOR 4

PURIFIED SUBSTRATE ASSAY

By

Suzanne C. Estry

Released during platelet aggregation is a heparin neutralizing protein known as platelet factor 4. The physiologic function of this protein is not completely understood, but it does exhibit anti-heparin activity *in vivo*. Measurement of this activity has been the subject of this study. With current techniques the biological activity has been difficult to quantitate because of the variability in blood cofactors necessary to assay platelet factor 4 activity. The new assay developed is a thrombin clotting time utilizing a purified substrate which helps to eliminate this variability. The purified substrate is composed of predetermined standardized amounts of fibrinogen and antithrombin III. Used with this purified substrate is a standardized concentration of thrombin. The end point of the thrombin clotting time is the thrombin catalyzed generation of fibrin from fibrinogen. The activity of thrombin is decreased by the presence of antithrombin III-heparin complex and this complex is in turn regulated by the competition of platelet factor 4 for heparin. The final thrombin clotting time is a reflection of platelet factor 4 activity only if all other factors are present in optimum controlled amounts. This new Biological Platelet Factor 4 Purified Substrate Assay takes into account this need for optimum levels of all other factors.

DEDICATION

To my husband, Doug, for his encouragement and advice and to my family for their support.

ACKNOWLEDGMENTS

I wish to express my appreciation to the following people:

Joan C. Mattson, M.D., my major professor, for all her technical guidance, and personal encouragement given to me throughout my Master's program.

Thomas G. Bell, D.V.M., Ph.D., for his technical assistance and for serving as a member on my committee.

Earl W. Campbell, Jr., M.D., for his willingness to serve as a member on my committee.

Martha T. Thomas, M.S., M.T.(ASCP), my academic advisor, for her guidance in my graduate study.

Belinda J. Oxender for her excellent typing.

To all the people who generously donated their blood to this worthy cause.

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INTRODUCTION

Platelets play an important role in hemostasis not only in the formation of a platelet plug but also in the release of certain components which aid in clot formation and eventually resolution. Platelet factor 4 is a platelet specific heparin neutralizing protein and is released when platelets aggregate. Preliminary studies by other investigators have established that plasma levels of platelet factor 4 are significantly increased in clinical situations involving *in vivo* systemic platelet aggregation and suggest that platelet factor 4 may function as a sensitive parameter to study localized thrombotic events, such as myocardial infarction.

The research project was designed to develop a biological assay for platelet factor 4. In doing this, it became obvious that an important naturally occurring inhibitor in the blood system known as antithrombin III must be taken into consideration. Antithrombin III can inhibit certain coagulation factors and uses heparin as a cofactor. Since these two proteins are closely interrelated, one cannot be evaluated without consideration of the other.

LITERATURE REVIEW

Platelet Factor 4

History

The heparin neutralizing activity of platelets was first described by Conley et al. in 1948. They observed that thrombocytopenic patients had a prolonged heparin effect as compared to those patients with normal platelet counts (1). In 1951, Van Creveld and Paulssen described a platelet factor that neutralized heparin. This heparin neutralizing activity was initially thought to be associated with platelet procoagulant activity (2, 3). It was soon shown to be distinct from platelet factor 3 by Jürgens in 1954 (4). This antiheparin activity was termed platelet factor 4 (PF4) by Deutsch et al. in 1955 (5). This first attempt at isolation and characterization of PF4 was made by Deutsch and Franke, 1957; they concluded that PF4 had characteristics of a protein (6).

Biochemistry

Research work done on PF4 during the sixties centered around perfection of techniques for isolation and characterization of the protein. Deutsch et al. in 1961, using DEAE-chromatography, increased the concentration of PF4 activity 100-fold in comparison with previous work (7). In 1964, Poplawski and Niewiarowski succeeded in isolating PF4 from pig platelets in higher concentration, but platelet factor 2 (PF2) was present as a contaminant in their end product (8). They ultimately

achieved further purification and complete separation from all other platelet factors by fractionation on DEAE-cellulose (9).

Niewiarowski et al., in 1965, demonstrated that their purified PF4 could neutralize antithrombin VI (fibrinogen breakdown products) (10). They concluded that the two activities, 1) antiheparin activity and, 2) the ability to neutralize antithrombin VI may reside in the same protein. Their purified PF4 did not influence the fibrinogen-fibrin conversion by thrombin indicating that PF2 and PF4 were not the same substance.

Farbiszewski et al. reported in 1969, that PF4 induces a para-coagulation reaction, a non-enzymatic clotting of soluble fibrin monomer complexes (11). However, this was later disproven in a study done by Käser-Glanzmann et al. in 1973 (12).

The question of where PF4 resides in the platelet remains a controversial point with no definite answer as yet. Using sucrose gradient fractions of platelet homogenates and testing each fraction for heparin neutralizing activity (HNA), Day et al. in 1973 concluded that PF4 might be localized in the platelet dense bodies (13). In 1974, Walsh and Gagnatelli assayed platelet release products for antiheparin activity to help determine how PF4 is stored and released (14). They found that the release of HNA from normal platelets by thrombin and collagen was slower than that of serotonin. Lysosomal enzymes were not released by collagen whereas under the same conditions HNA was released. They concluded that PF4 either could be stored in and released from dense granules by mechanisms different from those for other dense body constituents, or that PF4 could be stored and released from a morphologically similar but functionally distinct group of dense granules. Their evidence

seems to exclude the possibility that PF4 resides in alpha granules which contain lysosomal enzymes. Isolating PF4 from pig platelets and studying sucrose gradient fractions, Joist et al. indicated that PF4 was distributed among all of the subcellular fractions except for the dense granules (15). Two more studies in 1965, one by Broekman et al. (16) and another by DaPrada et al. (17) suggest that PF4 resides in the alpha granules. Another concept which needs experimental verification is that PF4 is attached in some way to the membrane of the platelet. This idea has been suggested by O'Brien et al. (18), Moore et al. (19), and Mui (20) based on the finding that some antiheparin activity was found in fractions containing mostly membranes and in those containing mitochondria and granules of lesser density.

Methods for isolation and purification of PF4 along with its characterization improved during the seventies. One such experiment by Barber et al., has shown that PF4 is released in the form of a high molecular weight proteoglycan-PF4 complex (21). This complex dissociates at high ionic strength into an active component (MW 29,000) and a proteoglycan carrier. Upon further purification, the carrier has been shown to consist of chondroitin 4-sulfate with a molecular weight of 59,000. The molecule contains four chondroitin 4-sulfate chains (MW 12,000) in covalent linkage to a single polypeptide. The complete complex has a molecular weight of 350,000 which suggests that four moles of PF4 are bound per mole of proteoglycan and that the carrier occurs in the form of a dimer consisting of eight moles of PF4 and two moles of proteoglycan. The isolated chondroitin 4-sulfate moieties combine with PF4 at a binding ratio of one mole of PF4 per carbohydrate chain. Heparin completely displaces PF4 from both the saturated

proteoglycan and chondroitin 4-sulfate complexes. The relative binding capacities of glycosaminoglycans for PF4 as shown by Barber et al. are: heparin [100], heparitin sulfate [75], chondroitin 4-sulfate [50], dermatan sulfate [50], and chondroitin 6-sulfate [50]. Hyaluronic acid does not combine with PF4 (21). Handin and Cohen (22) also studied the ability of the sulfated glycosaminoglycans to bind to the protein by employing the use of [^{3}H] heparin. Their results differed slightly from those of Barber et al. (21). They concluded that dermatan sulfate has a higher binding capacity than chondroitin 6-sulfate and chondroitin 6-sulfate has a greater binding capacity than chondroitin 4-sulfate (22).

Evidence from other works indicate that the active component of the complex (PF4) exists, at physiologic ionic strength and pH, as a tetramer (19) with a molecular weight of the subunit ranging from 6,000 to 11,600 (19, 22, 23). Handin and Cohen have presented evidence that it is the lysine residues on the protein that interact with the heparin (22).

The amino acid analysis of PF4 has been reported in several different publications with similar results (19, 22, 23, 24). Table 1 lists the amino acid composition given by Handin and Cohen (22).

Clinical Implications

Although PF4 has been demonstrated to neutralize heparin added to plasma *in vitro* and its level in plasma has been shown to be increased in certain pathological states, the role of this protein in normal or abnormal hemostasis is unknown. The presence of PF4 with its heparin neutralizing ability in the circulation may have potential

Table 1. Amino acid composition of heparin-neutralizing protein.

amino acid	residue/mole	amino acid	residue/mole
Alanine	5	Lysine	7
Arginine	3	Methionine	0
Aspartic acid	6	Phenylalanine	1
Cysteine	2	Proline	4
Glutamic acid	9	Serine	4
Glycine	4	Threonine	4
Histidine	2	Tryptophan	0
Isoleucine	4	Tyrosine	1
Leucine	8	Valine	3

significance in modulation of coagulation reactions, particularly the interaction of heparin with antithrombin III. In addition, the fact that PF4 can bind sulfated aminoglycans present on cell surfaces and in plasma suggests that it may also play a role in other physiologic processes.

There have been several attempts to develop assays to measure the plasma level of PF4 in order to define the role of PF4 in hemostasis and thrombosis. The different assays currently in use can be divided into two main approaches, 1) immunologic determination of PF4 protein, and 2) biological assays of heparin neutralizing capacity.

