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STUDIES OF HUMAN FACTOR VIII/von WILLEBRAND FACTOR

by

Mark N. Bobrow

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1982

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 2, 1982
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Chairman of Examining Committee

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Abstract

STUDIES OF HUMAN FACTOR VIII/von WILLEBRAND FACTOR

by

Mark N. Bobrow

Adviser: Professor Aaron Lukton

A method was developed to produce a murine antibody in ascitic fluid to human factor VIII/von Willebrand factor. The murine antiserum was comparable to a commercial rabbit antiserum when used to quantitate factor VIII related antigen by immunoelectrophoresis and enzyme immunoassay.

The structure of factor VIII/von Willebrand factor was examined by circular dichroism spectroscopy. F VIII/vWF was found to have approximately 50% unordered or random coil structure and less than 15% α helical structure. It appears to be a structurally stable protein since changes in its CD spectrum were minimal after heating, and in the pH range 5-9. F VIII/vWF most likely contains many disulfide bonds which are responsible for its stability. This is evident in the change in its CD spectrum on the addition of dithiothreitol.

The interaction between F VIII/vWF and heparin was investigated. F VIII/vWF bound to heparin at physiological pH and ionic strength. By increasing the ionic strength, F VIII/vWF eluted retaining all of its biological activities. From the pH dependence, and the effect of calcium and citrate on the interaction, it appears that it occurs by two independent mechanisms. One is through a positively charged residue on F VIII/vWF and the other involves intrinsic calcium.

The replacement of calcium by terbium was undetectable by fluorescence.

To my parents.

Acknowledgement

I cannot, nor will I, even attempt to list all the people to whom I owe thanks for the help given me over the past several years. I will, however, list the people who were instrumental to the progress of this thesis on a daily basis.

Professor Aaron Lukton, my thesis advisor and friend, rescued me from the jaws of mediocrity. Harry Rubin first interested me in biochemical research and has guided me since. My wife, Cindy, managed to bear with me and give me all the support I needed. And, finally, I never would have finished without Bill (William C.) Knoop's tireless help.

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Abbreviations

CD	circular dichroism
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGTA	ethyleneglycol-*bis*(β-aminoethyl ether) N,N'-tetraacetic acid
IBS	imidazole buffered saline
NPP	p-nitro phenyl phosphate

I. INTRODUCTION

A. The History of Blood Coagulation

The earliest theory of blood coagulation, the classical theory, was derived from the work of Hammarsten (1), Schmidt (2), and Morawitz (3). This theory postulated the existence of four clotting factors: thromboplastin derived from damaged tissue, prothrombin and fibrinogen from plasma, and calcium. Following tissue damage, thromboplastin is released and reacts with prothrombin in the presence of calcium to form thrombin, which then reacts with fibrinogen to form fibrin (Fig. 1). There was no clear evidence whether or not any of these factors were enzymes until Bettleheim and Baily (4) and Lorand (5) showed that thrombin splits fibrinogen into major fragments which polymerize and minor fragments which remain in solution. Sherry and Troll (6), using synthetic substrates, showed that thrombin is an esterase.

In 1935, the one-stage prothrombin time test was introduced by Quick (7). This test was based on the four factor theory. If thromboplastin, fibrinogen, and calcium were present, then the clotting time of the system would reflect the concentration of prothrombin. The first indication of an additional clotting factor came from Owren (8) in 1947. Investigating a patient with a prolonged one stage prothrombin time, Owren found that this could be corrected by the addition of normal plasma from which all the prothrombin had been removed by adsorption with aluminum hydroxide. Owren concluded that normal plasma contains a factor which was lacking in this patient and was necessary for the interaction between prothrombin and thromboplastin. He called it factor V, being the fifth clotting factor. In 1950, Owren (9) renamed it proaccelerin on the basis of its function.

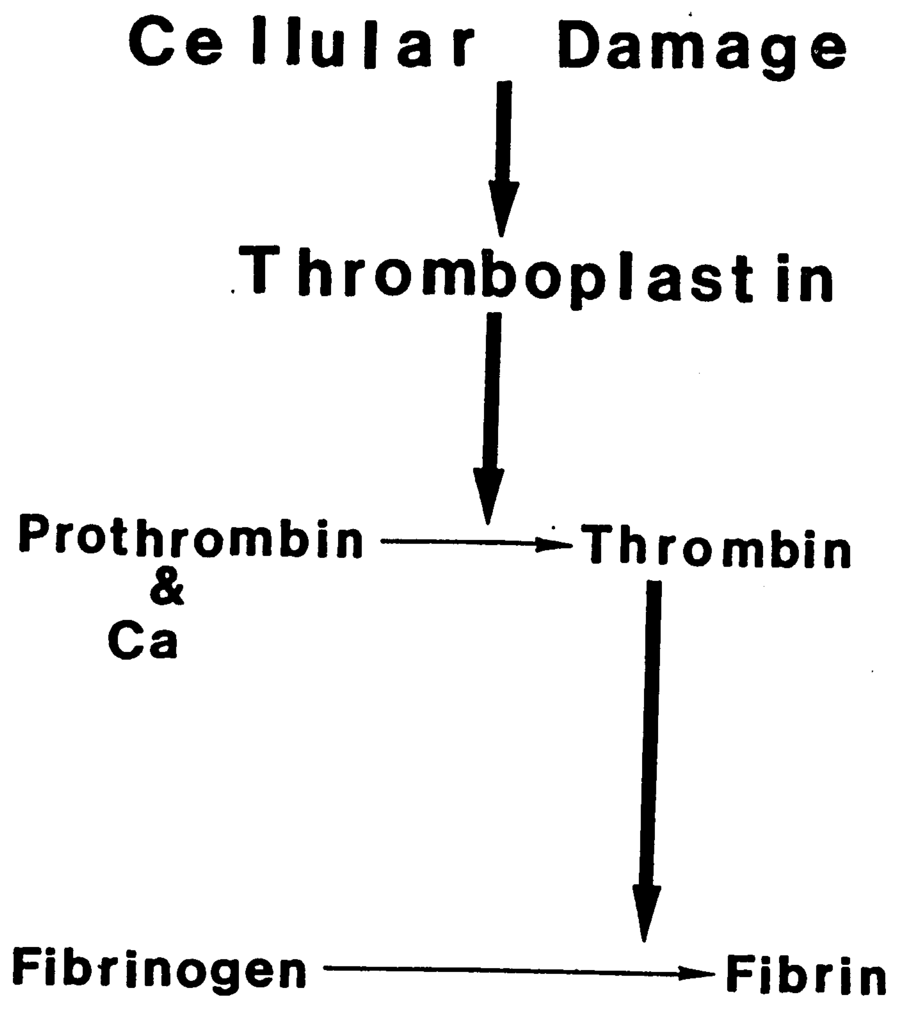


Figure 1 : The Classical Theory of Blood Coagulation.

In 1942, Witts and Hobson (10) proposed to use Russell's viper venom and phospholipid in lieu of brain extract in the one-stage prothrombin time test. Russell's viper venom exhibited a powerful coagulant activity with added phospholipid on recalcified plasma. This worked fine on normal plasma, yielding results which were in agreement with those of the brain extract. However, in patients receiving dicoumarin drugs, the venom test tended to give shorter clotting times. Since the dosage of dicoumarin administered depended upon the results of the test, this resulted in higher doses of dicoumarin being given.

In 1948, Owen and Bollman (11) found that normal serum accelerated the thrombin generation of plasma from patients being treated with dicoumarin. Since normal serum contains little or no prothrombin or factor V, this suggested that the serum contained another factor which was deficient in the patient's plasma. In 1949, de Vries (12) showed that normal serum shortened the prothrombin time of plasma which had been adsorbed with barium sulfate. On the basis of these studies, Owen postulated the existence of an additional factor which he named proconvertin. This resembled prothrombin in that it was stable, adsorbed by inorganic gels, and its in-vivo levels were reduced by dicoumarin drugs. However, unlike prothrombin, it was present in serum. Owen believed that, during clotting, proconvertin was activated by thromboplastin and accelerin to form convertin which reacted with prothrombin. In 1951, Koller (13) performed experiments on a substance which he called factor VII (the designation factor VI was given to a hypothetical derivative of factor V). This factor VII turned out to be identical to Owen's proconvertin. Factors V and VII had to be included in the classical theory. This was done by showing that they accelerated

the conversion of prothrombin to thrombin.

The major inadequacy of this clotting scheme was the failure to explain the clotting defect in hemophilia. It was observed that the coagulation time of hemophilic bood was grossly prolonged as measured in glass tubes, while the one-stage prothrombin time was normal. At first, it was thought that hemophilic platelets failed to release a thromboplastic component on contact with a foreign surface (14), but further studies showed that hemophilic platelets functioned normally in normal blood. It was also shown that the clotting defect in hemophilic blood could be corrected by adding normal platelets; however, the correction was not done by the platelets, but by the small amount of normal plasma which contaminated them (15). Pohle and Taylor (16) in 1937 showed that a concentrated globulin material from normal plasma reduced the clotting time of hemophilic blood in vitro and when administered in-vivo. Minot and Taylor (17) in 1947 found that the active factor was present in Cohn fraction I and III2 of normal plasma, but absent in these fractions of hemophilic plasma. Thus, it was concluded that normal plasma contains a factor which is lacking in hemophilic plasma which did not correspond to any factors in the classical theory (factors I-VII). This plasma factor, which was called antihemophilic globulin (AHG), could not be placed into the existing classical theory since it seemed to play no part in the thromboplastin-prothrombin reaction or subsequent reactions. The function of AHG was postulated to occur prior to the appearance of thromboplastin (18,19). This idea received some support when the thrombin generation test was used in 1953 (20). This test showed that there is about a three-minute delay from the time that normal blood comes in contact with glass till

there is a generation of thrombin. When brain thromboplastin was added, the delay time was shortened but the slope of the thrombin generation was the same. This indicated that a few minutes after glass contact, a thromboplastin activity appeared in the blood which is similar to that of tissue thromboplastin. When the thrombin generation test was applied to hemophilic blood, a greatly prolonged delay phase was observed. This long delay was shortened by adding AHG, and the degree of shortening was proportional to the concentration of AHG in the final mixture.

In 1953, Biggs and Douglas (21) developed the thromboplastin generation test. This test used plasma which was adsorbed to remove prothombin and factor VII, serum which was prothrombin free but had factor VII, normal platelets, and calcium. By using either the adsorbed plasma, the serum, or the platelet component from the patient and completing the system with the remaining components from normal blood, a clotting defect in one component could be detected. The majority of hemophilic patients tested with the thromboplastin generation test gave results indicating a fault in the plasma component. This indicated a deficiency in AHG, since a factor V deficiency could be ruled out because of a normal one-stage prothrombin time test. However, in a series of patients, the serum component was found to be abnormal (22). At first, this suggested that there was a deficiency in factor VII, but finding a normal one-stage prothrombin time test refuted this idea. It was believed that another serum factor existed which was required for intrinsic thromboplastin generation, but not for prothombin generation; and some patients suffering from hemophilia lacked not AHG, but this new serum factor. This new factor was called Christmas factor

after the patient who's investigation led to the discovery. Similar observations were made by Aggeler (23) in the United States. His group called the factor "plasma thromboplastin antecedent" (PTC).

In 1955, Biggs (24) developed the thromboplastin generation test into a quantitative assay for AHG activity. This allowed a quantitative study of the AHG levels in hemophiliacs and a correlation between the AHG levels and susceptibility to bleeding. Results indicated that patients with less than 1% of the normal level of AHG incurred deep tissue hemorrhages, intractable bleeding after minor injuries, and there was joint involvement. In patients with 1-5% AHG activity, there was still serious bleeding after minor injuries, but joint involvement was less severe. When a level of 40-50% was reached, there was no evidence of abnormal bleeding (25). Until this time, the best treatment for hemophilic bleeding was massive blood transfusions. Four pints of fresh blood administered to a hemophiliac adult in 8-10 hours caused about a 10% rise in the AHG level. A liter of plasma, which could be given in 45 minutes, raised the AHG level by 15-20%. To maintain these levels, two doses had to be administered daily. This method could never provide high enough AHG levels to allow major surgery, but was used for dental extractions and to control joint or tissue bleeding (26).

Attempts were then made to produce an AHG concentrate. Since an enormous amount of human blood would be needed, the use of animal blood was considered. Ox blood could be obtained in large quantities which contained from 5-8 times the AHG content of human blood. An ox plasma concentrate, of which one gram had an activity equivalent to 5-8 liters of fresh human plasma, was developed in 1955 (27). One disadvantage of using animal preparation is its immunogenic potential. Sensitization

could occur, leading to allergic reactions or anaphylaxis. So the use of animal AHG was confined to desperate situations where the life of the patient was in danger.

The first trial of this material was on three volunteers who had dental extractions. The patients had their teeth extracted without undue bleeding or obvious ill effects (28). In 1957, there were fourteen reports using animal AHG (29), including four cases of accidental injury and three of unavoidable major surgery. One patient who was bleeding from a shotgun wound in the back became sensitive to the bovine AHG but was saved by being administered with pig AHG to which he responded satisfactorily (30). By 1966, many cases of successful major surgery on hemophiliacs were recorded. In 1964, the development of a human AHG concentrate from cryoprecipitate led to the increased use of human AHG and the use of animal AHG declined (31). Similarly, effective Christmas factor concentrates were used in patients suffering from this disease who required major surgery (32-34).

In 1953, a hemorrhagic state which differed from hemophilia and Christmas disease was described (35). This bleeding disorder was postulated to be caused by a deficiency in a new factor which was named plasma thromboplastin antecedent (PTA). Two years later, a defect due to a deficiency of a factor which initiates changes in blood following glass contact was described (36). This was named Hageman factor after the patient who was investigated. A still unexplained finding is that patients deficient in Hageman factor show little or no coagulation dysfunction.

An important development occurred when it was found that two patients exhibited prolonged one-stage prothrombin times with both

Russell's viper venom and brain thromboplastin (37,38). Deficiency of factor VII would not account for the prolongation with venom, and prothrombin deficiency was ruled out by a two-stage assay. These patients' serum was defective in the thromboplastin generation test, but had a normal amount of Christmas factor. It was concluded that these patients were deficient in yet another clotting factor which was named "Stuart-Prower factor" after both patients. The function of Stuart-Prower factor was for the formation of plasma thromoboplastic activity and for the activation of prothrombin by Russell's viper venom or brain extract.