Immunologic Assays

In 1974, Gjesdal developed an electroimmunoassay based on the Laurell rocketry technique (25). One problem encountered with this procedure is that it cannot detect PF4 in platelet poor plasma without first concentrating the plasma, though serum levels can be measured.

A radial immunodiffusion method has been developed by Niewiarowski et al., 1976, using the Mancini technique (26). They tested a normal individual's platelet free plasma and platelet rich plasma which had

been treated to cause release of PF4. The results from this study showed that PF4 was a sensitive marker of platelet release *in vitro*.

Another experimental method is the radioimmunoassay (RIA) (27, 28, 29). Bolton and associates found an increased level of PF4 in patients who had received prosthetic heart valves (28). Handin et al. used the RIA to compare the levels of PF4 in normal individuals to those of patients with coronary artery disease, chest pain with no myocardial infarction, and chest pain with myocardial infarction. They found a significant increase of PF4 level in all groups of patients studied, with those of myocardial infarction being the highest, 5-fold (29).

From these results and others obtained by using the immunologic technique to measure PF4, it seems that the quantitation of such a protein might be a useful diagnostic tool. These assays, however, do not assess the biological activity of the protein molecule measured. The biological activity may be an equally important diagnostic tool for determining how PF4 functions physiologically. The currently available system for measuring biological activity of PF4 involves the addition of known quantities of heparin to plasma and the measurement of the capacity of the plasma to neutralize heparin's anticoagulant effect. The heparin effect may be quantitated by utilizing a thrombin time assay or by measuring the rate of inactivation of Factor Xa.

The assay for Factor Xa inactivation is based on the ability of residual heparin, not neutralized by PF4, to potentiate the inactivation of Factor Xa by a plasma inhibitor (30, 31). There is evidence that, antifactor Xa, is antithrombin III (32). If this is true, the method of Factor Xa inactivation adds another step to that of the

method of a thrombin clotting time. Even though the sensitivity of Factor Xa-inhibitor-heparin reaction seems to be much higher (30), than that of the thrombin-antithrombin III-heparin reaction, the end point of both assays is a clot formation which must include thrombin and fibrinogen.

Many variations to the thrombin time assays for PF4 have been developed. The sequence of events in this kind of assay is as follows: 1) heparin is added to the test system, 2) PF4 binds up (neutralizes) the heparin, 3) any residual heparin will complex with the antithrombin III, and 4) the antithrombin III-heparin complex can inactivate the thrombin. The amount of thrombin inactivation is measured by the amount of time it takes to convert fibrinogen to a fibrin clot. One of the simplest assays of this type was developed by O'Brien and associates in 1974 (33). In the test system, heparin is added to the test plasma and a thrombin clotting time is done. The results are reported in seconds and a shortening of the clotting time means a higher level of heparin neutralization. O'Brien and associates used this Heparin Thrombin Clotting Time (HTCT) to study different groups of patients. They found a shortening of HTCT in myocardial infarctions, atherosclerosis, deep vein thrombosis, and secondary diffuse intravascular coagulation (33, 34). There was a prolonged HTCT in patients with idiopathic thrombocytopenic purpura (33). Concerning the patients with myocardial infarctions, the HTCT returned to a normal level within one to three months after infarction (34).

A thrombin clotting time method developed by Harada and Zucker utilizes a series of heparin dilutions added to the test plasma (35). With each dilution of heparin, a thrombin clotting time is done. The

heparin concentration giving a clotting time of over 20 seconds is used as the end point. After this point the clotting time becomes unreliable because the PF4 capacity to bind heparin has been exceeded. The excess heparin complexed with antithrombin III completely inactivates the thrombin. The results of the assay are reported out as units of heparin neutralized.

In 1976, Dana and associates modified the assay of Harada and Zucker (35) by the addition of normal pooled plasma "substrate plasma" in order to provide a surplus of fibrinogen and antithrombin III (36). The substrate plasma and test plasma are added together along with serial dilutions of heparin. This mixture is incubated, after which thrombin is added and a clotting time determined. The results are reported in units of heparin neutralized per milliliter. Utilizing this method, Dana et al. found a decrease of heparin neutralizing activity (HNA) in patients with bone marrow depression, idiopathic thrombocytopenic purpura, and myeloproliferative disorders with thrombocytosis (36). The conditions in which an increased HNA was found included diffuse intravascular coagulation, secondary thrombocytosis, acute and chronic coronary insufficiency, and chronic coronary artery disease (36, 37).

Another technique for assaying PF4, described by Fuster et al., uses a heparin thrombin clotting time in which a ratio of clotting times of the test plasma to that of distilled water is utilized (38). The ratio is plotted on the ordinate with percent of PF4 on the abscissa. Results obtained with serum are used as 100% PF4, results obtained with platelet poor plasma from a thrombocytopenic patient (less than 5,000 mm^3 platelet count) are used as 0% PF4. Also in

this procedure the test plasma has been heated at 60 C for 10 minutes to remove the fibrinogen and antithrombin III which is not needed. The pooled normal substrate plasma utilized in this test contributes optimal amounts of antithrombin III and fibrinogen (38).

Saleem and Fretz in 1977 created another modification to the basic heparin thrombin clotting time in which they used a lyophilized commercial plasma for the substrate plasma (39). The lyophilized plasma is a normal pooled plasma utilized as a normal control in routine coagulation procedures. The authors claimed improved sensitivity and precision of the assay due to the fact the lyophilized plasma had a low sensitivity to heparin, permitting use of larger concentrations of heparin that still had reproducible clotting times.

Protamine sulfate has the ability to neutralize heparin. This substance was used by Poplawski and Niewiarowski (1965) to standardize their assay of measuring antiheparin activity (40). The method involved a thrombin clotting time in which a serial dilution of protamine sulfate is substituted for the test specimen in calculating a standard curve. The clotting time of the test specimen is compared to the curve of the protamine sulfate with results being expressed in mean equivalents of protamine sulfate standard solution. Bovine citrated platelet poor plasma is used as a substrate plasma.

In 1977, Levine et al. measured PF4 activity by the method of Poplawski and Niewiarowski (40) using hexadimethrine bromide (Polybrene ®) as a standard instead of protamine sulfate (41). The authors stated that Polybrene ® proved completely stable when diluted whereas protamine lost activity progressively after dilution and was stable for less than one hour. This assay was used to measure

PF4 activity in platelet poor plasma prepared from platelet rich plasma which had been subjected either to controlled wall impact surface injury or to shear stress force in a jet stream injection device. The results indicated that this assay was a sensitive index of mechanical platelet damage.

The assays mentioned thus far have dealt with measuring either the antiheparin activity of PF4 or its antigenic levels. Another unique assay has been developed to measure the effect of PF4 on collagenase activity (42). Collagenase degrades the protein collagen at neutral pH and physiological temperature. Regulation of collagen metabolism by collagenase is important in both normal and disease states (42). Collagenase activity can be measured by the release of ^{14}C glycine peptides from guinea pig skin collagen (43). It has been shown that PF4 inhibits human skin collagenases which suggests a possible physiological role for this protein in connective tissue metabolism (42).

Antithrombin III

History

Before antithrombin III can be discussed, a little background on antithrombins in general must be given. In 1905, Morawitz first applied the term "antithrombin" to a substance in the circulating blood which inhibited thrombin by progressive inactivation (44). In 1939, Brinkhouse and associates, also reported a substance which in conjunction with heparin prevented thrombin formation (45). Throughout the years different antithrombins have been described and at one time there were thought to be six different antithrombins.

ANTITHROMBIN I: Seegers demonstrated in 1947 that fibrin could adsorb thrombin (46).

ANTITHROMBIN II: Astrup and Darling, 1943, described a heparin cofactor with antithrombin activity (47).

ANTITHROMBIN III: Astrup and Darling, 1943, also reported an antithrombin which inhibited thrombin progressively without heparin (47).

ANTITHROMBIN IV: Seegers and associates, 1952, described a progressive antithrombin seen only in ether-treated defibrinated plasma having no other antithrombin activities (48). Others have not been able to confirm this finding.

ANTITHROMBIN V: Loeleger and Hers, 1957, talked about this antithrombin as the cause of a hemorrhagic diathesis in a patient with rheumatoid arthritis (49).

ANTITHROMBIN VI: Niewiarowski and Kowalski, 1958, characterized this as a degradation product of fibrinolysis (50).

In 1967, Abildgaard from Norway showed that antithrombin II and antithrombin III are the same protein (51). This inhibitor is now called only antithrombin III or antithrombin-heparin cofactor. The term, antithrombin II, is no longer in usage. Since antithrombin III is a physiologically more significant natural inhibitor than the other known antithrombins and since it has a progressive and irreversible action on thrombin with its interaction with heparin, it has been studied extensively.

Biochemistry

Antithrombin III (AT III) is an alpha-2-globulin with a molecular weight of approximately 64,000 (52, 53). The mode of action of AT III has been extensively studied by Rosenberg (32). He has demonstrated that AT III can inactivate not only thrombin but also other serine proteases of the coagulation-fibrinolytic system including coagulation factors XIIa, XIa, Xa, IXa, and plasmin of the fibrinolytic system. When AT III and thrombin or other serine proteases

interact, a stable complex is formed. Complex formation has been documented by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Both AT III and thrombin migrate in SDS-PAGE as single bands with molecular weights of 62,000 and 33,800, respectively. When these two proteins are incubated together these two single bands disappear and a complex with a molecular weight of 88,700 is formed.