In 1944, Robbins (39) noticed that plasma contained a factor which in the presence of calcium causes fibrin to become insoluble in urea. After subsequent study of this factor (40-42), it became known as "fibrin stabilizing factor." Its function is to form stronger bonds between fibrin molecules.

Until an international committee met from 1957-1959, there was much confusion as to the terminology to be used when referring to the clotting factors. This committee decided on a universal system of nomenclature using the Roman Numeral System begun by Owen (Table 1).

At this time, it was not known how these clotting factors interacted. However, there was the idea of two types of thromboplastin. Tissue thromboplastin required factors V, VII, Stuart-Prower factor, and calcium for its reaction with prothrombin. Plasma thromboplastin seemed to arise as a result of interactions between factors VIII, IX, V, PTA, Stuart-Prower factor, Hageman factor, calcium, and platelets. The factors involved in the tissue thromboplastin system came to be known as the extrinsic system, and those involved in the plasma

Table 1

The Roman Numeral System for Nomenclature of Clotting Factors

Factor	Name
I	Fibrinogen
II	Prothrombin
III	Thromboplastin
IV	Calcium
V	Proaccelerin
VII	Proconvertin
VIII	Antihemophilic Factor
IX	Christmas Factor
X	Stuart—Prower Factor
XI	Plasma Thromboplastin Antecedent
XII	Hageman Factor
XIII	Fibrin Stabilizing Factor

thromboplastin system as the intrinsic system. Both the intrinsic and extrinsic systems lead to the conversion of prothrombin to thrombin (Fig. 2).

Information on how the factors of the extrinsic system interacted emerged when, in 1959, it was shown that the concentration of factor VII influenced the rate of formation of the prothombin activator, while the concentration of factor X influenced the amount formed (43). From this and other studies (44), it seemed that a reaction between tissue factor, factors VII, X, and calcium resulted in the formation of a product which then reacted with factor V to form a prothrombin activator. It was also shown that venom required factors V and X, phospholipid, and calcium to activate prothrombin (45) and that factor X was a substrate in the venom activated system (46).

MacFarlane, in 1961 (47), showed that venom reacts with factor X to form a new product which then reacts with factor V and phospholipid to form prothrombin activator. In 1962, it was shown (48) that the activity of the venom was due to an esterase which cleaves the factor X molecule forming an active product (Xa) which itself is an esterase.

Nemerson and Spaet, in 1964 (49), using tissue extracts from which the phospholipid was removed, showed that these extracts activated factor X in the presence of factor VII and, then, the subsequent addition of phospholipid and factor V was needed for prothrombin activation (Fig. 3). Since factors V and X and phospholipid were also needed for the intrinsic prothrombin activator, it was believed that factor X might be activated in a similar way by the various plasma factors. When a mixture of factors VIII, IX, X, and activated XI and XII react, factor Xa is formed and the amount formed is determined by

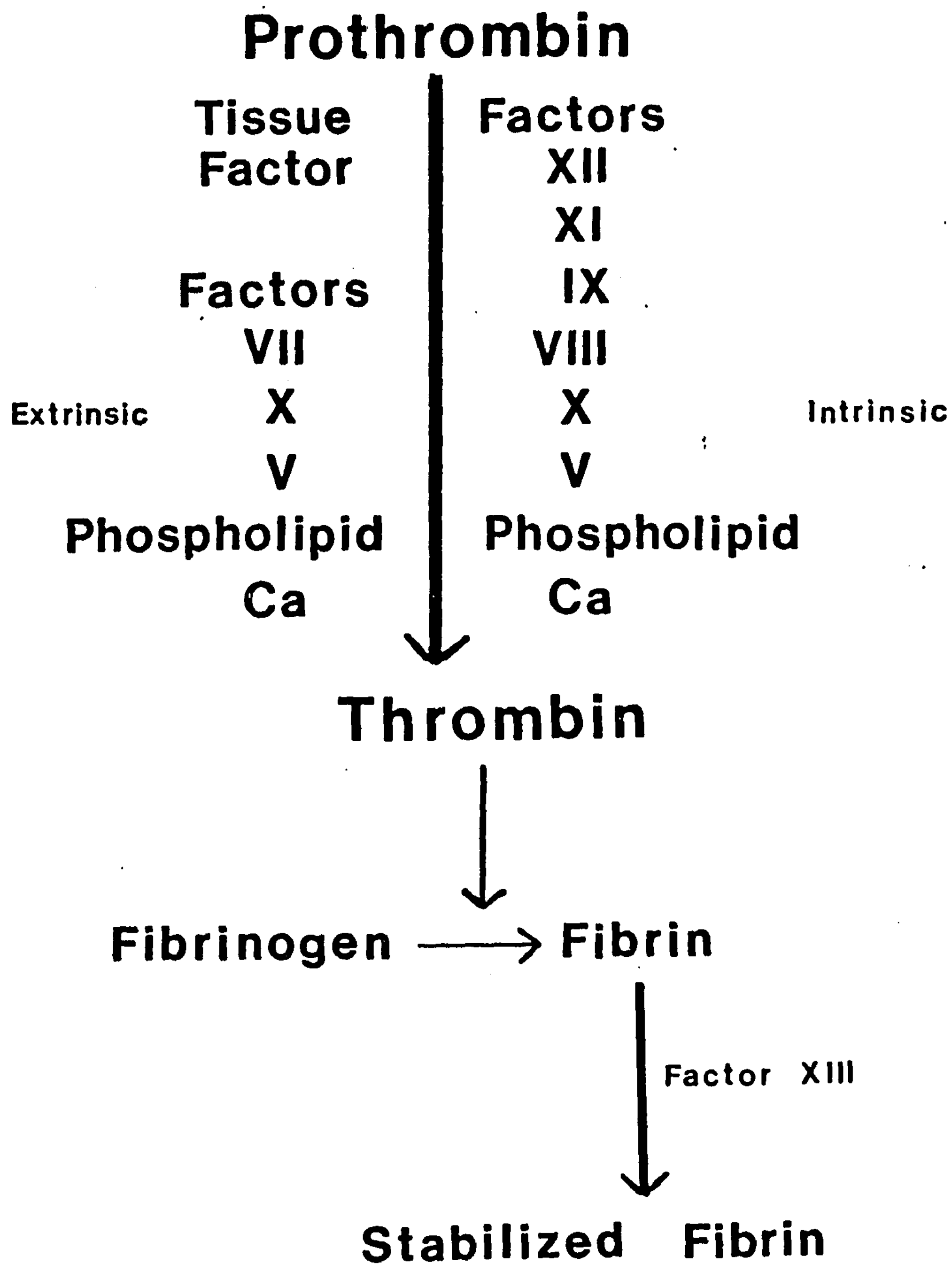


Figure 2 : The Clotting Factors Involved in the Intrinsic and Extrinsic Systems. (26)

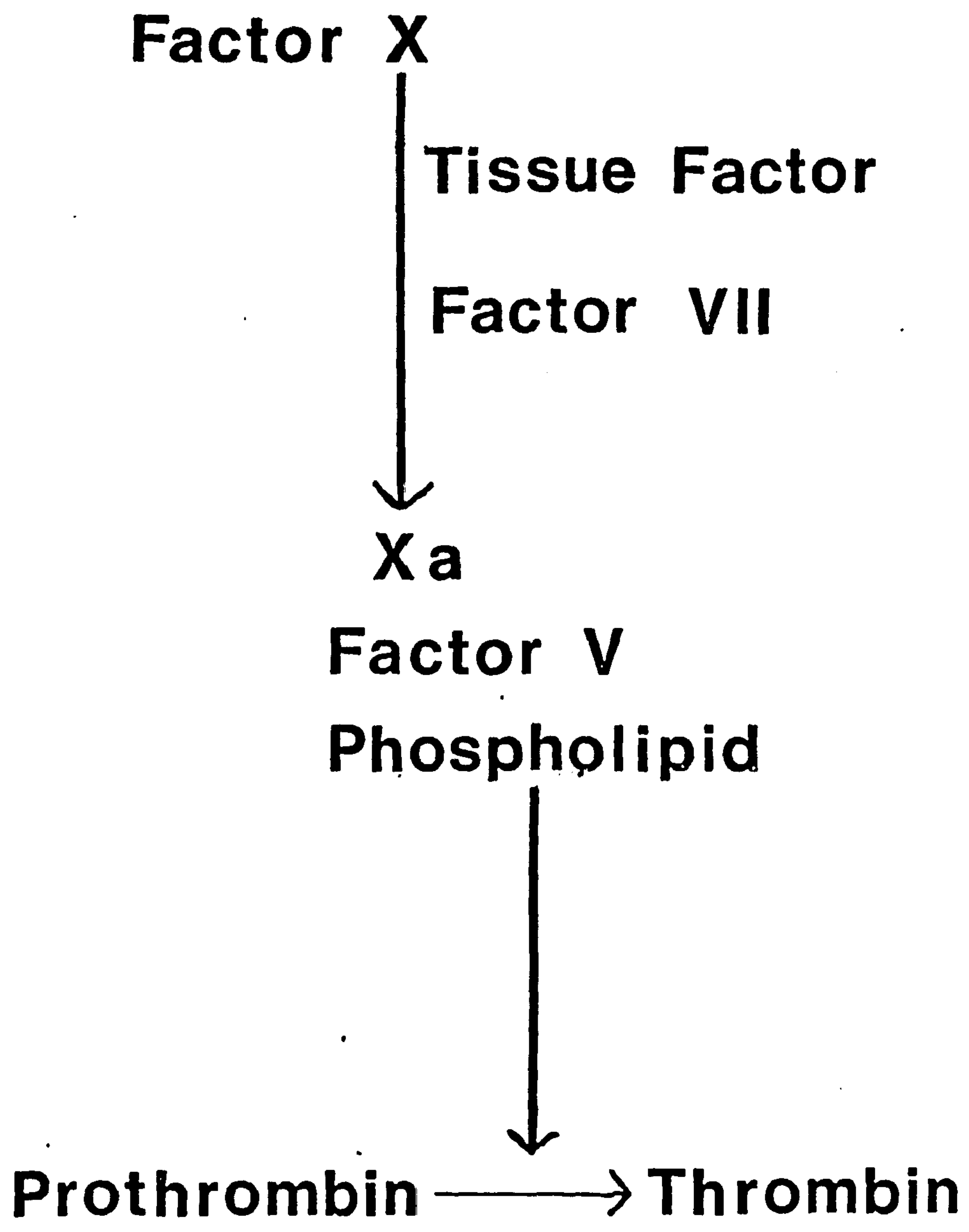


Figure 3 : Prothrombin Activation by the Extrinsic System. (26)

the concentration of factor VIII. If Russell's viper venom is added to this, there is no further activation of factor X (50). Thus, it was concluded that factor X was activated in a similar way by the intrinsic system and by Russell's viper venom or tissue thromboplastin.

In 1957 (51), it had been shown that factor XII was the factor activated by glass contact. It had also been shown that activated factor XII activated factor XI (52) which activated factor IX (53). Biggs et al., in 1965 (54), indicated that activated factor IX reacts with factor VIII and the product caused activation of factor X. MacFarlane (55) summed up the intrinsic reactions in what he called an enzyme cascade for blood coagulation. In 1965 (56), he published the complete coagulation scheme (Fig. 4). In this scheme, MacFarlane accounts for the auto-catalytic effects of thrombin on factors V and VIII and the effect of thrombin on platelets which consists of a release reaction.

In 1965, Hathaway (57) described another factor deficiency which he called Fletcher factor. However, Hathaway's experiments were not clarified until it was shown that another protein, prekallikrein (Fletcher factor), participated in the contact phase. It was shown by various workers (58-60) that factor XIIa activated prekallikrein and kallikrein, in turn, activated factor XII (58). This gives rise to an amplificaton of the contact phase. Collagen, glass, or some other surface triggers the conversion of factor XII to factor XIIa which will activate prekallikrein to kallikrein. The kallikrein will then activate more factor XII.

There is evidence (61-64) to indicate that an activated factor VIII is not the entity that activates factor X but a complex between factor IXa, factor VIII, calcium ions, and phospholipid is responsible

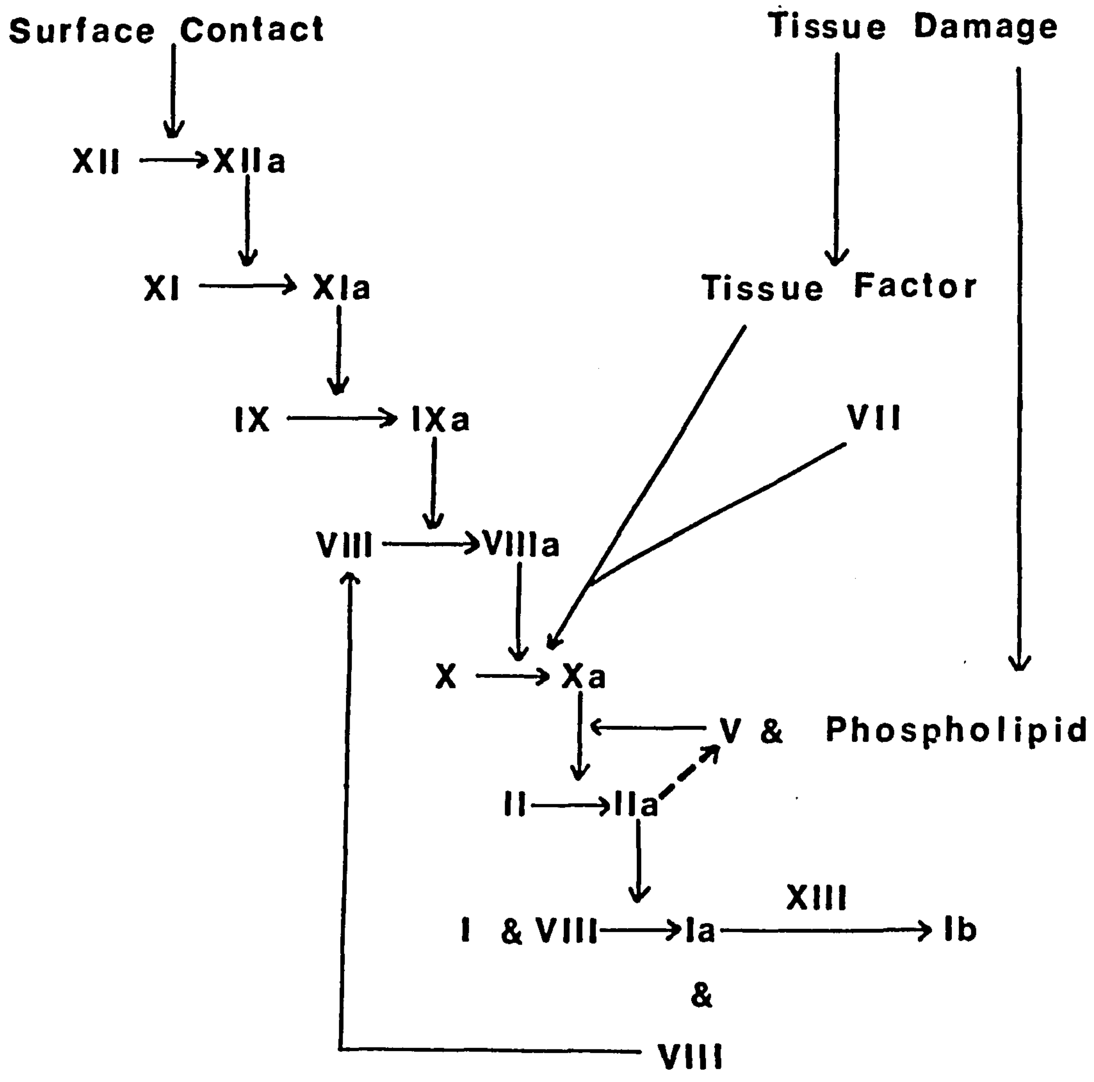


Figure 4 : The Enzyme Cascade for Blood Coagulation as Described by MacFarlane (56).

for the catalysis. These modifications in the intrinsic system are depicted in Fig. 5 (65).