If thrombin is treated with di-isopropyfluorophosphate which alkylphosphorylates the unique serine residue, no interaction between AT III and thrombin takes place (32). Rosenberg has shown that it is the arginine residue on the antithrombin that is involved in the interaction of serine proteases. Inhibition of the serine proteases can be accomplished with or without heparin as a cofactor. In the case of thrombin, if heparin is not present, AT III can inactivate a larger quantity of thrombin but at a much slower rate than if heparin were used as a cofactor. In the presence of heparin, inactivation of thrombin is almost instantaneous. It has been shown by Rosenberg that heparin binds to lysyl residues on the AT III. The dramatic increase in reaction rate is probably due to a heparin dependent conformational alteration of the AT III which renders the reactive arginine site more accessible to the active serine center of thrombin (32).

Clinical Implications

With the recognition that AT III complexed with heparin is responsible for neutralization of thrombin and other serine proteases, it has become evident that it is a very important feedback system in the coagulation-fibrinolytic process. A decrease in plasma AT III

levels is of major clinical importance since such a decrease is thought to promote thrombogenesis. There are two categories of decreased AT III, one of which is inherited and another of which is acquired and found in certain pathological conditions.

Inherited

Studies done on several families reporting congenital deficiencies of AT III have supported the thesis that low levels of AT III can lead to thrombosis. In 1965, Egeberg reported a comprehensive study on the coagulation system in members of a Norwegian family with a high incidence of thrombotic disease in which episodes most often occurred as deep venous thrombosis in the leg (54). Since the procoagulant factor activities were within the normal range, attention was centered around AT III. Plasma AT III activity was abnormally low in members with a history of thrombosis and in some of their children. The average level for all members was 72% of normal and 50% of normal for those with a history of thrombosis. These episodes occurred most often with trauma, surgery, pregnancy, and inflammation. It was seen in this family that AT III deficiency can manifest itself with thrombosis in children as well as adults. As the deficiency was found in both males and females and seemed to be inherited from either parent, Egeberg concluded that the inheritance pattern was one of autosomal dominance. Other family studies have reported similar data to that of the Norwegian family (55, 56).

Acquired

With the acquired states of AT III deficiency, the data is less conclusive. Not all patients exhibit the same finding with the same

disease states. There appears to be a tendency toward a decrease in AT III activity in the circulating blood in coronary-artery disease (57), hepatic disease (58), venous thrombosis (59, 60), extensive pancreatic carcinoma (61, 62) and disseminated intravascular coagulation (63). There are also reports of reduced AT III levels and an increased risk of thrombotic manifestations in women taking oral contraceptives (63, 64). However, AT III levels are not uniformly depressed in these groups of patients. The question of why AT III activity is decreased in some but not all patients having these conditions has not been resolved.

MATERIALS AND METHODS

SUBSTRATE AND TEST PLASMAS

Preparation

The substrate platelet poor plasma was prepared by collecting blood from normal subjects in plastic syringes. Blood samples were anticoagulated with a 1:10 ratio by volume of 3.8% trisodium citrate. Blood was then centrifuged at 2500 rpm for 10 minutes. The platelet poor plasma was removed with a plastic pipette and placed in plastic test tubes.

The test plasma was prepared in a similar manner. After separation, 1 ml aliquots of test plasma were heated at 60 C for 10 minutes and recentrifuged to obtain the defibrinated supernatant. If the sample was not to be assayed immediately, the plasma was aliquoted and stored at -30 C before heating at 60 C.

Anticoagulants

In tests on the prevention of platelet release, several anticoagulants were evaluated. In addition to citrate, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis (β-aminoethyly ether) N, N'-tetraacetic acid (EGTA) were studied. Both were obtained from Sigma Chemical Company, St. Louis, Missouri, and used at concentrations ranging from 0.2 mg/ml to 3 mg/ml. An anticoagulant cocktail developed by Robert I. Handin was also used which included the following:

Acid citrate dextrose (ACD) solution, NIH formula A

EDTA	1 mM
Prostaglandin E_1	1 uM
Adenosine	1 mM

One milliliter of the Handin ACD cocktail was added to 5 ml of whole blood.

Absorption of Plasma

Platelet factor 2 accelerates the conversion of fibrinogen to fibrin (66). Calcium phosphate can absorb PF2 but not PF4. In some studies calcium phosphate was added to the heat inactivated plasma at a ratio of 1 mg to 1 ml of test plasma. The mixture was heated at 37 C for 10 minutes with frequent mixing and centrifuged to obtain supernatant.

Alpha-2-macroglobulin

In tests of the effect of other antithrombins, performed only with the newly developed assay, α 2-macroglobulin was evaluated. Purified α 2-macroglobulin, obtained from the National Red Cross, Bethesda, Maryland was used at a concentration of 3 mg/ml. It was added either to the imidazole buffered saline to be tested with the purified substrate alone or added to the defibrinated test plasma.

PLATELET FACTOR 4 ASSAYS

Platelet Factor 4 Thrombin Clotting Time (PF4 TCT)

The PF4 TCT was a modification of a thrombin clotting time described by Penner (65). The thrombin used was Fibrindex from Ortho Diagnostics, Inc., Raritan, New Jersey. It was diluted in 0.1 M calcium chloride until a TCT of 8-9 seconds was achieved. Citrated saline was made by adding one part of 3.8% trisodium citrate to five

parts of 0.9% sodium chloride. Upjohn's sodium heparin (1000 units/ml) was used; 1 ml of 1000 u/ml was diluted in 100 ml of citrated saline giving a stock of 10 u/ml. This heparin stock was diluted further in citrated saline to obtain dilutions from 1 u/ml to 6 u/ml in 1 u/ml increments. The pooled substrate and test plasma were prepared as previously described.

The test procedure requires the construction of a PF4 TCT standard curve. The heparin concentrations previously prepared were diluted 1:10 in substrate plasma giving final concentrations of 0.1 u/ml to 0.6 u/ml of heparin in 0.1 u/ml increments. A TCT was done on each heparin dilution. After a reasonable curve was obtained using an individual substrate plasma, a pooled substrate plasma was obtained from 10 normal male donors to produce a final constant curve. The curve was plotted on semi-log paper with the clotting time (seconds) on the ordinate and the units of heparin concentrations on the abscissa.

In the performance of the assay of the test plasma, the substrate plasma with a final concentration of 0.3 u/ml of heparin was used. The reaction mixture consisted of 0.2 ml of the substrate plasma-heparin mixture, and 0.1 ml of test plasma. The test mixture was incubated at 37 C for 3 minutes. One-tenth milliliter of thrombin was then added and a clotting time determined.

The results were reported as units of heparin neutralized per ml by the PF4 in the test plasma. The results were calculated by first determining the heparin concentration of the test plasma. This was obtained from the standard curve utilizing the clotting time of the test plasma. The heparin concentration of the test plasma was then

subtracted from the known amount of heparin (0.3 u/ml) added to the substrate plasma.

Modification of Plasma Heparin Neutralizing Activity Assay, Dana et al. (36)

One vial of thrombin (Fibrindex) was diluted with 3 ml of veronal buffer (pH 7.35) to give a final concentration of 16.7 u/ml. Upjohn's sodium heparin (1000 u/ml) was diluted in 0.9% sodium chloride to give concentration from 0.1 u/ml up to 1.0 u/ml in increments of 0.1 u/ml.

Substrate plasma and test plasma were prepared as previously described. The assay was performed using the Fibrometer, BBL Bioquest, Cockeysville, Maryland. A standard curve using pooled substrate plasma was prepared using substrate plasma with increasing heparin concentrations. The reaction mixture consisted of 0.1 ml veronal buffer, 0.1 ml pooled substrate plasma and 0.05 ml of saline (BLK) or heparin dilution. This reaction mixture was incubated for one minute at 37 C. Fifty microliters of diluted thrombin was then added and the time required for clot formation recorded. This procedure was repreated with increasing concentrations of heparin until a clotting time of over 20 seconds was obtained. The test plasma was assayed in the exact same way except that 0.1 ml of defibrinated test plasma was used in place of the 0.1 ml veronal buffer.

The standard (substrate) and test curves were plotted on linear graph paper with the clotting times on the ordinate and the units of heparin on the abscissa. The units of heparin, as read on the standard curve at 20 seconds, were subtracted from the units of heparin as read on the test plasma curve at 20 seconds. This value was reported as units of heparin neutralized.

BIOLOGICAL PF4 PURIFIED SUBSTRATE ASSAY

The subject of this thesis is the development of a biological assay for PF4. The assay as finally developed is described here. This assay uses only purified plasma components in place of a pooled substrate plasma. First, a concentration of 12.5 u/ml of thrombin was obtained by diluting thrombin (Fibrindex) in 4 ml of imidazole buffered saline. Fibrinogen from General Diagnostics, Morris Plains, New Jersey, was reconstituted with distilled water to a concentration of 6 mg/ml. Dilution of the heparin in increments of 0.05 u/ml were prepared as described for previous assays. Antithrombin III obtained from the American National Red Cross in Bethesda, Maryland or purified by a modification of the technique of Thaler et al. (67) was used at a concentration of 1.35 mg/ml.