This cascade fails to explain one important phenomenon. Deficiency in factors VIII or IX causes severe bleeding disorders, while deficiency in factors XII, XI, and Fletcher factor may be asymptomatic. This has been explained with the finding that the product of tissue factor and factor VII activates factor IX (66,67). Therefore, the contact phase involving Fletcher factor, factor XII, and factor XI can be bypassed.

Recent work (68) has clarified the events that take place to initiate the activation of the intrinsic pathway. It appears that factor XII in the presence of a surface and high molecular weight kininogen converts prekallikrein to kallikrein. The kallikrein formed, in the presence of high molecular weight kininogen, converts the surface bond factor XII to factor XIIa. The factor XIIa generated converts factor IX to IXa and the cascade continues (Fig. 6). Therefore, factor XII, once thought of as an inactive or weakly active zymogen, is actually the initiator of the clotting cascade.

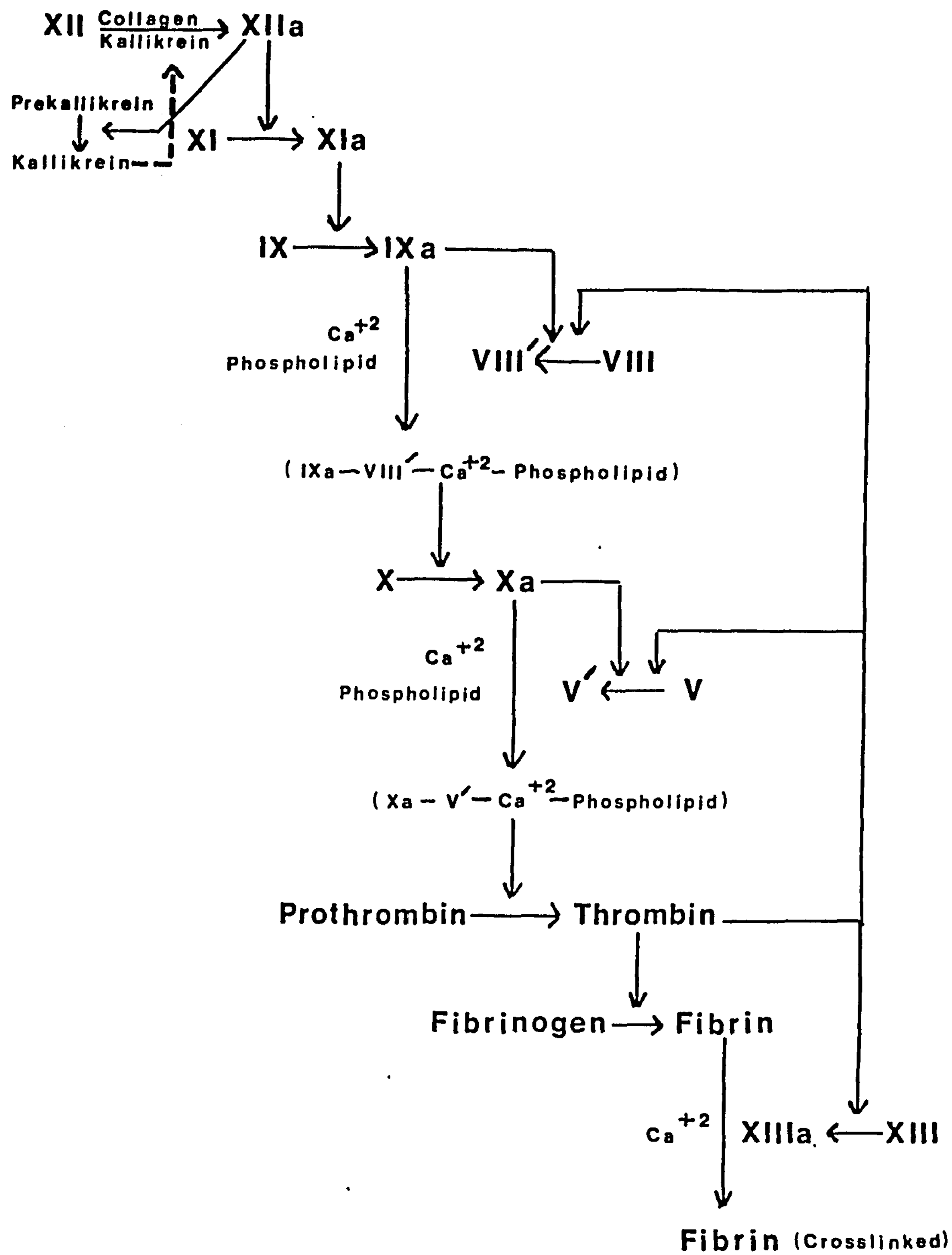


Figure 5 : The Modified Intrinsic Clotting Scheme. (65)

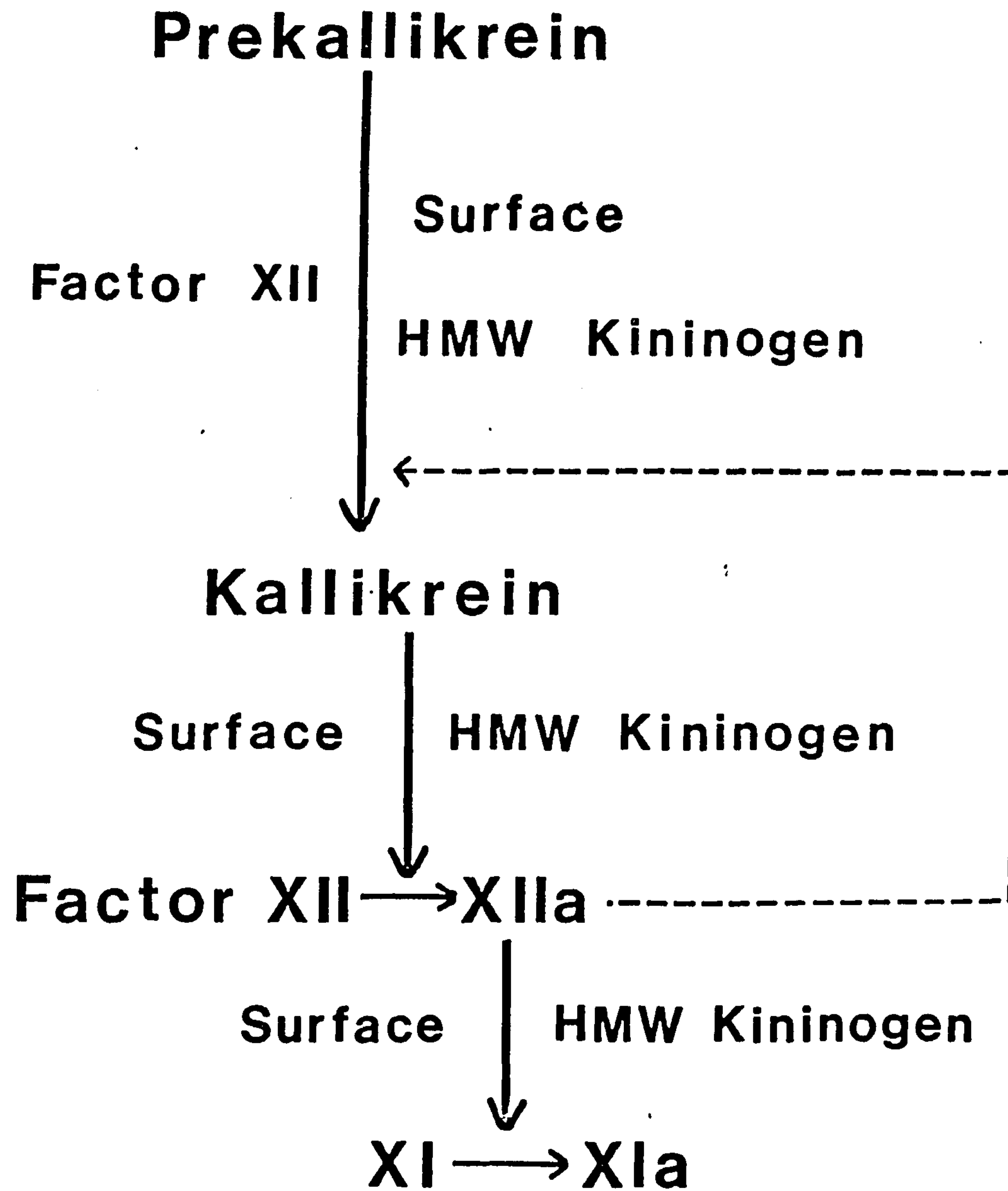


Figure 6 : The Contact Phase of Blood Coagulation.

B. Biochemical Mechanisms of Blood Coagulation

The activation of factor XII leads not only to intrinsic clotting, but also to fibrinolysis and kinin formation. Four proteins and a negatively charged surface are involved in the initial reactions called the contact phase, or contact activation of factor XII. The four proteins are factor XII, prekallikrein, high molecular weight kininogen, and factor XI.

Activation of factor XII is associated with its cleavage into two peptides (Table 2). Serine protease activity is associated with the small fragment. The large fragment is responsible for binding to a negatively charged surface. Alteration of positively charged guanido groups of arginine residues with phenyl glyoxal hydrate inhibits this interaction, suggesting the involvement of at least one arginine residue in binding. Binding to a surface apparently causes a conformational change in factor XII, since bound factor XII is activated five hundred times faster than free factor XII. This is also demonstrated by the change in the circular dichroism spectrum of factor XII in the presence of a negatively charged surface (69).

Prekallikrein and factor XI circulate complexed with high molecular weight kininogen. High molecular weight kininogen is composed of a single polypeptide chain containing a histidine rich region which is responsible for binding of the complex to a negatively charged surface. When binding occurs bringing prekallikrein in close proximity to factor XII, activation of prekallikrein takes place. The kallikrein formed is then able to activate factor XII. It is not clear where the binding site for prekallikrein is on high molecular weight kininogen, but the factor XI binding site is located between the histidine rich region and the

Table 2

Activation Products of the Clotting Factors

Factor	Species	Zymogen Form	Molecular Weight	Cleavage Products	Activity
XII	Human	single chain	80,000	52,000	surface binding
				28,000	serine protease
XI	Human	two identical chains linked by a disulfide bond	80,000 each chain	46,000 } each	
				27,000 } each	serine protease
IX	Bovine	single chain	55,400	27,300	serine protease
				16,000	
				alpha form	
X	Bovine	two non-identical chains linked by a disulfide bond	38,000	27,000	serine protease
			17,000	17,000	
				beta form	
				23,000	serine protease
				17,000	
II	Bovine	single chain	72,000	32,000	serine protease
				5,721	

carboxy terminal end.

There are two forms of activated factor XII. The alpha form occurs as a result of cleavage inside an intramolecular disulfide loop, yielding an active fragment linked to a surface bound inactive fragment, which then activates factor XI. The beta form occurs as a result of cleavage just outside the disulfide loop, liberating an active fragment. This enzyme readily activates prekallikrein, and the kallikrein formed is quickly dissociated from the surface (70,71).

Kallikrein cleaves high molecular weight kininogen in two places liberating a nonapeptide, bradykinin, while leaving the rest of the molecule surface bound complexed with factor XI. Circulating kallikrein also converts plasminogen to plasmin, resulting in fibrinolysis. Kallikrein also hydrolyzes arginine and lysine esters and is inhibited by diisopropylflourophosphate (DFP), and phenylmethyl sulfonylfluoride (PMSF). Therefore, the products of the kallikrein pathway, bradykinin and plasmin, are disseminated into the fluid phase, while the fibrin forming pathway, the alpha form of factor XIIa and factor XI, remain surface bound.

Factor XI is composed of two identical polypeptide chains linked by disulfide bonds. When activated by factor XIIa, each fragment is cleaved into a small and large fragment with the small fragment possessing activity. It is not certain whether factor XIa contains two active sites per molecule or whether it falls into the "half of the sites reactivity" classification, and thus only one site is functional. The physiological substrate for factor XIa is factor IX, but it also hydrolyzes arginine and lysine esters and is inhibited by DFP and PMSF.

Factor IX is a single chain glycoprotein containing γ-carboxyglutamic acid. γ-Carboxyglutamic acid is involved in calcium binding, which aids

in the interaction of the protein with phospholipids. Activation of factor IX by factor XIa is a two-step process that requires a divalent metal ion. Calcium ions are the most effective, but other ions are also active. Initially, factor XIa cleaves factor IX at one site yielding a light chain and heavy chain linked by a disulfide bond. This molecular species is inactive. In the second step, a small peptide is split from the amino terminal end of the heavy chain resulting in the formation of factor IXa.

Factor IXa is a serine protease with a reactive serine located in the heavy chain. The amino terminal region of factor IX is similar to the amino terminal regions of prothrombin and the light chain of factor X. In addition, the amino terminal regions of the heavy chains of factors IXa, Xa, and thrombin, as well as their active site regions, are homologous. They also contain γ-carboxyglutamic acid. It seems, therefore, that these three proteins have evolved from a common ancestral gene.