Assayed with this procedure were either test platelet poor plasma, prepared as previously discussed, or purified PF4 obtained from the American Red Cross, Lansing, Michigan. Purified PF4 in a concentration of 13.5 ug/ml was diluted at 1:10, 1:20, and 1:40 for use in this assay.

Both a standard curve (obtained with purified substrate components which lacked PF4) and a test curve (obtained with test plasma containing PF4) were done. For the standard (0 PF4) curve, 0.05 ml of fibrinogen, 0.05 ml of AT III, 0.1 ml imidazole buffered saline, and 0.05 ml saline (BLK) or heparin dilution were pipetted into a prewarmed fibro cup. This mixture was incubated for one minute at 37 C and the fibrometer timer started with the addition of 0.05 ml of thrombin. This procedure was repeated, using increasing heparin concentrations until the clotting time was over 45 seconds. The same

steps were followed as above for the test plasma except that 0.1 ml of defibrinated test plasma was used in place of 0.1 ml imidazole buffered saline.

In the calculation of results, all curves were brought to zero by subtracting the clotting time of the zero heparin from the clotting times of each one of the increasing heparin dilutions. This was done for both the standard (0 PF4) curve and test plasma curves. Curves were then plotted on linear graph paper with the zeroed clotting times on the ordinate and heparin concentrations on the abscissa.

Example:

u/ml Heparin	Standard Curve Clot Times	(0 PF4) zeroed	Test Plasma #1 Clot Times	Zeroed	Test Plasma #2 Clot Times	Zeroed
0	12.2 sec.	0	16.8	0	14.7	0
0.10	13.7	1.5	17.2	0.4	15.8	1.1
0.20	18.7	6.5	19.2	2.4	16.3	1.6
0.25	20.3	8.1	22.2	5.4		
0.30	34.8	22.6	23.7	6.9	18.2	3.5
0.35	49.2	37.0	27.3	10.5		
0.40	>120		34.3	17.5	19.8	5.1
0.45			38.8	22.0		
0.50			49.7	32.9	24.8	10.1
0.55			>120			
0.60					28.3	13.6
0.70					37.3	22.7

An example of the curves are shown in Figure 1.

After the curves were plotted, the results were determined by reading the units of heparin directly off the curves at 15 seconds. The units of heparin obtained from the standard (0 PF4) curve were then subtracted from the units of heparin obtained from the test plasma curve. The results were reported as units of heparin neutralized per ml.

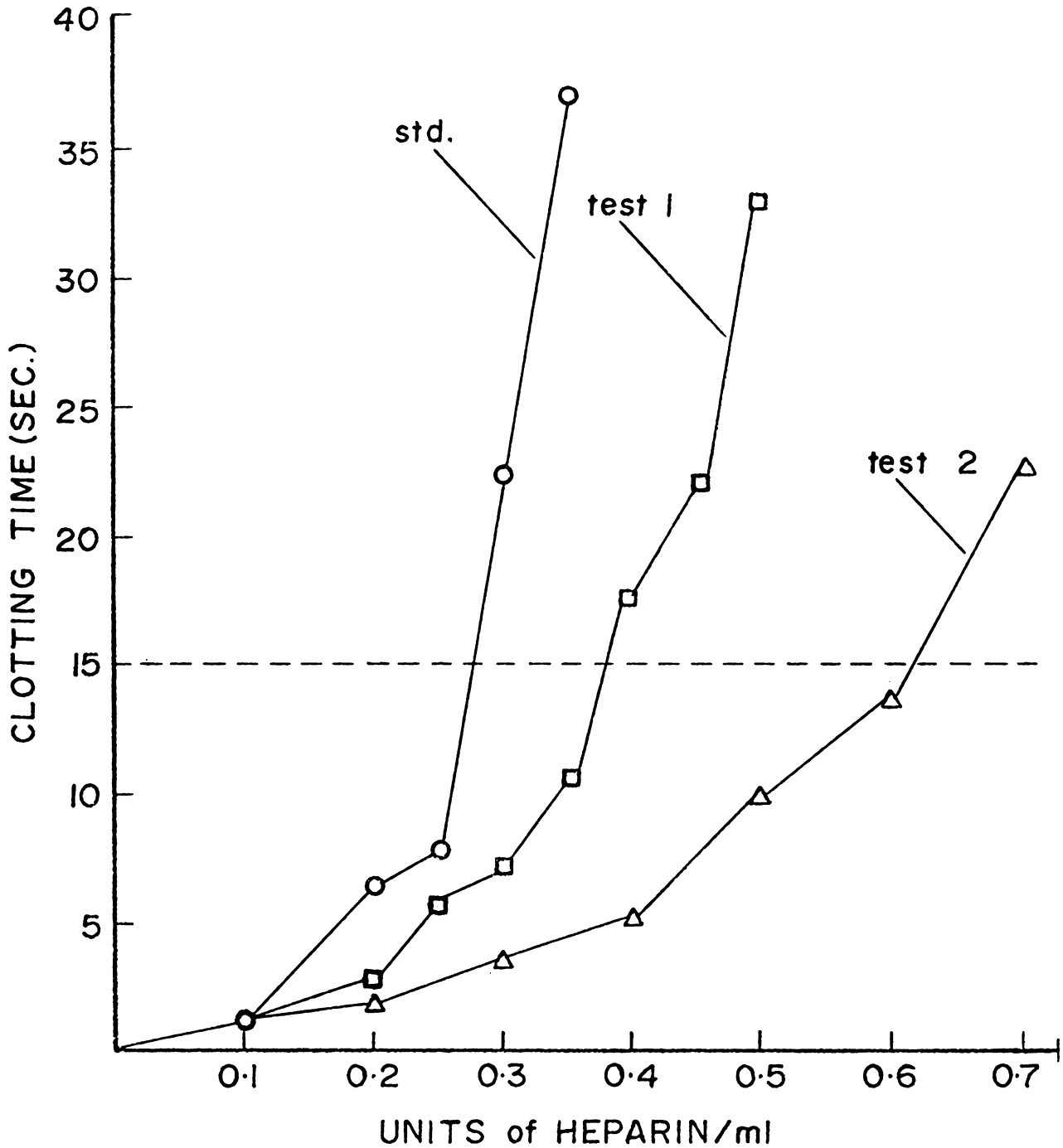

Figure 1. Curves from the Biological PF4 Purified Substrate Assay.

FIBRINOGEN CURVE

The reagents needed for construction of a fibrinogen curve are thrombin and fibrinogen. One vial of thrombin (Fibrindex was diluted with 5 ml of imidazole buffer (pH 7.2) to a concentration of 10 u/ml. The fibrinogen (Ab Kaba from Sweden) used in this study contained 1.2 grams citrate and 0.4 grams sodium chloride per gram of fibrinogen. It was reconstituted with distilled water to a concentration of 6 mg/ml and further diluted with 0.9% sodium chloride to the following concentrations: 5 mg/ml, 4 mg/ml, 3 mg/ml, 2.5 mg/ml, 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml.

A thrombin clotting time was performed using each concentration of fibrinogen on the Coagulation Profiler CP-7, Bio-Data Corporation, Willow Grove, Pennsylvania. The reaction mixture consisted of 0.1 ml of fibrinogen to which 0.1 ml of thrombin was added after 5 minutes incubation at 37 C. The time and rate of clot formation was recorded by the instrument on linear graph paper.

ASSAY FOR ANTITHROMBIN III (AT III)

The biological activity of AT III was measured by two different assays; the Thrombo-Screen AT III Kit by Pacific Hemostasis Laboratories, Inc., Los Angeles, California, and the Antithrombin III Assay described by Penner (65).

RESULTS

Effect of Anticoagulants on Thrombin Clotting Time Assays for PF4

The following results were obtained using the modified assay of Penner's thrombin clotting time. Table 2 shows that there is no difference in sensitivity between the two different anticoagulants used but there is an improvement by heating the plasma to 60 C for ten minutes. The improvement was not consistently found with every sample, however, this could be a reflection of the inherent variability of the method. In each instance serum levels of PF4 were determined to obtain a theoretical 100% value of PF4 following platelet activation.

Table 2. Plasma PF4 levels* with EDTA+ and sodium citrate as anticoagulants and heating of specimen utilizing modification of Penner's method.

Sample	EDTA	EDTA Heating	Citrate	Citrate Heated	Serum
1	0.17	0.14	0.145	0.145	0.47
2	0.16	0.155	0.175	0.15	0.50
3	0.16	0.115	0.13	0.13	0.43
4	0.09	0.05	0.105	0.095	0.41
5	0.065	0.085	0.085	0.12	0.37
6	0.195	0.115	0.195	0.15	0.45
7	0.145	0.13	0.135	0.16	0.42
8	0.10	0.75	0.14	0.105	----

*Results in units of heparin neutralized/ml.
+Concentration added, 1 mg/ml of whole blood.

Other comparisons of anticoagulants involved the use of EDTA and EGTA. There were no consistent results that indicated an improvement in measuring baseline plasma levels of heparin neutralizing activity (Table 3) by use of any one anticoagulant.

Table 3. Plasma PF4 levels* using unheated and heated plasmas with EDTA+ and EGTA+ as anticoagulants utilizing modification of Penner's method.