Clinical evidence indicates that deficiencies in factors XII or XI are either asymptomatic or are manifested as mild bleeding disorders. On the other hand, a deficiency in factor IX results in severe bleeding. It would seem likely then that factor IX can be activated by a mechanism independent of factor XIa. Such a mechanism has been elucidated (66). A mixture of factor IX, factor VII, tissue factor, and calcium ions generates factor IXa activity. If any of the components are excluded, factor IXa is not formed. When the activation is followed by SDS-polyacrylamide gel electrophoresis, a 55,000 Dalton band (native factor IX) is replaced by bands of 27,000 and 17,000 Daltons (heavy and light chains of factor IXa).

Factor VIII forms a complex with factor IXa, phospholipid, and

calcium ions. It is this complex which catalyzes the next reaction, the activation of factor X. Factor VIII acts as a cofactor in the complex with factor IXa catalyzing the activation of factor X. Factor IXa alone can activate factor X but at a much slower rate.

In the extrinsic system, factor VII and tissue factor combine to activate factor X. Tissue factor is a tissue lipoprotein found in brain, lung, placenta microsomes, liver, spleen, kidney, and endothelial cells. It is composed of 30-45% phospholipid by weight. Treatment of tissue factor with sodium deoxycholate separates it into protein and phospholipid (72). The protein or phospholipid alone lack activity; however, when recombined, activity is restored.

Bovine factor VII is composed of a single polypeptide chain and contains γ-carboxyglutamic acid. Unlike prothrombin and factor X, factor VII incorporates DFP. Factor VII and VIIa both incorporate the same quantity of DFP. After incorporating DFP, factor VII can no longer be activated, and factor VIIa becomes inactive.

Factor VII can be activated by factor XIIa (73), IXa (73), thrombin (74), and factor Xa (74). In the absence of phospholipid, both thrombin and factor Xa activate factor VII at the same rate. In the presence of phospholipid, the rate of activation by factor Xa increases four hundred fold, while the activation by thrombin is not affected. Activation of factor VII by factor Xa converts the single chain zymogen to a two-chain form. Further cleavage of factor VIIa by factor Xa results in the release of 12,000 Dalton peptide from the carboxy terminal end of the heavy chain with the concomitant inactivation of factor VIIa. Normal plasma contains a low level of factor VIIa activity. It is not certain whether this is due to a low concentration of circulating factor VIIa or whether the zymogen is active.

Bovine factor X is composed of a heavy and a light chain linked by disulfide bonds. All of the carbohydrate in factor X is bound to the heavy chain. Human factor X appears to be similar to bovine factor X since it is also composed of two chains with similar molecular weights (75).

The physiological activation of factor X is catalyzed by the complex of factor IXa, factor VIII, calcium, and phospholipid in the intrinsic system, and by factor VII and tissue factor in the extrinsic system. Non-physiological activators include a protease from Russell's viper venom and trypsin. The initial step in activation involves the cleavage of an 11,000 Dalton fragment from the heavy chain, and gives rise to an active enzyme designated as the alpha form of factor Xa (76). Following the initial cleavage, a second autocatalytic cleavage occurs, removing a 4,000 Dalton fragment from the carboxy terminal end of the heavy chain (77). The loss of this fragment, which contains one-third proline by weight, gives rise to the beta form of factor Xa. The alpha and beta forms of factor Xa have equivalent coagulent activity.

The activation of factor X can occur by a second pathway which is the reverse of the first. Cleavage of the 4,000 Dalton fragment from the carboxy terminal end of the heavy chain gives rise to the beta form of factor X which lacks enzymatic activity. This reaction is catalyzed by factor Xa and lipid. The conversion of the beta form of factor X to the beta form of factor Xa occurs by cleavage of the 11,000 Dalton fragment from the amino terminal end of the heavy chain. This reaction is also catalyzed by factor Xa and lipid. Factor Xa is a serine protease with an active serine located in the heavy chain, and it is inhibited by DFP. The role of the light chain of factor Xa has not yet been elucidated.

Factor V is a plasma glycoprotein that functions as a cofactor for factor Xa in the conversion of prothrombin to thrombin. Calcium ions and phospholipid are also required for the reaction. Stable preparations of factor V have only been obtained from bovine plasma. Bovine factor V is composed of a single polypeptide chain. It is stabilized by calcium ions and loses activity in the presence of chelating agents. Treatment of factor V with Russell's viper venom or thrombin results in an increase in activity with a decrease in molecular weight. This activated factor V binds to prothrombin whereas the precursor does not. Although the exact role of factor V is not known, it is believed to act as a substrate binding protein in the factor Xa, factor V, phospholipid, and calcium ion complex.

Human and bovine prothrombin are both single polypeptide chains containing γ-carboxyglutamic acid. Activation of bovine prothrombin by factor Xa occurs in two steps. First, there is cleavage of a 35,000 Dalton peptide (prothrombin fragement 1-2) from the amino terminal end of prothrombin. The product of this cleavage is prethrombin II. Cleavage of prethrombin II by factor Xa inside a disulfide loop forms thrombin, which is composed of a heavy and a light chain linked by the disulfide bond. The heavy chain contains the active serine residue.

Thrombin can also activate prothrombin. Thrombin cleaves a 23,000 Dalton fragment (fragment 1) from the amino terminal end of prothrombin. The product of this cleavage is prethrombin 1. Cleavage of a 13,000 Dalton fragment (fragment 2) from the amino terminal end of prethrombin 1 by factor Xa gives rise to prethrombin 2. Prethrombin 2 is then converted to thrombin by factor Xa.

Human fibrinogen is a glycoprotein composed of three pairs of

nonidentical polypeptide chains held together by disulfide bonds. In the conversion of fibrinogen to fibrin, thrombin cleaves four arginylglycine bonds: one from the amino terminal end of each alpha chain and one from the amino terminal end of each beta chain. The cleavage of these four bonds results in the release of two fibrinopeptides A and two fibrinopeptides B. However, the initial event, the formation of a fibrin monomer, occurs by thrombin activation of only one alpha chain of fibrinogen. This monomer rapidly and irreversibly self associates to form a dimer. Further polymerization occurs as a result of subsequent activation of intact alpha and beta chains (78).

Factor XIII is a tetramer composed of two pairs of nonidentical polypeptide chains, "a" and "b," held together by noncovalent bonds. Factor XIII isolated from platelets is an "a" chain dimer. Activation of factor XIII by thrombin involves the cleavage of an arginylglycine bond in the amino terminal region of each of the "a" chains. This reaction which occurs in the absence of calcium ions results in the release of two activation peptides, and gives rise to an inactive tetrameric intermediate. In the presence of calcium, the tetrameric intermediate dissociates into a catalytic dimer composed of two modified "a" chains which exhibits transglutaminase activity, and a noncatalytic dimer composed of two "b" chains.

Factor XIIIa catalyzes the formation of intermolecular glutamyllysine linkages between fibrin molecules. The initial reaction results in the fibrin molecules crosslinked through gamma-gamma chain linkages. The gamma chain crosslinking is followed by a slower alpha-alpha chain crosslinking. Approximately six crosslinkages are formed per mole of fibrin, four between alpha-alpha chains, and two between gamma-gamma chains. After crosslinking, the fibrin polymer is very insoluble and

resistant to lysis. Factor XIIIa is inhibited by selective alkylation of one sulfhydryl group, which suggests that it is one of the "half of the sites reactivity" enzymes.

There are many common features of the clotting proteins. There is sequence homology between factors IX, X, and prothrombin as well as their activated forms, factors IXa, Xa, and thrombin. These factors in addition to factor XIIa are all serine proteases.

There appears to be a requirement for the clotting proteins to interact with a surface. Physiologically, this would be the platelet membrane; in in vitro analysis, phospholipid mixtures are substituted for platelets. Factors IX, X, VII, and prothrombin all contain modified glutamic acid residues (γ-carboxyglutamic acid). The presence of this amino acid enables the proteins to bind calcium, which functions in the binding of these proteins to surfaces. Vitamin K is a cofactor in the post-translational modification (carboxylation) of the glutamic acid residues. Inhibition of this reaction renders the clotting factors nonfunctional. High molecular weight kininogen has a histidine rich region which functions in surface binding. Factor XI and prekallikrein become surface bound through their interaction with high molecular weight kininogen. Factor XII contains functional arginine residues which are implicated in surface binding. It is not clear whether factor V and factor VIII procoagulant interact directly with surfaces or interact indirectly through factors IXa and Xa.

Factor V and factor VIII procoagulant have some common features. Both of these proteins act as cofactors for serine proteases (factors IXa and Xa), with calcium and phopholipid also required for the reaction. Both are stabilized by calcium ions and lose activity in the presence of chelating agents. And finally, both have increased activity in the

presence of thrombin, with a concomitant decrease in molecular weight.

C. Factor VIII/von Willebrand Factor

The first recorded description of hemophilia and its genetic transmission is in the fifth century Talmud and in subsequent Rabbinic writings (79). The Talmud states that a mother must not circumcise her third child if the first two died as a result of bleeding from circumcision. In later writings, this was expanded to say that the first two sons need not be from the same husband. It was thus recognized that transmission of the disease was by the mother.

The first accurate account of hemophilia in the modern medical literature was given by John Conrad Otto in 1803 (80). In 1828, the name "hemophilia" was used to describe this disorder (81). The whole blood clotting test was invented in 1893 (82), and this was used to diagnose hemophilia. This test was used until it was observed that the rate of prothrombin conversion to thrombin is delayed in hemophilic blood, prompting the development of more sensitive tests.

It was shown, in 1911, that a fraction of normal plasma could shorten the prolonged clotting time of hemophilic blood (83). This fraction was named antihemophilic globulin (84). The clotting time of a hemophilic could also be shortened when transfused with normal blood (85). This was later demonstrated to be due to a more rapid conversion of prothrombin to thrombin (86). The plasma component that is deficient in hemophilia was called antihemophilic factor (AHF), and factor VIII (87).

In 1926, Eric von Willebrand described a bleeding disorder afflicting a young girl and 23 of her 66 family members (88). This disorder, which affected both sexes, was distinguishable from the known hemostatic abnormalities at the time. von Willebrand thought that this disorder was due to abnormal platelet function, and it was considered to be a platelet

disorder until it was found, in 1953 (89-91), that patients with this disorder had a deficiency of factor VIII.

When patients with von Willebrand's disease are infused with normal plasma, their factor VIII levels are increased and their prolonged bleeding times are shortened (92). It has also been shown (93-95) that the factor VIII deficiency and the prolonged bleeding time could be corrected by the infusion of hemophilic plasma. The term "von Willebrand factor" is given to the plasma factor deficient in von Willebrand's disease.

There are three biological activities associated with the VIII/von Willebrand factor protein: procoagulant activity (that which is deficient in hemophilia), von Willebrand activity (that which, when absent, causes abnormal platelet function), and antigenic activity (the ability of the protein to be precipitated by a monospecific antiserum). Differential diagnosis of hemophilia and von Willebrand's disease can be made by quantitating the factor VIII antigenic material which is present in hemophilic but absent in von Willebrand plasma, and by testing for abnormal platelet function in von Willebrand's disease. One test for platelet function is the aggregation of platelet rich plasma by the antibiotic, ristocetin (96). Ristocetin aggregates normal and hemophilic platelet rich plasma, but fails to aggregate the platelet rich plasma of patients with von Willebrand's disease.

The nature of the interaction between the factor VIII procoagulant and the von Willebrand factor remains to be determined. Using standard purification techniques, both activities (proteins) copurify as the factor VIII complex. Attempts to isolate the procoagulant have indicated that less than one percent of the mass of the factor VIII complex gives rise to procoagulant activity (96). Since the von Willebrand factor

comprises the bulk of the factor VIII complex, it is generally agreed that studies of the complex reflect the properties of the von Willebrand factor (97).

Although there is evidence to indicate that the factor VIII complex is composed of a single molecular species (98,99), there is an abundance of data indicating that it is composed of two different proteins.

The two proteins can be separated by gel filtration in the presence of 1.0 M NaCl or 0.25 M $CaCl_2$ (100-104), ion exchange chromatography (105), aminohexyl Sepharose chromatography (106), antibody affinity chromatography (107,108), and other techniques. They possess different antigenic determinants, and thus give rise to antibodies that specifically interact with each protein. Using these antibodies, specific immunoassays have been developed to quantitate each protein (109,110). The procoagulant and von Willebrand factor biological activities are independent of each other, and their plasma concentrations vary independently under certain conditions. And, finally, the two proteins are under different genetic control. Hemophilia (procoagulant deficiency) is sex linked inherited while von Willebrand's disease (von Willebrand factor deficiency) is inherited autosomally.

Factor VIII Procoagulant (F VIII:C)

Investigations into the effect of ionic strength on factor VIII led to the belief that, under suitable conditions, factor VIII dissociates. The sedimentation rate of bovine (111) and human factor VIII (112) was shown to decrease when the ionic strength was increased. Changes in the chromatographic behavior of bovine factor VIII when the NaCl concentration was increased indicated that the factor VIII activity was associated with a lower molecular weight species (113). Owen and Wagner (100)

showed that canine F VIII:C could be dissociated and separated on a Bio Gel A-15M column in the presence of 0.25 M $CaCl_2$. Rick and Hoyer (101) examined the changes in human factor VIII when subjected to sucrose density ultracentrifugation in the presence of 1.0 M NaCl and agarose gel filtration in 0.24 M $CaCl_2$. When plasma was centrifuged in 0.14 M NaCl, F VIII:C and antigenic activities were identified in fast sedimenting fractions. When the centrifugation was done in the presence of 1.0 M NaCl, the F VIII:C was shifted to the slowly sedimenting fractions, while the antigenic activity remained in the fast sedimenting fractions. When subjected to chromatography on Sepharose 6B in 0.12 M NaCl, both activities eluted in the void volume. When the column was equilibrated with 0.24 M $CaCl_2$, the antigenic activity was recovered in the void volume, but the F VIII:C eluted in later fractions. Although the low molecular weight procoagulant activity did not have antigenic activity, it was inactivated by human anti factor VIII (which is procoagulant specific) and rabbit anti factor VIII antisera.

Weiss and Hoyer (102) dissociated and chromatographically separated factor VIII in 0.8 M NaCl. They showed that the high molecular weight fragment, which had antigenic activity, also possessed von Willebrand factor activity.

It was not certain whether the two components in the native state were held together by noncovalent bonds or whether there was a covalent interaction which was broken by proteolysis during purification. This uncertainty was clarified when it was shown that the dissociation occurs in the presence of proteolytic inhibitors (103,104). Furthermore, it has been demonstrated that reassociation of the two components occurs when the dissociating agent is removed (114).

Purification and Characterization

Until recently, F VIII:C has not been characterized after separation and studies of F VIII:C have been made on the factor VIII complex. Human F VIII:C was purified 3500-fold by first immobilizing the factor VIII complex from plasma on a column which had anti factor VIII covalently coupled to it (108). The F VIII:C was then eluted with a calcium gradient. The molecular weight of the protein was calculated to be 285,000 (115) from its gel filtration properties on Sephadex G-200 and from sucrose density gradient centrifugation.

A 320,000-fold purification was obtained for F VIII:C from bovine plasma (116). Starting with 125 liters of plasma, 0.4 mg of protein were isolated by ammonium sulfate and glycine precipitation, followed by DEAE-Sephadex, Sulfate-Sepharose, Sephadex G-200, and factor X-Sepharose chromatography. The molecular weight estimated by gel filtration on Sephadex G-200 was calculated to be 250-300,000. On sodium dodecyl sulfate-urea polyacrylamide gels, the protein migrated as a triplet with molecular weights of 85,000, 88,000, and 93,000. In the absence of denaturing agents, the protein did not enter the gel. In the presence of 2-mercaptoethanol, the electrophoretic pattern remained the same. Incubation of the protein with thrombin resulted in a 30-fold increase in its activity. When analyzed by gel electrophoresis, the triplet was converted to a combination of a doublet with molecular weights of 69,000 and 73,000, and bands of 55,000 and 38,000. Activation of the factor VIII procoagulant protein with factor Xa in the presence of calcium and phospholipid, resulted in the same electrophoretic pattern as after thrombin activation.

Human F VIII:C was purified 5,000-fold (117) from plasma by limited reduction with 1 mM dithiothreitol, Sepharose 4B chromatography, alkylation with iodoacetamide, Sephadex G-100 and polyelectrolyte resin

chromatography. In a second paper by the same group (118), a 17,000-fold purification was achieved by limited reduction with 1 mM dithiothreitol, Sepharose 4B chromatography, reaction of the protein with pyridyl disulfide, polyelectrolyte resin chromatography, and thiol disulfide interchange chromatography using thiopropyl Sepharose. The protein was eluted from this column with dithiothreitol, and was alkylated by collection into iodoacetamide. The molecular weight of the reduced and alkylated protein in both cases was approximately 115,000. Although the von Willebrand factor loses activity in 1 mM dithiothreitol, F VIII:C appears to have at least one disulfide bond which can either be reduced or modified and is non-essential for activity. It has also been shown (99) that F VIII:C activity is not lost when the factor VIII complex is incubated with 0.05 M 2-mercaptoethanol. In contrast to this, the von Willebrand factor activity was rapidly lost. There are, however, essential thiol groups in F VIII:C since modification with p-chloromercuribenzoate inactivates it (119).

Thrombin Activation

F VIII:C is activated by thrombin (115,116,120-123). In the presence of thrombin, procoagulant activity rapidly increases and, then, gradually decreases. Vehar and Davie (116) have demonstrated that, when bovine F VIII:C is exposed to thrombin, there is a reduction in molecular weight of each of the chains. Hoyer and Trabold (115) have shown that thrombin activated F VIII:C has a molecular weight of 116,000 as determined by gel filtration and ultracentrifugation studies. This is in contrast to a molecular weight of 285,000 which they have reported for the unactivated protein. Although the exact mechanism of inactivation of activated F VIII:C is unknown, there is evidence to indicate that thrombin is not involved. When F VIII:C was activated by passage through

a column of immobilized thrombin (122), inactivation occurred even though thrombin was not present. After activation by thrombin, the addition of thrombin inhibitors (115,123) had no effect on the inactivation. Hultin and Jesty (123) give evidence to indicate that the inactivation of activated F VIII:C has the characteristics of first order decay.

Other Properties

When plasma is treated with EDTA, there is a loss of F VIII:C activity (124). EDTA treated factor VIII complex loses F VIII:C activity without losing von Willebrand factor activity (121). The loss of procoagulant activity is irreversible as incubation with calcium or other metals did not restore activity. When the factor VIII complex was separated by gel filtration in the presence of radioactive calcium, all the radioactivity coeluted with F VIII:C (125). It seems, therefore, that calcium not only binds to F VIII:C, but is necessary for its structural integrity.

Carbohydrate analysis of F VIII:C has not yet been done, but both bovine (116) and human (108) F VIII:C have been shown to bind to concanavalin A.

F VIII:C can be quantitated by specific immunoassays using the sera from hemophiliacs that develop inhibitors and from individuals who form autoantibodies to F VIII:C (126-128). These antisera interact specifically with F VIII:C, but do not precipiate the protein.

The site of synthesis of F VIII:C is not yet known. However, there is evidence to indicate that it is released by the liver (129-132). Owen and Bowie (132), studying the generation of clotting factors in rat liver perfused with a synthetic blood substitute, have indicated that the liver only releases F VIII:C in the presence of the von Willebrand factor.

von Willebrand Factor (vWF)

Biochemical studies of the von Willebrand factor have been done on the factor VIII complex. Molecular weight determinations by gel filtration and sedimentation equilibrium indicate that the protein has a molecular weight in excess of 1,000,000 (133-135). When electrophoresed in 5% polyacrylamide gels in the presence of sodium dodecyl sulfate and 6M guanidine or urea, no protein enters the gel. On reduction with mercaptoethanol or dithiothreitol, a single band is detected on SDS polyacrylamide gels with a molecular weight estimated to be 200,000-240,000 (133-135). It has recently been demonstrated that the 1,000,000 dalton protein polymerizes as a multimeric set with molecular weights ranging from 1-20 x 10^6 daltons (99, 136-138). There is evidence to indicate that only the large polymers interact with platelets (139). Therefore, it appears that polymerization of vWF to form large aggregates is essential for normal platelet function.

Carbohydrate

There is a good deal of evidence to indicate that carbohydrate plays an important role in the function of vWF. Removal of sialic acid residues has been shown to cause an increase (140,141), a decrease (142, 143) or no change (144) in platelet aggregating activity. Removal or oxidation of a penultimate galactose reduces platelet aggregating activity (144,145). When the oxidized galactose was reduced, activity was restored. Alteration or removal of carbohydrate residues does not affect procoagulant activity.

Sialic acid is involved in the in vivo survival of F VIII/vWF. The asialo protein is cleared by rabbit liver with a $T_{1/2}$ of 5 min as opposed to 240 min. for the normal protein (142). It has also been demonstrated that the liver asialo-glycoprotein lectin binds asialo F VIII/vWF, but not

native or asialo-agalacto F VIII/vWF (145).

An attempt to correlate carbohydrate content of F VIII/vWF with disease has led to conflicting results. Gralnick et al. (146) have shown that F VIII/vWF from each of five von Willebrand patients did not stain with periodic acid Schiff stain for carbohydrate. In two of these patients, the sialic acid content of F VIII/vWF was decreased. In contrast, Zimmerman et al. (147) have found that F VIII/vWF from fifteen of sixteen von Willebrand patients stained with periodic acid Schiff reagent. In another study (148), F VIII/vWF from von Willebrand patients had a decreased interaction with concanavalin A as compared to normal F VIII/vWF. The antigenic properties of carbohydrate modified F VIII/vWF are the same as normal F VIII/vWF when using rabbit antiserum. Therefore, if von Willebrand F VIII/vWF is carbohydrate deficient, this cannot account for the decreased quantity of factor VIII related antigen in von Willebrand disease.

Platelet Binding Sites

The presence of binding sites for F VIII/vWF on human platelets has been demonstrated by using ^{125}I F VIII/vWF and washed human platelets (149). The amount of F VIII/vWF that bound to the platelets was dependent on the ristocetin concentration, and binding was linear for ristocetin concentrations up to 1 mg/ml. The time for binding to reach equilibrium was dependent upon the number of platelets. When a fixed concentration of ristocetin and platelets were used, binding of F VIII/vWF increased as the concentration of F VIII/vWF increased, but this eventually reached a plateau. This shows that the binding is saturable. Scatchard analysis of the binding data give a dissociation constant, K_d, of 0.46 nM for a ristocetin concentration of 1 mg/ml and 0.5 nM for a ristocetin concentration

of 0.5 mg/ml. The calculated total number of binding sites per platelet was 31,000 in the presence of 1 mg/ml ristocetin, and 13,700 in the presence of 0.5 mg/ml ristocetin. These were based upon the assumption that F VIII/vWF is univalent with a molecular weight of 1.1×10^6 Daltons. In examining the specificity of binding, it was found that only unlabeled human or bovine F VIII/vWF competed with labeled F VIII/vWF. The addition of 2 mM $CaCl_2$, NaCl, or EDTA had little effect on binding. However, the addition of 2 mM EGTA resulted in an 86% inhibition of binding. Since EDTA had no effect, the inhibitory effect of EGTA is most likely due to some property of the molecule other than its Ca^{+2} chelating activity. In studying the binding in the pH range of 5.3 to 9.0, maximum binding occurred between pH 7.0 and 7.5. When platelets were treated with increasing concentrations of chymotrypsin, binding of F VIII/vWf pregressively decreased, indicating that the receptor for F VIII/vWF is proteinaceous in nature.

Following the identification of platelet binding sites for F VIII/vWF, a study of whether binding of F VIII/vWF correlates with function (platelet aggregation) was undertaken (150). It was reasoned that ristocetin causes F VIII/vWF binding sites to become accessible and that the number of binding sites is dependent upon the ristocetin concentration. If platelet aggregation is dependent upon binding, then the number of binding sites made accessible by ristocetin should correlate with the rate or extent of ristocetin induced platelet aggregation over a range of ristocetin concentrations. Using ristocetin at concentrations from 0.2 to 1 mg/ml, it was shown that the initial velocity of platelet aggregation was directly proportional to the amount of binding. It was also

demonstrated that, when platelets were exposed to different concentrations of chymotrypsin or trypsin, binding of F VIII/vWF and platelet aggregation were reduced concomitantly and progressively. There was also a direct correlation between the reduction in binding of F VIII/vWF and the reduction in the initial velocity of ristocetin induced platelet aggregation by heparin, EGTA, and other inhibitors.

In another study, the correlation between binding and platelet aggregation was examined for carbohydrate modified F VIII/vWF (151). Binding was determined by the ability of unlabeled modified (or normal) F VIII/vWF to inhibit binding of ^{125}I F VIII/vWF. It was found that, after removal of sialic acid, platelet aggregation was reduced to 39%. Subsequent oxidation of penultimate galactose residues further reduced aggregation to 19% of normal. Reduction of the oxidized galactose with KBH_4 restored aggregating activity to 33.3%. In the binding study, it was found that the concentration required to inhibit 50% of the binding of ^{125}I F VIII/vWF to 5 x 10^6 platelets was 2.0 μg/ml for native F VIII/vWF, 14.8 μg/ml for asialo, 66 μg/ml for galactose oxidized, and 30 μg/ml for KBH_4 reduced-galactose oxidized F VIII/vWF. The inhibition of binding was further analyzed by using Lineweaver-Burk plots to determine the type of binding, and Dixon plots to determine the inhibition constant, K_i. Examination of the inhibition of binding by Lineweaver-Burk plots indicates that the inhibition is competitive. Upon removal of sialic acid, the K_i increased from 1.1 nM to 12.5 nM. Oxidation of the penultimate galactose further increases the K_i to 53.8 nM. Reduction of the oxidized galactose brought the K_i to 18.9 nM. To correlate binding and aggregation, the log of platelet aggregating activity was plotted against the log of the K_i for each form of F VIII/vWF. The linear relation found between these two parameters indicates that platelet

aggregating activity is a function of receptor binding affinity.

In examining whether F VIII/vWF could interact with other cells, it was found that some binding to red blood cells and polymorphonuclear leukocytes occurred (149). It was felt that the interaction was non-specific because binding could be progressively diminished with additional washes. With the finding that F VIII/vWF has blood group oligo saccaharide structures attached covalently (152), interactions with other cell types could occur through ABO blood group interactions.

The physiological significance of ristocetin induced platelet aggregation and binding of F VIII/vWF to platelets is not clear. However, in light of the fact that there is reduced ristocetin induced aggregation in von Willebrand's disease, the phenomenon most likely has physiological significance. To add credence to this, there is evidence to indicate that glycoprotein I of the platelet membrane is the receptor for F VIII/vWF. There is a disorder, the Bernard-Soulier syndrome, in which glycoprotein I is deficient and the resulting platelet abnormality can be linked to a decreased interaction with F VIII/vWF (153-156).

Other Properties

Amino Acid analysis of F VIII/vWF (133-135) indicates that it contains very low amounts of tyrosine and tryptophan, and there are no free sulfhydryl groups. The molecule also contains approximately 20% aspartic acid and glutamic acid, and approximately 10% lysine and arginine.

Antibodies against F VIII/vWF raised in rabbits precipiate F VIII/vWF and inhibit F VIII/vWF-platelet interactions. The antisera vary in their inhibitory affects on F VIII:C (157). The immunoprecipitation properties of the antisera allows for the quantification of factor VIII related antigen by Laurell immunoelectrophoresis, counter immunoelectro-

phoresis, and radioimmunoassay (158-159).

Using immunofluorescence to detect the site of synthesis, VIII related antigen was detected in endothelial cells of arteries, arterioles, capillaries, veins, megakaryocytes, platelets, and the medium from cultured human umbilical cord endothelial cells (160-163).

D. Purpose of Research

The purpose of this work was to study some of the physical properties of F VIII/vWF by circular dichroism and fluorescence spectroscopy. In addition, the interaction between F VIII/vWF and heparin was investigated in order to gain a better understanding of how F VIII/vWF interacts with membrane surfaces. A method was developed for the production of antibodies in mice to F VIII/vWF in order to have a source of antibody for the quantitative assays of F VIII/vWF.