Sample	EDTA	EDTA Heated	EGTA	EGTA Heated
1	0.195	0.115	0.055	0
2	0.145	0.13	0.060	0
3	0.10	0.075	0.170	0.105
4	0.125	0.10	0.135	0.125

*Results in units of heparin neutralized/ml.
+Concentration added, 1 mg/ml of whole blood.

There seemed to be an inhibitory effect or a decrease in values with EGTA, so an experiment utilizing various concentrations of EGTA was done. This was to see if there was an optimum level of EGTA and to rule out the possibility that either a too high or too low concentration caused the inhibitory effect (Table 4).

The anticoagulant cocktail developed by Robert Handin was evaluated to determine if it was superior to other anticoagulants in its ability to protect platelets from activation during preparation of the sample for assaying. Blood was drawn and placed into either the Handin cocktail or standard 3.8% sodium citrate. Samples were then treated identically. The heparin neutralizing activity of the sample in the anti-release cocktail was higher than that in the standard

citrate anticoagulant. This indicated that the cocktail was not beneficial in reducing release due to handling (Table 5). Again the serum levels were determined to obtain a theoretical value for 100% PF4 following platelet activation.

Table 4. Plasma PF4 levels* with varying concentrations of EGTA utilizing modification of Penner's method.

Concentration of EGTA mg/ml	Specimen Unheated	Specimen Heated
0.2	specimen clotted	-----
0.6	0.165	0.015
1.0	0.140	0.05
2.0	0.12	0.05
3.0	0.135	0.015

*Results in units of heparin neutralized/ml.

Table 5. Comparison of Handin's anti-release cocktail with sodium citrate on plasma PF4 levels* utilizing modification of Penner's method.

Sample	Citrate Unheated	Heated	$CaHPO_4$	Cocktail Unheated	Heated	$CaHPO_4$	Serum
1	0.11	0.095	0.11	0.175	0.140	0.135	0.63
2	0.135	0.115	0.185	0.155	0.095	0.140	0.60

*Results in units of heparin neutralized/ml.

Effect of Other Plasma Constituents

Platelet factor 2 (PF2) accelerates the conversion of fibrinogen to fibrin (66). Since calcium phosphtae can absorb PF2 but not PF4, calcium phosphate was added to plasma to determine the extent of PF2 influence on the PF4 assay. Samples were treated the same except that, after heating, one aliquot was treated with calcium phsophate with the results shown in Table 6. The absorption of PF2 from the plasma did not influence the measurement of PF4 in this assay.

Table 6. Plasma PF4 levels* before and after plasma absorption with calcium phosphate+ utilizing modification of Penner's method.

Sample	Citrate	Citrate $CaHPO_4$	EDTA	EDTA $CaHPO_4$	EGTA	EGTA $CaHPO_4$
1	0.095	0.05	0.05	0.08	-----	-----
2	0.12	0.11	0.085	0.10	-----	-----
3	0.15	0.15	0.115	0.125	0	0.055
4	0.16	0.13	0.13	0.125	0	0.075
5	0.095	0.110	-----	-----	-----	-----
6	0.11	0.11	-----	-----	-----	-----
7	0.095	0.10	0.02	0.04	-----	-----
8	0.10	0.08	-----	-----	-----	-----
9	0.085	0.085	-----	-----	-----	-----
10	0.10	0.095	-----	-----	-----	-----
11	0.105	0.115	0.075	0.085	0.105	0.105
12	0.12	0.15	0.10	0.09	0.105	0.105
13	0.095	0.11	-----	-----	-----	-----
14	0.13	0.085	-----	-----	-----	-----

*Units of heparin neutralized/ml.
+Concentration added, 1 mg/ml of defibrinated plasma.

Effect of Antithrombin III in the Test Plasma on PF4 Values

Although, apparently at the threshold of reproducibility for this method, the results from measuring PF4 levels on unheated plasma versus heated plasma seemed to indicate an improved sensitivity due to the loss of antithrombin III. To further support this, an experiment was performed measuring the biological activity of antithrombin III. Plasmas were heated at 56 C to defibrinate the plasma (method procedure) and 60 C to remove antithrombin III activity. The antithrombin III values given in Table 7 show that there is a definite loss of antithrombin III activity after heating the plasma to 60 C for 10 minutes.

Table 7. The effects of incubation at 60 C on antithrombin III levels* of activity measured by method of Penner.

Sample	Heated at 56 C+ for 5 minutes	Heated at 60 C for 10 minutes
1	100	40
2	145	35
3	150	40
4	137	35
5	85	50

*Values are in percent of normal.
+To defibrinate plasma.

Since the antithrombin III biological activity dropped on the majority of samples to about one third of the original level, samples were heated for longer periods of time to determine if the loss of activity was dependent on time of incubation (Figure 2). Optimum

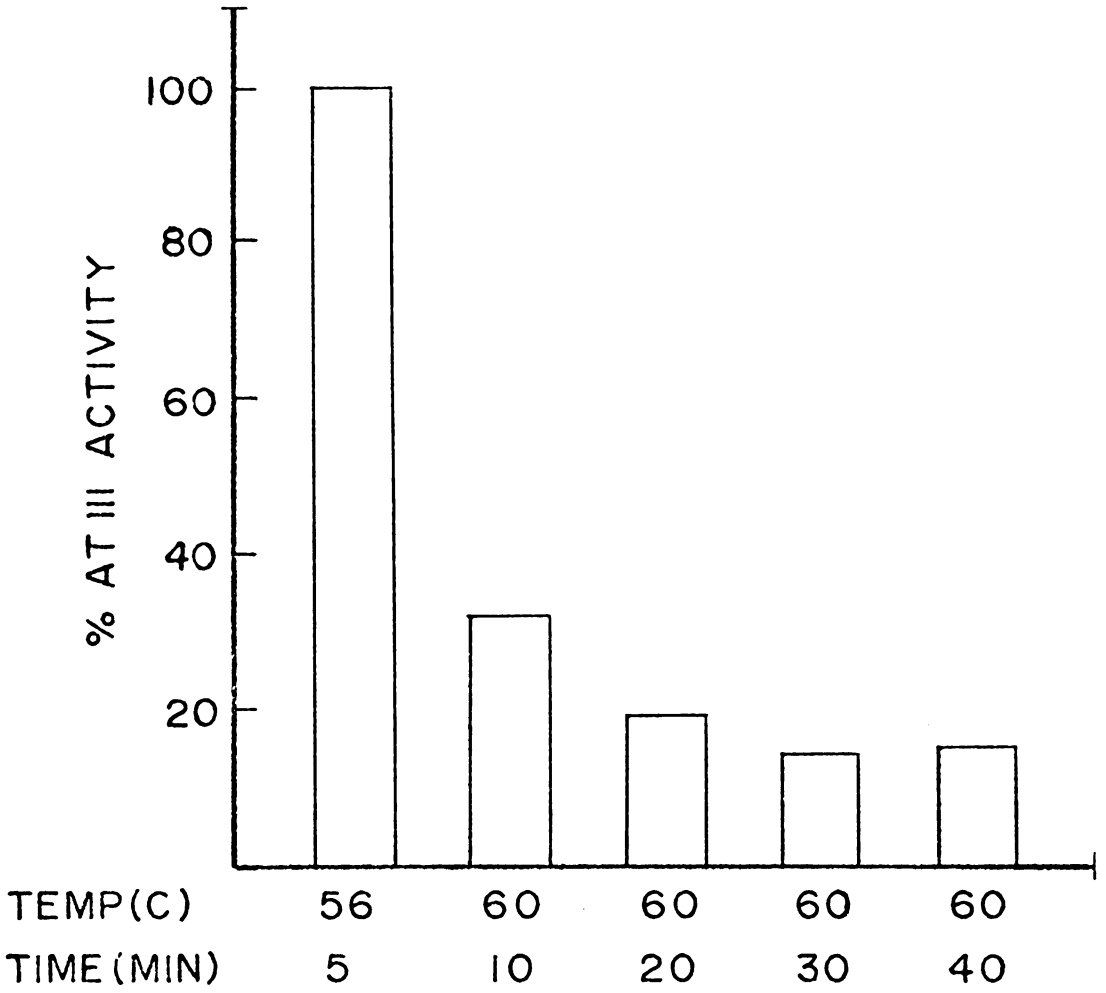

Figure 2. The effect of incubation times on the levels of antithrombin III activity measured by method of Penner.

inhibition of antithrombin III was achieved after 30 minutes. Longer incubation did not cause the antithrombin III to fall further.

In a separate experiment, plasmas were heated to a higher temperature (65 C) to determine if further reduction in antithrombin III could be achieved. Proteins denatured at 65 C making it impossible to assay the samples. Therefore, 60 C appears to be the optimum temperature for antithrombin III inactivation.

Sensitivity and Reproducibility of Available PF4 Assays

The sensitivity of the modified method of Dana et al. was tested. A group of ten normal subjects were assayed with the results given in Table 8.

Table 8. Plasma PF4 levels* from ten normal subjects utilizing Dana et al. method.

Range	0.095 to 0.520
Mean	0.287
S.D.	0.129

*Units of heparin neutralized/ml.

The same variation in values found with normal controls were also noted in the substrate plasma curves (Table 9).

Table 9. Units of heparin neutralized per milliliter in the substrate plasma curves obtained by method of Dana et al.

Number of Curves	13
Range	0.16 to 0.46
Mean	0.335
S.D.	0.093

To further test the reproducibility and variability of the modified Dana et al. assay, three normal subjects were used. Three venipunctures (15 minutes apart) were done on each individual with each sample being number coded. After all nine samples were drawn, they were treated identically and tested for plasma heparin neutralizing activity. The results given in Table 10 show that there is a great deal of variation from sample to sample from the same individual.

Table 10. Plasma PF4 levels* on three samples from a normal subject utilizing Dana et al. method.

	Sample		
Subject	1	2	3
1	0.55	0.45	0.39
2	0.39	0.48	0.40
3	0.47	0.43	0.51

*Units of heparin neutralized/ml.

The Development of a Controlled Purified Substrate

In order to develop a more sensitive and reproducible biological assay, it was felt that a carefully controlled substrate was

essential. It was decided to utilize purified plasma components which provide optimum amounts of the needed plasma reactants. In the development of the purified substrate, the optimum concentration of the components (fibrinogen and antithrombin III) had to be determined. Initially, a fibrinogen curve was constructed, using the Bio-Data Coagulation Profiler CP-7. This instrument not only gave results of the thrombin clotting time in seconds (Figure 3) but also provided the maximum rate of velocity at which the fibrinogen was polymerizing (Figure 4). From the clotting times it seemed that a concentration of at least 3 mg/ml was necessary for the assay. However, utilizing the maximum velocity of the fibrinogen curve, the highest concentration (6 mg/ml) tested appeared best.

The antithrombin III obtained from the National Red Cross had a protein concentration of 13.5 mg/ml and an activity of 43 u/ml. In order to find the best concentration for the assay, the antithrombin III was tested at various dilutions. From the initial results (Tables 11 and 12) it appeared that the amounts of thrombin and antithrombin III had to be adjusted to find the optimum amounts for this system. Adjustment of the antithrombin III (Table 12) gave only a small range of measurable clotting times before total thrombin inactivation resulted in no clot formation.

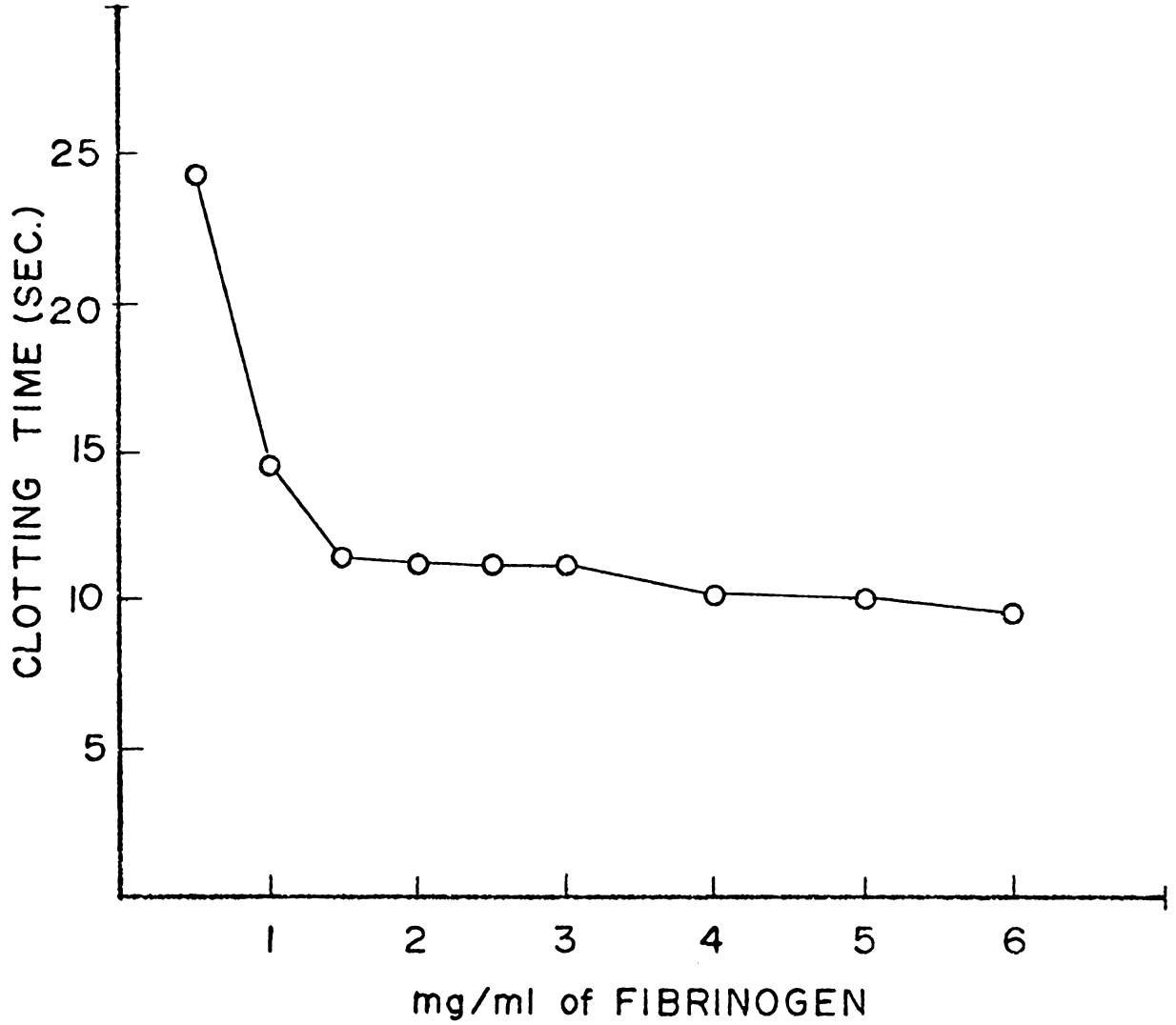

Figure 3. Effects of increasing concentration of fibrinogen on the thrombin clotting time.

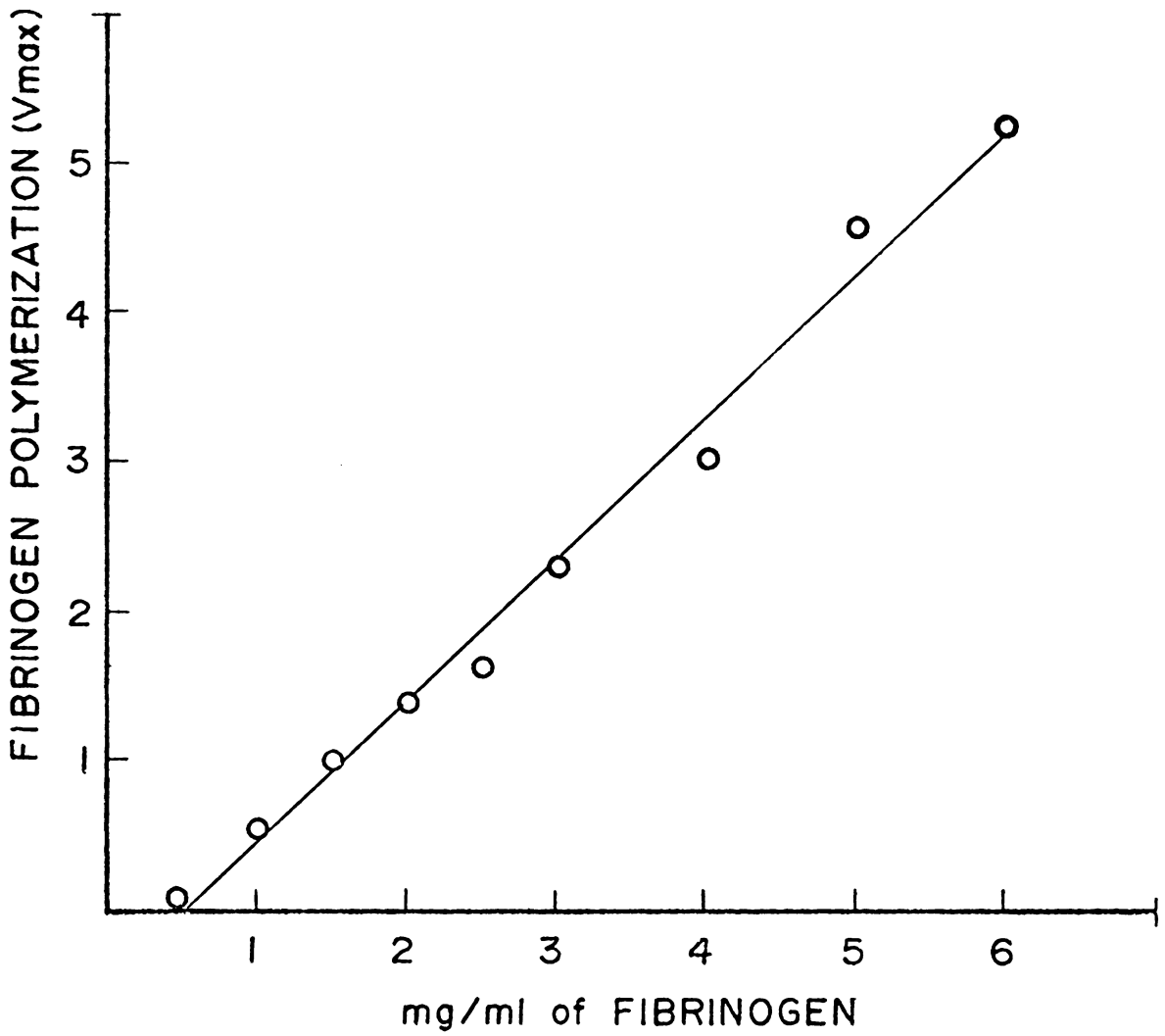

Figure 4. Effects of increasing concentration of fibrinogen on the maximum velocity of fibrin formation.