II. PRODUCTION OF ANTIBODIES TO FACTOR VIII/von WILLEBRAND FACTOR IN THE ASCITIC FLUID OF C57BL/6J MICE

A. Introduction

Quantitative abnormalities of F VIII/vWF in von Willebrand's disease and hemophilic carriers are detected by immunoassay. Rabbits have been a prime source of antibodies for the determination of factor VIII related antigen by Laurell immunoelectrophoresis and enzyme immunoassay. The use of rabbits requires adequate facilities for their housing. Successful bleeding of rabbits may also be difficult and, at times, results in death.

Production of ascitic fluid containing antibodies in mice was first described by Munoz (164). It was found that, after intraperitoneal injections with either bovine serum albumin or ovalbumin mixed in Freund's adjuvant, the mice developed large amounts of fluid containing antibodies to the antigens.

In a study by another group (165), it was found that substantial quantities of ascitic fluid containing antibodies could be obtained by injecting mice with antigenic materials derived from either *Staphylococcus aureus* or *Salmonella enteriditis* mixed with incomplete Freund's adjuvant. In subsequent work by others (166-168), it was observed that the response of the mice varied from mouse to mouse, with the strain of mice, and with the method of injection.

Tung et al. (169) have made a thorough investigation of the effect of adjuvant to antigen ratio, amount and time course of injection, and strain of mice, in order to maximize ascitic fluid production and antibody concentration. They found that maximum antibody and ascitic fluid production occurred when eight-week old A/J mice were injected on days 0, 14, 21, and 35 with 0.2 ml of a 9:1 mixture of complete Freund's adjuvant to antigen with the antigen at a concentration of 25 mg/ml. The requirement for such a large concentration of

antigen poses limitations to the application of the technique. By modifying their method, an antiserum to F VIII/vWF was produced with the concentration of F VIII/vWF being 100-200 μg/ml. The antiserum was used to quantitate factor VIII related antigen by Laurell immunoassay, and the results obtained were compared to those using a commercial rabbit antiserum. The anti F VIII/vWF antibody was isolated by affinity chromatography and its subclass was identified. An attempt was also made to develop a specific one-reagent fluorescence assay for F VIII related antigen.

B. Experimental Methods

Purification of F VIII/vWF

F VIII/vWF was purified by a modification of the method of Rubin et al. (170). A 30 ml vial of Hemofil (Hyland) was reconstituted with 10 ml of imidazole buffered saline (IBS) (0.05 M imidazole, 0.15 M NaCl, pH 7.35). The reconstituted concentrate was adsorbed with 0.1 ml of $Al(OH)_3$ (Amphogel, Wyeth) per ml concentrate to remove factors II, IX, X, and XI. The insoluble matter was removed by centrifugation at 5,000 XG for 10 minutes at 25°C. The supernatant was poured off, and to it was added 0.1 units of Malayan Pit Viper venom (Arvin) per ml of supernatant (171). This was allowed to clot overnight. The clot was broken with a glass rod, and the liquid was separated from the insoluble material by centrifugation at 15,000 XG for 15 minutes at 25°C. The supernatant (4-5 ml) was applied to a 1.6 x 100 cm Bio Gel A-15M (Bio Rad) column, eluted downward with IBS at a flow rate of 8 ml per hour and collected in 3 ml fractions. The void volume eluate was pooled, dialyzed against 0.15 M NaCl overnight, and stored at -20°C until used.

Factor VIII Procoagulant Assay

Factor VIII procoagulant activity was measured by the one-stage APTT method as described by Mitchell et al. (172). Factor VIII deficient plasma (Dade, 0.1 ml), 0.1 ml of test material or control plasma, and 0.1 ml of activated cephaloplastin reagent (Actin, Dade) are mixed and incubated at 37° for 2 minutes. $CaCl_2$ (0.02 M, 0.1 ml) is added and the time it takes for a clot to form is recorded. To prepare a standard curve, the time in seconds is plotted versus the log of the percent factor VIII procoagulant activity. A factor VIII procoagulant activity of 100% (1 U/ml) is defined as a 1/5 dilution of pooled normal plasma.

Inhibition of coagulant activity was measured in Bethesda units as described by Kasper et al. (173). This involves incubating one part control plasma with one part antiserum for two hours at 37°C and then measuring the residual procoagulant activity. An antiserum containing 1 Bethesda unit of inhibitor will reduce procoagulant activity by 50%.

Protein Assays

Protein concentration was determined by the microbiuret method (174) using bovine serum albumin as a standard.

Production of Antisera

Eight week old male or female C57BL/6J mice (Jackson Laboratories) were injected intraperitoneally (with a 25 gauge, 5/8 inch long needle) with 0.2 ml of a 2:1 mixture of complete Freund's adjuvant (Difco) to the factor VIII solution. The injections were given on days 0, 14, 28, 35, 42, 49, and 56, with the production of noticeable ascitic fluid beginning between days 49 and 63. Additional weekly or biweekly injections were given until the development of adhesions made injection difficult. When sufficient ascitic fluid developed (usually weekly, starting with the 49th day), fluid was tapped by inserting a 20 gauge 1.5 inch needle (without syringe) into the abdominal cavity and allowing the fluid to drain. This fluid was immediately centrifuged for 5 minutes at 5,000 XG at 4°C and then frozen.

On thawing, clots formed which were broken with a Pasteur pipette. These were removed by centrifuging for 10 minutes at 15,000 XG. The fluid was heated at 56°C for 30 minutes and centrifuged for 15 minutes at 15,000 XG. The antiserum was absorbed by the method of Zimmerman et al. (175). It was incubated overnight at 37°C with a fraction of plasma soluble in 3% ethanol and insoluble in 8% ethanol at -3°C. The precipitate which formed after the incubation was removed by

centrifugation for 15 minutes at 15,000 XG. The antiserum was heated at 56°C for 30 minutes and centrifuged for 15 minutes at 15,000 XG. This antiserum was stored frozen in 2-5 ml aliquots.

Isolation of Anti F VIII/vWF Antibody

F VIII/vWF was immobilized on agarose as follows. The void volume fractions from the Bio Gel A-15M chromatography step were pooled and concentrated to 5 ml using Aquacide II (Calbiochem). It was then dialyzed overnight at 25°C against bicarbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.5). CNBr (0.5 g)(Eastman) was added to 5 ml of Sepharose 4B (Pharmacia) in 7 ml of water; the pH was maintained at 11 with 4 M NaOH for 12 minutes. The activated Sepharose was washed with 500 ml of cold bicarbonate buffer on a sintered glass funnel. The activated Sepharose was poured into a beaker, the F VIII/vWF solution was added, and the volume brought to 15 ml with bicarbonate buffer. Coupling proceded for two hours at 25°C with constant stirring. After two hours, the Sepharose was washed with bicarbonate buffer until the eluate had no absorbance at 280 nm. Residual active sites were blocked by adding 30 ml of 1 M ethanolamine pH 9 and incubating for two hours at 25°C. The resin was washed with 15 ml of glycine-HCl buffer (0.5 M glycine, 0.1 M NaCl pH 2.4), 15 ml of borate buffer (0.055 M sodium borate, 1 M NaCl pH 8.0), 15 ml of acetate buffer (0.1 M sodium acetate, 1 M NaCl pH 4.0), and phosphate buffer (0.1 M sodium phosphate, 0.15 M NaCl pH 7.5) until the eluate had a pH of 7.5. The resin was packed into a 0.9 x 15 cm column (Pharmacia) and kept at 4°C.

The antiserum was brought to 50% saturation with ammonium sulfate and then centrifuged for 10 minutes at 7,500 XG at 4°C. The precipitate was removed and dialyzed against the phosphate buffer. 2 ml of this

material was applied to the immobilized F VIII/vWF resin and eluted with the phosphate buffer until the eluate had no absorbance at 280 nm. The bound protein was eluted with 3 M NaSCN in 0.1 M sodium acetate buffer pH 6.0, dialyzed against 0.15 M NaCl, and then stored at 4°C. The column was regenerated by washing as previously described.

Qualitative Immunodiffusion and Immunoelectrophoresis

Immunodiffusion was performed by coating a 2.5 x 7.5 cm strip of Gel Bond Film (FMC) with 3 ml of 1% agarose (Bio Rad) in Tris-Tricine buffer (Bio Rad Immunoelectrophoresis Buffer IV). Wells (3 mm diameter) were filled with appropriate solutions and allowed to develop for 24-48 hours. The gels were washed for 48 hours in 0.3 M NaCl, 48 hours in 0.15 M NaCl, dried, and stained with acid fuchsin. Immunoelectrophoresis gels were prepared as were the immunodiffusion gels. A 3 mm well was filled with reconstituted Hemofil. The gel was subjected to electrophoresis at 3 ma for 1.5 hours. Troughs were cut and filled with antiserum and allowed to develop for 24-48 hours. The gels were washed, dried, and stained as previously described for immunodiffusion.

Laurell Immunoelectrophoresis

Quantitative immunoelectrophoresis was performed by the method of Laurell (176). Eleven ml of 1% agarose in Tris-Tricine buffer pH 8.6 containing antiserum (usually 0.4-0.8%) was layered on a 10 x 75 cm piece of Gel Bond Film. Antigen was placed into 3 mm wells, and electrophoresed at 3 ma for 16 hours. The gel was dried and stained with coumassie blue.

Enzyme Immunoassay

Enzyme immunoassay was performed by modifying the method of Ness and Perkins (177). This method involves immobilizing F VIII on polystyrene

tubes (1, Fig. 7). Unoccupied sites are blocked by adding bovine serum albumin. Test or control material (containing F VIII) is incubated with mouse anti F VIII, and this mixture is added to the tubes. Immobilized F VIII and free F VIII compete for the antibody (2, Fig. 7). A solution containing rabbit antimouse antibody coupled to alkaline phosphatase is added (3, Fig. 7). The substrate p-nitrophenylphosphate is added (4, Fig. 7), and the alkaline phosphatase converts it to p-nitrophenol. The absorbance is then read at 405 nm (p-nitrophenolate ion). The quantity of enzyme catalyzed product formation is proportional to the concentration of immobilized anti-factor VIII, and inversely proportional to the original plasma factor VIII concentration.

1. Purified factor VIII was dialyzed against 0.2 M sodium carbonate buffer pH 9.2. This solution (0.2 ml) was added to 12 x 75 Falcon tubes #2052, incubated at room temperature for 30 minutes and then removed from the tubes.

2. Bovine serum albumin (BSA) (0.5%) in carbonate buffer pH 9.2 was added (0.5 ml) and incubated at room temperature for 15 minutes. The BSA solution was removed and the tubes washed 2 times with 0.2% BSA in 0.15 M sodium phosphate buffer - 0.15 M NaCl pH 7.2 (PBS-BSA).

3. Serial dilutions of plasma were made in PBS-BSA, and 0.2 ml of these dilutions were incubated with 0.1 ml of murine antiserum diluted 1/200 in PBS-BSA overnight at 4°C. This mixture (0.2 ml) was added to the tubes and incubated at room temperature for one hour. This mixture was discarded and the tubes were washed 3 times with PBS-BSA.

4. The conjugate was prepared by adding 0.3 ml (1.5 mg) of alkaline phosphatase (Sigma Type VII) to a volume of the IgG fraction of rabbit antimouse IgG (USB) which contained 0.5 mg of protein, and dialyzing at 4°C against PBS overnight. Glutaraldehyde (10 µl) was added to a final

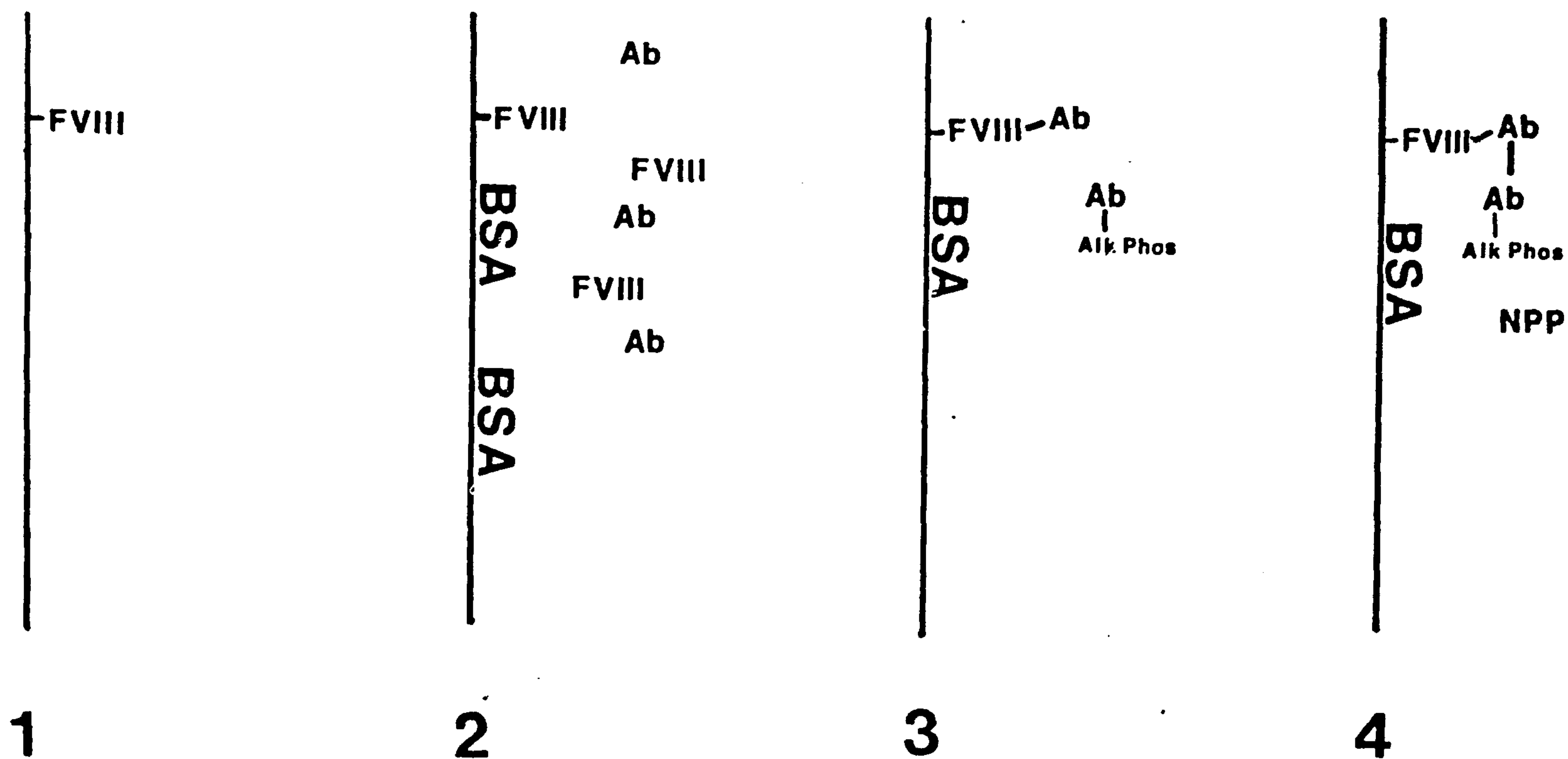


Figure 7 : Enzyme Immunoassay. 1. Immobilization of F VIII. 2. Competition Between Free and Immobilized F VIII for the Antibody. 3. Addition of Rabbit Anti Mouse IgG Coupled to Alkaline Phosphatase. 4. Addition of the Substrate p-nitrophenyl phosphate.

concentration of 0.2% and allowed to react for two hours at room temperature. This was then dialyzed against PBS at 4°C overnight and diluted 1/200 with PBS-BSA.

Conjugate (0.2 ml) was added to the tube and incubated for two hours at room temperature. The conjugate solution was removed and the tubes were washed 3 times with PBS-BSA.

5. The substrate p-nitrophenyl phosphate (Sigma) was prepared just before use at a concentration of 1 mg/ml in 0.05 M sodium carbonate buffer pH 9.8 with 0.001 M $MgCl_2$.

0.2 ml of substrate was added and incubated for one hour. 1.5 ml Of 1 M sodium phosphate pH 9.0 was added, and the absorbance of each tube was read at 405 nm.

Fluorescence Assay

N-(1-pyrene)maleimide was synthesized as described by Weltman et al. (178) (Fig. 8). 2.1 g of 1-aminopyrene (Aldrich) in 24.5 ml of ice cold tetrahydrofuran was mixed with 1 g of maleic anhydride in 12.4 ml of ice cold tetrahydrofuran. This reacted overnight at 4°C with constant stirring. The bright yellow precipitate was washed with tetrahydrofuran and then dried.

To a solution of 22.66 ml acetic anhydride (24.54 g) containing 0.245 g of sodium acetate, 1.8 g of the precipitate [N-(1-pyrene)maleamic acid] was added. The suspension was heated at 100°C for 45 minutes with stirring. After cooling to room temperature, the mixture was poured into 100 ml of ice water, and the precipitate [N-(1-pyrene)maleimide] was collected by suction filtration. The product (0.56 g) was collected after 2 x recrystallization from ethanol and drying. The melting point was determined to be 218-220°C. The melting point for N-(1-pyrene)-maleimide given by Weltman et al. (178) is 223-225°C.

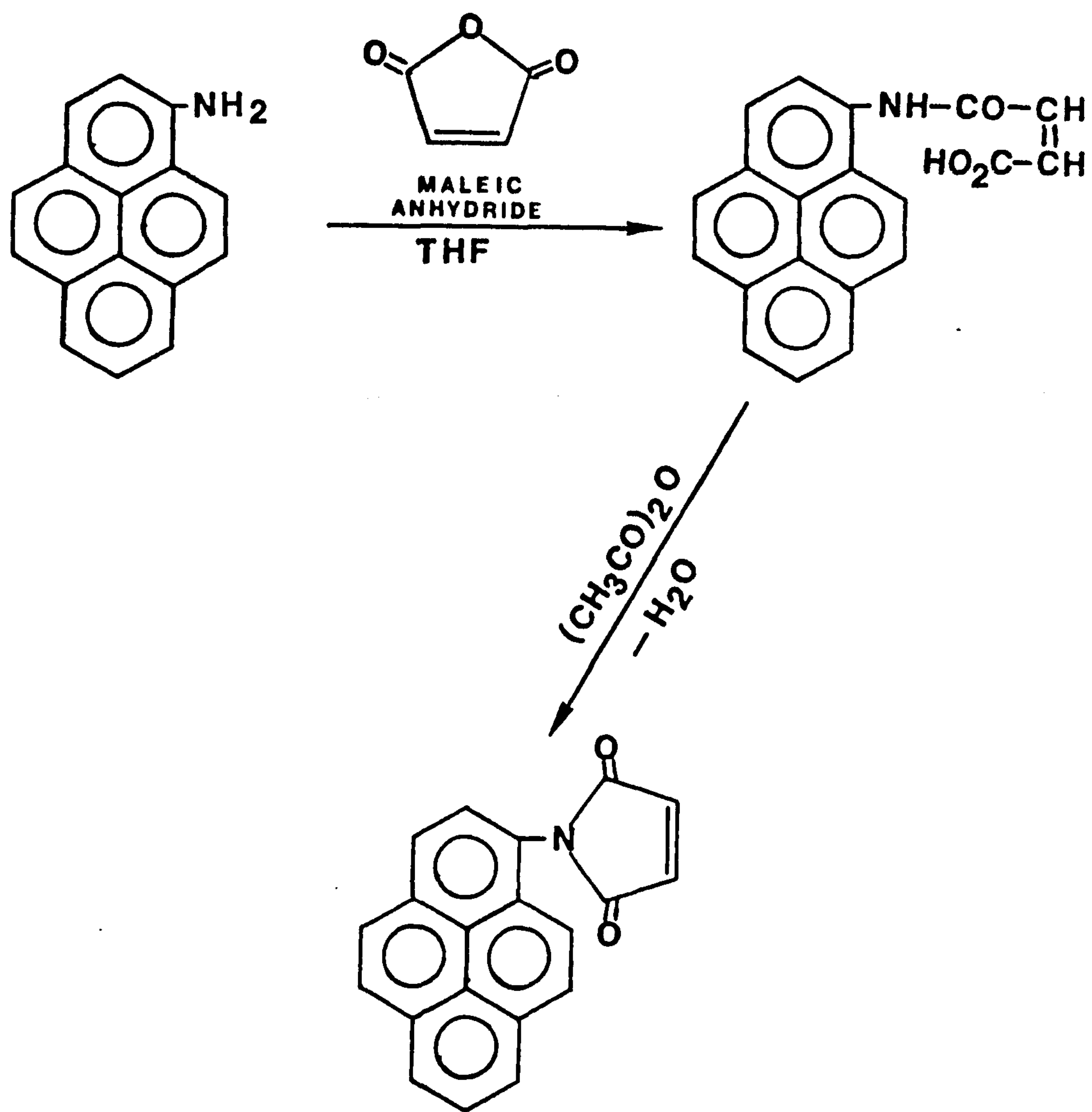


Figure 8 : The Synthesis of N-(1-pyrene) maleimide.

N-(1-pyrene)maleimide was conjugated to the isolated antifactor VIII antibody (from the immobilized F VIII resin) and to a fraction of the antiserum which was insoluble in 50% saturated ammonium sulfate by the method of Liburdy (179). The fluorescence emission at 390 nm (λex 345nm) was monitored for the conjugated antibodies alone, and in the presence of purified F VIII/vWF or plasma.

C. Results and Discussion

Purification of F VIII/vWF

The starting material used in the purification of F VIII/vWF, Hemofil, was developed as a therapeutic treatment for hemophilia. Hemofil contains approximately 25 times the activity of F VIII:C as that of plasma. This permits large quantities of F VIII:C to be administered without a large increase in blood volume. Hemofil is prepared from pooled cryoprecipitate which is obtained from citrated human plasma (180-183). Fibrinogen is selectively precipitated from the redissolved cryoprecipitate with polyethylene glycol. The F VIII/vWF is then precipitated by adding glycine. The precipitate is redissolved in citrated saline and lyophilized.

Fig. 9 is the elution profile for the gel filtration of $Al(OH)_3$ and Arvin treated Hemofil on Bio Gel A-15M. Only units of F VIII:C are indicated in the figure. However, all activities coeluted in the void volume. When this material is electrophoresed into 5% polyacrylamide gels in the presence of 1% sodium dodecyl sulfate and 5 M urea, no protein enters the gel (Fig. 10). Upon reduction with 0.1 M 2-mercaptoethanol, a single band is evident within the gel (Fig. 10).

Production of Antisera

In a thorough investigation of the effect of adjuvant to antigen ratio on the production of ascitic fluid in mice, Tung et al. (169) have found that a 9:1 ratio of adjuvant to antigen was needed to maximize ascitic fluid production. However, in the concentration of F VIII/vWF available (100-200 μg/ml), a 9:1 ratio did not elicit an antibody response. A 2:1 ratio was required to produce antibody. In this investigation, C57BL/6J and four other strains of mice were used (A/J, A/HeJ, SWR/J and RF/J). The four strains failed to produce adequate

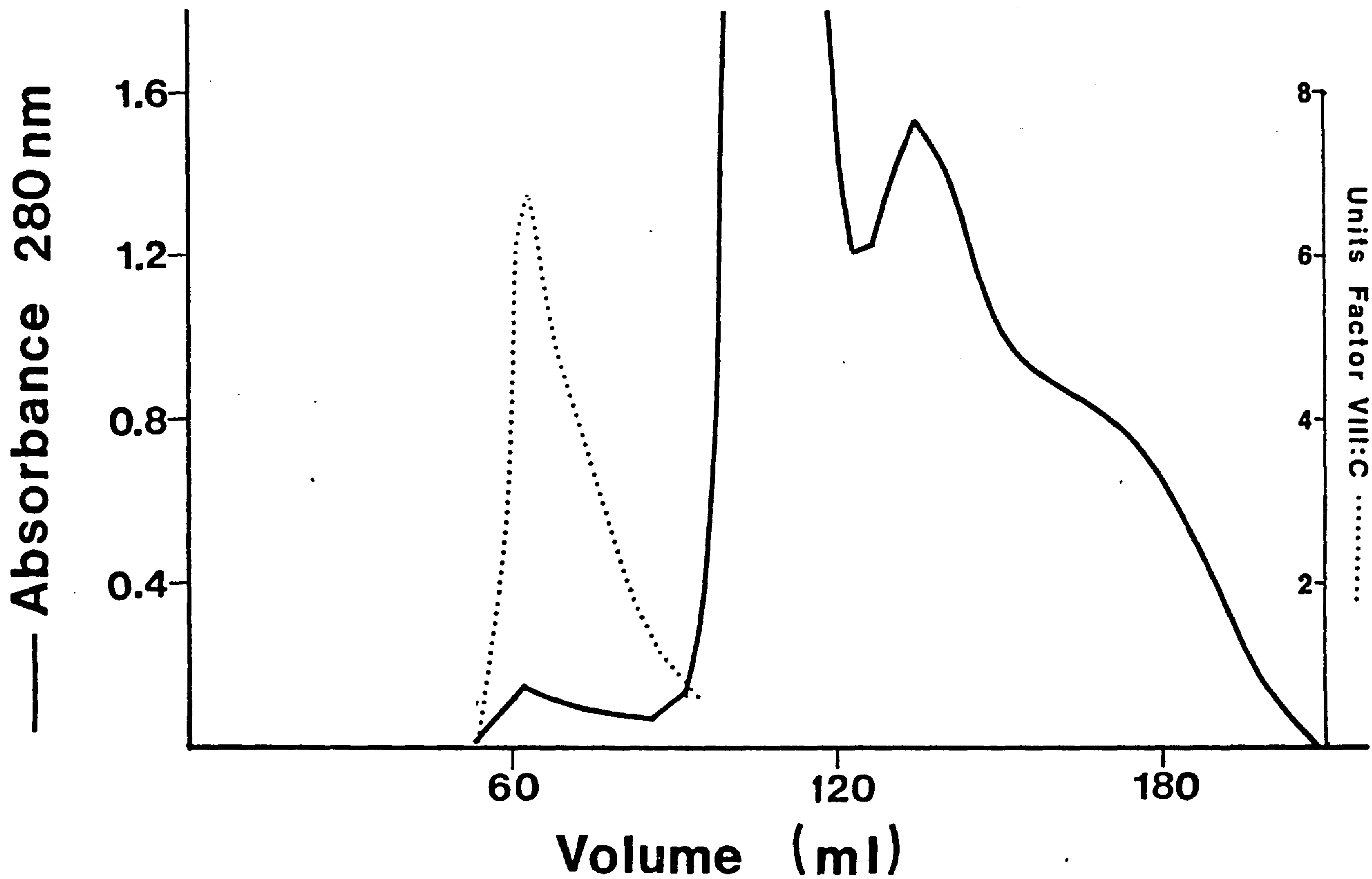


Figure 9 : The Gel Filtration of $Al(OH)_3$ and Arvin Treated Hemofil on a 1.6 X 100 cm Column of Bio Gel A-15M.

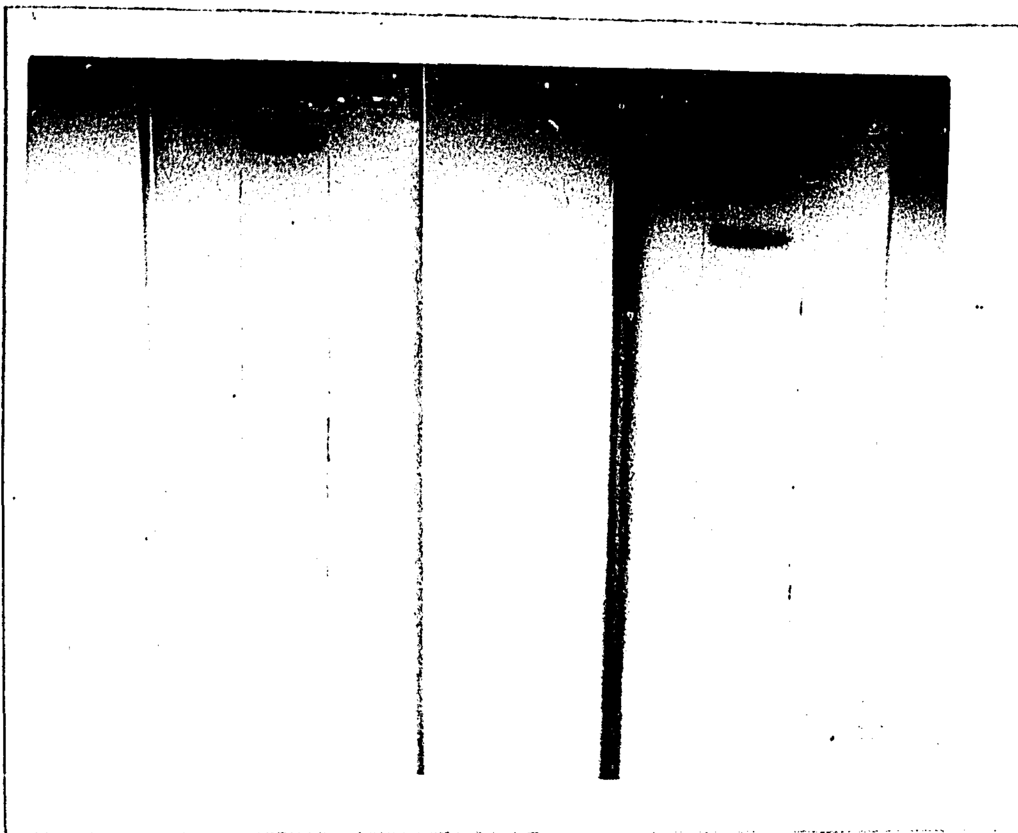


Figure 10 : 5% Polyacrylamide Gel Electrophoresis of F VIII/vWF in the Presence of 1% SDS and 5M Urea. A. Non-reduced. B. Reduced with 0.1M 2-Mercaptoethanol.

volumes of ascitic fluid using an adjuvant to antigen ratio of 2:1. Using a 9:1 ratio, all strains produced larger volumes of ascitic fluid, however, only the C57BL/6J mice produced large volumes of fluid using a 2:1 ratio of adjuvant to antigen.