Table 11. Effect of antithrombin III concentration on clotting times (seconds) utilizing the purified substrate assay.+

Fibrinogen mg/ml	AT III* undiluted	1:2	1:4	1:8	1:10
3	no clot	no clot	no clot	14.2	14.3
6	no clot	10	---	---	---

*13.7 mg/ml protein concentration.
+8 u/ml thrombin concentration.

Table 12. Effect of thrombin concentration (8 u/ml) with various dilutions of heparin on clotting times (seconds) utilizing the purified substrate assay.

Antithrombin III diluted 1:10 (1.35 mg/ml)

Fibrinogen mg/ml	u/ml of heparin			
	0	0.1	0.2	0.3
3	14.1	no clot	---	---
6	9.1	10.9	11.3	no clot

Antithrombin III diluted 1:20 (0.685 mg/ml)

Fibrinogen mg/ml	u/ml of heparin						
	0	0.1	0.15	0.17	0.19	0.20	0.25
6	10.0	9.6	11.4	12.9	12.1	18.1	no clot

Because it would be desirable to have a greater range of heparin dilutions with measurable thrombin clotting times, the thrombin concentration was altered (Table 13).

Table 13. Effect of increased thrombin concentration (12.5 u/ml) with various dilutions of heparin on clotting times (seconds) utilizing the purified substrate assay.

Antithrombin III diluted 1:10 (1.35 mg/ml)									
Fibrinogen mg/ml	u/ml of heparin								
	0	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.50
6	11.2	12.2	14.5	16.0	19.5	23.2	33.7	40.8	no clot

Biological PF4 Purified Substrate Assay

Based on the results of experiments described thus far, an improved assay has been developed which utilizes a purified substrate with 6 mg/ml of fibrinogen, 1.35 mg/ml of antithrombin III and 12.5 u/ml of thrombin. The following results are from this newly developed assay. In this assay, heparin clotting times are performed on the purified substrate alone with these results forming the baseline for the test. The results for the purified substrate (0 PF4) are given in Table 14. This set of results includes the use of two different preparations of antithrombin III, one from the National Red Cross, Bethesda, Maryland and another prepared at the American Red Cross, Lansing, Michigan. There is very little variation in the results.

Table 14. Units of heparin neutralized per milliliter by purified substrate alone.

Number of runs	12
Range	0.25 to 0.325
Mean	0.293
S.D.	0.021

A group of 10 normal subjects was assayed with this method (Table 15). The results indicate that the assay is very sensitive for there is a small range of the results.

Table 15. Plasma PF4 levels* from ten normal subjects utilizing the Bio PF4 purified substrate assay.

Range	0.035 to 0.17
Mean	0.105
S.D.	0.045

*Units of heparin neutralized/ml.

Four of these normal subjects were assayed on different occasions, to test the daily variance in an individual and to determine the reproducibility of the assay (Table 16).

Table 16. Repeated plasma PF4 levels* in normal subjects obtained on different days.

Subject	Sample 1	Sample 2	Sample 3
1	0.065+	0.045+	0.115
2	0.17	0.16	0.14
3	0.14	0.12	-----
4	0.09	0.105	-----

*Units of heparin neutralized/ml.
+Specimen was lipemic.

To further test the reproducibility of this procedure, three samples drawn a few minutes apart from a normal subject were number

coded and tested by the new Bio PF4 assay and the modified Dana et al. method. All of the blood samples were handled in exactly the same manner. After platelet poor plasma was obtained from centrifugation, platelet counts were done and the plasma aliquoted and frozen to be assayed by both methods at a later time. Platelet counts on the plasmas were 7,000/mm^3 or less. The results are given in Table 17.

Table 17. Comparison of plasma PF4 levels* of three samples from a normal subject using the Dana et al. method and the Bio PF4 purified substrate assay.

	Bio PF4 purified substrate assay			Dana et al. method		
	Sample			Sample		
Subject	1	2	3	1	2	3
1	0.13	0.14	0.14	0.17	0.225	0.25
2	0.09	0.09	0.105	0.125	0.23	0.195
3	0.10	0.10	0.12	0.135	0.33	0.25

*Units of heparin neutralized/ml.

Also tested with the new assay was purified PF4 with a protein concentration of 13.5 ug/ml. Platelet factor 4 is not soluble in low salt concentrations, so the purified protein had to be assayed in higher salt solutions. Unfortunately, high salt concentrations interfere with the test system so dilutions had to be used which maintained solubility but decreased the higher salt content. The results of testing a 1:10, 1:20, and 1:40 dilution of the purified PF4 are shown in Figure 5.

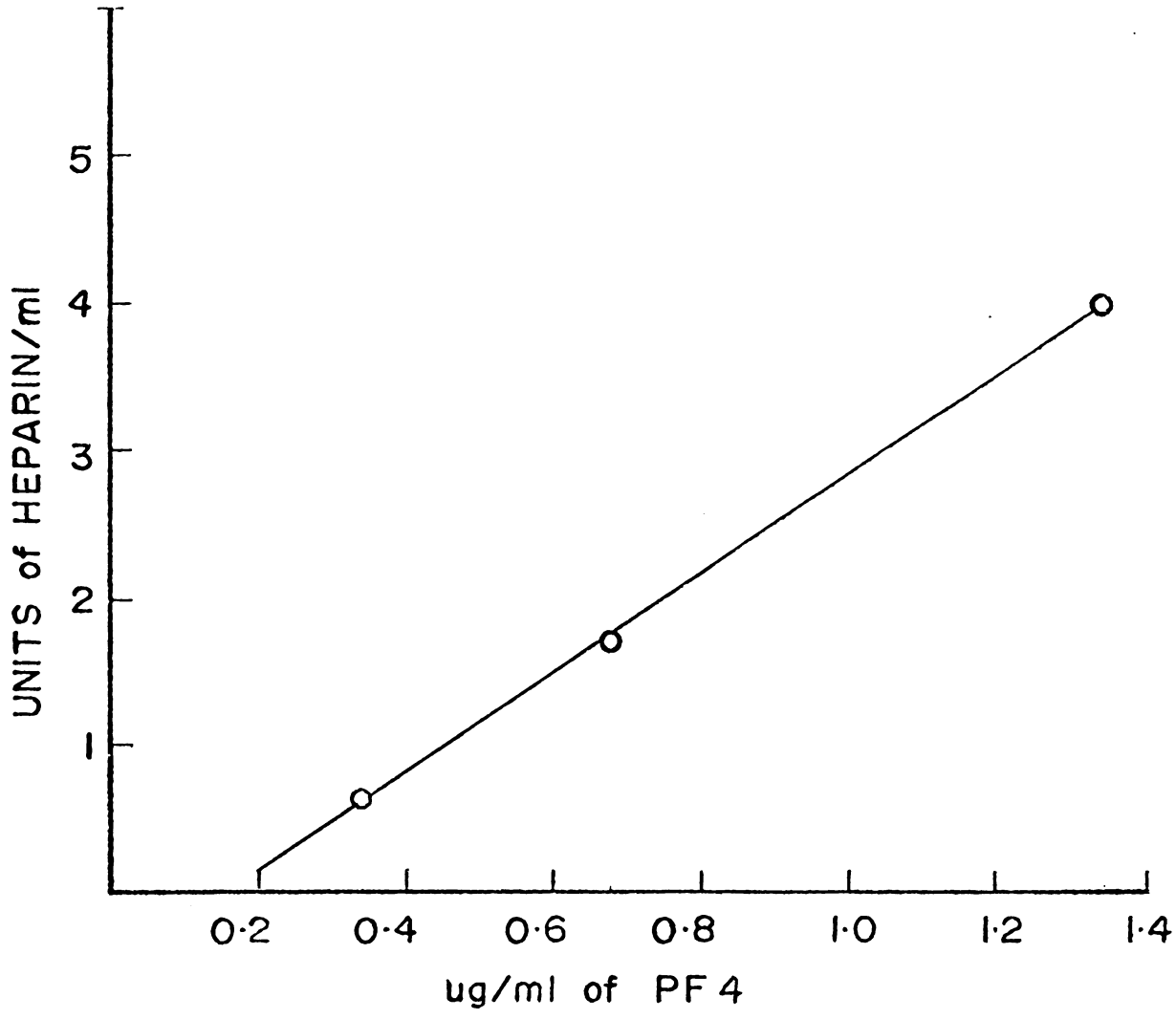

Figure 5. Units of heparin neutralized by serial dilutions of purified PF4.

From the graph (Figure 5), it is possible to interpolate results from the new assay expressed in units of heparin neutralized per milliliter into micrograms per milliliter of PF4 present in plasma. The results from Table 15 have been converted to micrograms per milliliter of PF4 and shown in Table 18.

Table 18. Units of heparin neutralized per milliliter expressed in micrograms per milliliter of PF4.

Normal Subjects	u/ml of heparin neutralized	ug/ml of PF4
Lower range	0.035	0.155
Upper range	0.17	0.195
Mean	0.105	0.175

Effects of Other Plasma Constituents

Even though antithrombin III is considered the main plasma inhibitor of thrombin, there are other antithrombins present in the plasma. In order to determine if plasma levels of α 2-macroglobulin would interfere with the new assay, it was added to the test system. The units of heparin neutralized per ml by the purified substrate alone was 0.33 u/ml, with 3 mg/ml of α 2-macroglobulin added to the purified substrate was 0.34 u/ml. This indicates no interference with α 2-macroglobulin with the use of the purified substrate.

Another experiment included the addition of α 2-macroglobulin to the test plasma before assaying. The results are as follows: 1) test plasma with no α 2-macroglobulin neutralized 0.045 u/ml of heparin, and 2) test plasma with 3 mg/ml of α 2-macroglobulin added neutralized 0.05 u/ml of heparin. Again, these results indicate no interference

with the new assay. The amount of α 2-macroglobulin added to the test plasma represents an elevated plasma level of α 2-macroglobulin.

DISCUSSION

The main problem inherent in all of the available assays for PF4 which use a pooled normal substrate plasma is the uncontrolled reaction of the plasma components. This reaction includes not only the blood coagulation factors but also the inhibitors of the coagulation system. When assaying for the heparin neutralizing activity of PF4, there can be interactions within the test system which can lead to unreliable results. The reactions involved in the measurement of PF4 heparin neutralizing activity are summarized as follows: 1) PF4 and the added heparin form a complex, 2) any residual heparin forms a complex with antithrombin III which causes rapid inactivation of thrombin, and 3) the remaining unneutralized thrombin will cause the conversion of fibrinogen to fibrin which is the end point. The important components (antithrombin III and fibrinogen) of the pooled substrate plasma, such as used in the Dana et al. procedure (36), would be in competition with the antithrombin III and fibrinogen in the test plasma for participation in the reactions of the test system. Even though, in the procedure of Fuster et al. (38), the test plasma is heated to remove the antithrombin III and fibrinogen, it can only be assumed that the pooled substrate plasma has optimum levels of antithrombin III and fibrinogen. In the procedure of Saleem et al. (39) utilizing a lyophilized commercial plasma as a substrate, there is still an assumption that there is an optimum level of antithrombin III and fibrinogen. In the newly developed assay utilizing the purified substrate, the

concentration of fibrinogen and antithrombin III have been tested to ensure optimum levels. Based on the results of these experiments, it is felt that the proper concentration of substrate constituents have been achieved for the reaction taking place.

Another consideration in the use of the pooled plasma for a substrate is the presence of other potential antithrombins and coagulation factors. It has been shown by Rosenberg (32) that antithrombin III can inactivate other serine proteases besides thrombin. So in the case of the pooled plasma, interactions between antithrombin III and other coagulation factors could cause interference in the enzymatic reaction of the test system. In the Dana et al. method, heparin neutralizing activity of the control group was reported to be quite low (0.1 ± 0.1 u/ml of heparin neutralized) (36). However, it was also reported that all test plasmas were screened for any unrecognized antithrombin activity; if any were found the samples were discarded and not tested. Heating the test plasma to remove antithrombin III and optimizing the antithrombin III concentration in the purified substrate assay, make it unnecessary to take into consideration variations in the level of antithrombin III in the test plasma. The reaction in the purified substrate assay is more defined than in any of the previously available biological systems. Unexpectedly higher PF4 levels might be present in substrate plasmas. This possibility is also avoided by the use of a controlled substrate which contains only antithrombin III and fibrinogen.

The results of testing the new assay indicate enhanced sensitivity and an improved reproducibility over existing methods. By assaying the plasma of a normal subject drawn at three different

intervals using both the Dana et al. method and the new method, it was possible to show that there is greater variability with the Dana et al. method than with the new assay. Even though the heparin neutralizing activity of PF4 can be detected by other biological procedures, it is clear that the use of plasma as a substrate introduces variables that contribute to a loss of sensitivity.

Statistical analysis of the data obtained from assaying test samples with the new assay and the Dana et al. method (Table 17) is used to compare the two methods. Two parameters used in analyzing the data are: 1) the student t test which compares the accuracy, and 2) the F test which compares the precision of the two methods. The results from the t test ($p < 0.005$) show a significant difference between the two assays, and indicate that the Dana et al. method is not as accurate. Having establishing that there is a significant difference between the methods and that the accuracy of the new assay is greater, a comparison of the precision of the two procedures was made using the F test. Results from the F test show again a significant difference at the 95% confidence level between the precision of the two methods. The values found for these two tests are: $df = 8$, $t = 4.727$, and $F = 10.24$.

Another indication of the improved precision of the new assay is the ability of this assay to use smaller increments of heparin. A difference of 0.05 u/ml of heparin neutralized can be detected with the use of the purified substrate. In the Dana et al. method even a change of 0.1 u/ml of heparin will sometimes not be reflected by a change in the clotting time. Small changes in heparin concentration, indicating the binding of PF4 to heparin, can be detected in the new assay.

Expression of results in all of the biological methods used is based on the amount of neutralized heparin added to the test system. Assaying purified PF4 with the controlled substrate introduces another possible way of expressing plasma PF4 activity. The results of the normal subjects expressed in units of heparin neutralized per milliliter (Table 15) have been converted to micrograms of PF4 by use of the curve obtained from assaying purified PF4 (Figure 5). These values are given in Table 18 with the mean of the normal subjects having a level of 175 ng/ml of PF4. Levine and Krentz, using a RIA method for assaying PF4, reported various levels depending on the speed of centrifugation of the specimen and kind of anticoagulant used. The range of values they reported, utilizing a citrate anticoagulant and centrifugation at 2400 g, was 48-136 ng/ml of PF4 (27). They also reported that the higher the speed of centrifugation, the lower the levels of PF4. Comparison of 175 ng/ml (new assay with specimen centrifuged at 625 g and citrate anticoagulant) to that of 88 ng/ml (mean for the RIA method with specimen centrifuged at 2400 g and citrate anticoagulant) suggests that the new biological assay might be as sensitive as the RIA methods. Several investigators (27, 28, 29) have measured the levels of PF4 in platelet poor plasma utilizing the RIA method. They used anti-release reagents in acid citrate dextrose as the anticoagulant. Values of PF4 in these cases ranged from 5 to 16 ng/ml depending on the speed on centrifugation of the specimen.

The experiment in which α 2-macroglobulin was added to the assay system to evaluate its antithrombin activity showed no inhibition. It has been reported that α 2-macroglobulin contributes significantly

to the inactivation of thrombin. However, the reaction time for this inhibition is much longer than with antithrombin III and α 2-macroglobulin does not use heparin as a cofactor (51). Since the assay utilizes short incubation times, any interference from α 2-macroglobulin should be avoided. Data supports this as no changes in PF4 values, expressed as heparin neutralization, could be detected when exogenous α 2-macroglobulin was added to the system.

There seems to be no improvement in the detection of PF4 with different attempts to reduce platelet release due to specimen handling. Even though the assay used to evaluate the different anticoagulants (citrate, EDTA, EGTA, Handin's cocktail) did not have a great sensitivity, no significant differences were found and 3.8% trisodium citrate was used throughout.

The absorption of the test plasma with calcium phosphate to remove PF2 did not cause a change in the heparin neutralizing activity of the test sample. Even though the role of PF2 is the acceleration of the conversion of fibrinogen to fibrin, the experiment suggests no interference by PF2 in the test system.

CONCLUSION

The development of the Biological Platelet Factor 4 Purified Substrate Assay has proven to be a sensitive and reproducible assay for measurement of PF4's heparin neutralizing activity. The precision of the assay has been optimized to the extent that results on individual test subjects show superior reproducibility with the controlled substrate than with any biological assay in general use. Even though the physiological function of PF4 is not completely understood, research indicates that it may be involved in coronary artery disease, thromboembolic disorders and in pathological states which exhibit thrombocytopenia and thrombocytosis. Utilization of the newly developed assay may help to elucidate the role of PF4 in these disease states.

APPENDIX

APPENDIX A

Preparation of General Reagents

0.9% Sodium Chloride

Dissolve 0.9 grams of sodium chloride in 100 ml of distilled water.

3.8% Trisodium Citrate

Dissolve 3.8 grams of trisodium citrate in 100 ml of distilled water.

0.1 M Calcium Chloride

Dissolve 14.7 grams of calcium chloride in 1000 ml of distilled water.

Acid Citrate Dextrose (ACD), NIH Formula A

22.0 grams of trisodium citrate,
8.0 grams of citric acid,
24.5 grams of dextrose, are dissolved in distilled water and diluted to 1 liter.

Veronal Buffer

11.75 grams of sodium diethyl barbital,
14.67 grams of sodium chloride, are dissolved in 1570 ml of distilled water. Mix, then add 430 ml 0.1 N HCl and pH to 7.35.

Imidazole Buffer (pH 7.2-7.4)

Dissolve 1.72 grams of imidazole in 90 ml of 0.1 N HCl and then dilute to 100 ml with distilled water.

Imidazole Buffered Saline

A 30% solution is prepared by combining 30 ml of imidazole buffer (pH 7.2-7.4) and 70 ml of 0.9% sodium chloride.

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LITERATURE CITED

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