The quantity of fluid produced by one group of 10 mice is listed in Table 3. The range of fluid was 17-57 ml, and the mean was 33.3 ml. The mortality rate of the C57BL/6J mice varied from 20-50%. This can be kept to a minimum by carefully monitoring the mice during the time they are producing fluid and ensuring that too much fluid does not accumulate by frequent tapping.

Characterization of the Antisera

Immunodiffusion studies of the antifactor VIII/vWF antibody which was isolated by affinity chromatography with immobilized factor VIII/vWF, show that it is an IgG 1 antibody (Fig. 11A, 11B). The identification of the antibody as an IgG 1 is in agreement with previous work. Three reports of monoclonal antifactor VIII/vWF indicate that the monoclonal antibody was predominantly IgG 1 with some being of the IgG 2b class (184-186).

Immunoelectrophoresis of Hemofil followed by diffusion against the antiserum (Fig. 12) yielded two precipitin lines. After incubation of the antiserum with a fraction of normal plasma, only a single precipitin line was evident. Both the absorbed and unabsorbed antisera gave one precipitin line in immunodiffusion studies against Hemofil.

Quantitative Immunoassays

The mouse antiserum was comparable to a commercial rabbit antiserum when used to quantitate Factor VIII related antigen by the method of Laurell (Fig. 13A, B). Fig. 13C shows the use of the mouse antiserum to quantitate factor VIII related antigen eluted from a Bio Gel A-15M

Table 3

Total Quantity of Fluid Produced by One Group of 10 Mice

Mouse	Sex	Total Fluid (ml)
1	M	54
2	M	21.5*
3	M	40
4	M	23.5*
5	F	49
6	F	57
7	M	17*
8	M	23*
9	M	26
10	F	22*

*Indicates that the mouse died prematurely.

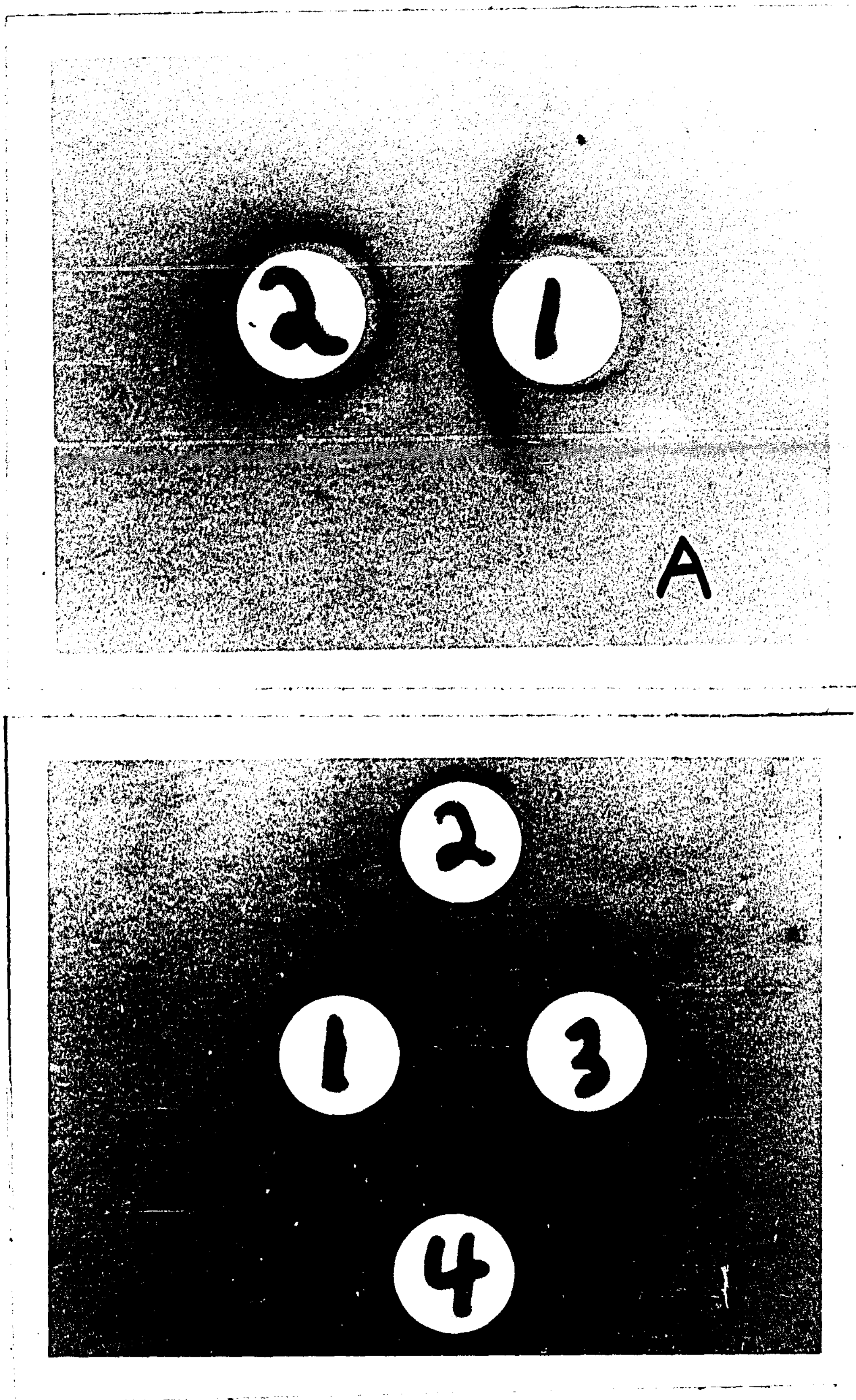


Figure 11 : Immunodiffusion Studies of the Antifactor VIII Antibody. A) Well 1 : Eluate from Immobilized F VIII/vWF Column. Well 2 : Anti Mouse IgG (USB). B) Well 1 : Eluate from Immobilized F VIII/vWF Column. Well 2 : Anti Mouse IgG 1 (Litton). Well 3 : Anti Mouse IgG2a (Litton). Well 4 : Anti Mouse IgG2b (Litton).

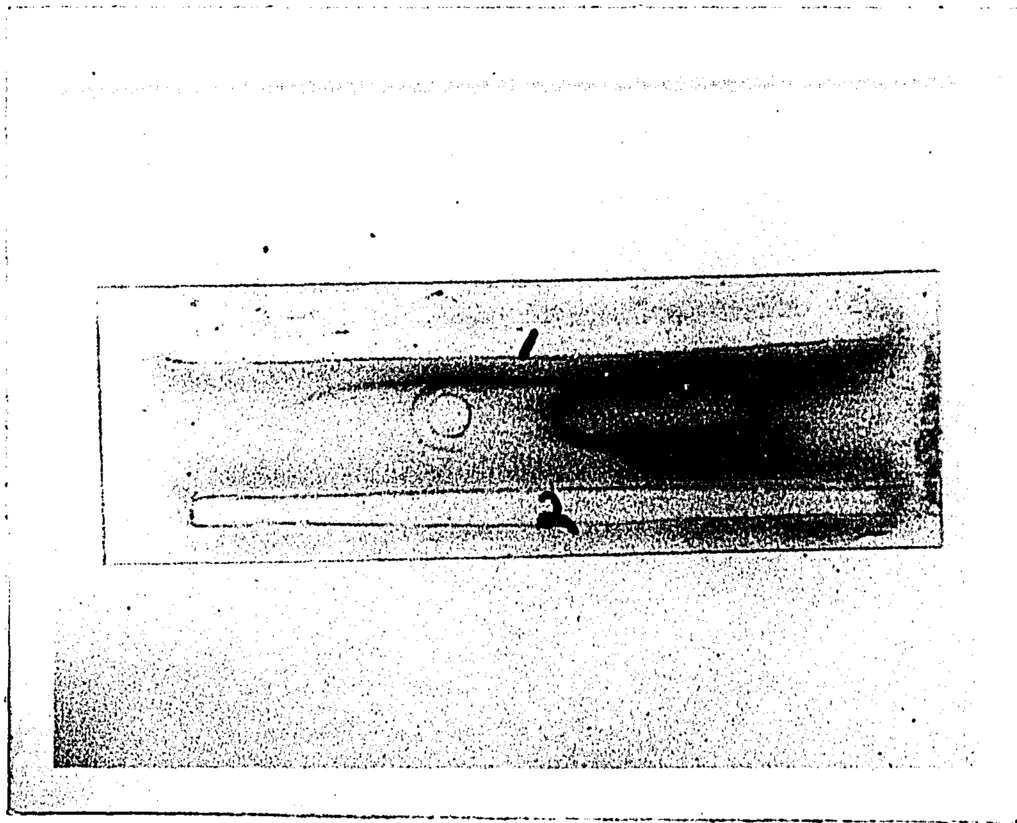
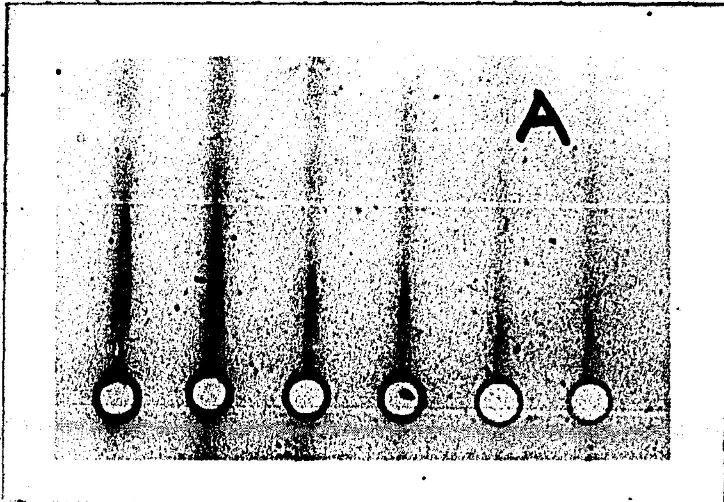
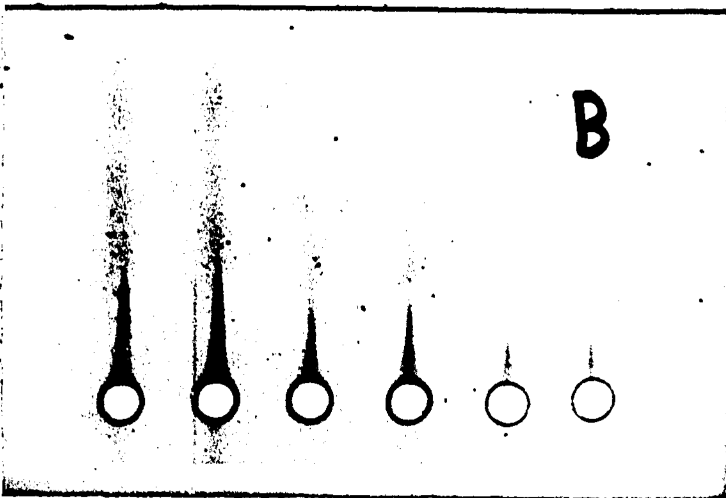


Figure 12 : Immunoelectrophoresis Study of the Mouse Antiserum. Reconstituted Hemofil was Placed in Well. Troughs Contain Mouse Antifactor VIII. 1) Before Absorption. 2) After Absorption.





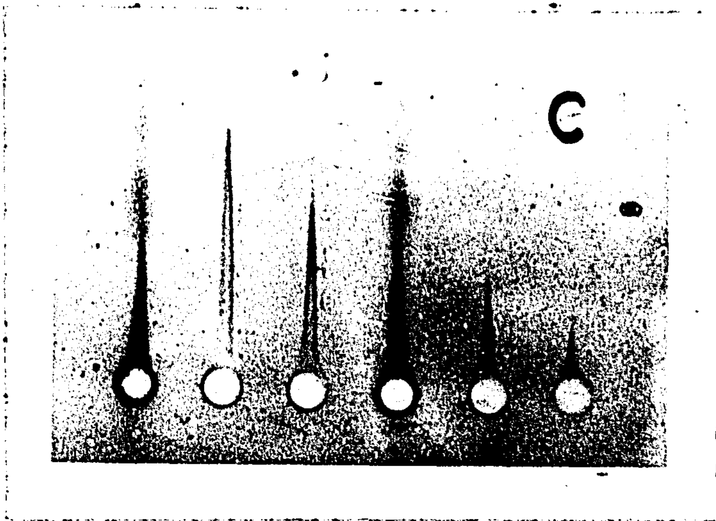


Figure 13 : Laurell Immunoelectrophoresis. A) Using Rabbit Anti F VIII (Calbiochem) at a Concentration of 0.6%. Normal Plasma was Placed in Wells; Duplicate Concentrations of 100%, 50%, and 25% were run. B) Same as A except that Mouse Anti F VIII/vWF at a Concentration of 0.4% was used. C) Analysis of Void Volume Eluate of Bio Gel A-15M Chromatography Using Mouse Anti F VIII/vWF. Wells 1 and 4 are Normal Plasma. Wells 2, 3, 5, and 6 are Serial Dilutions of the Column Eluate.

column. The commercial antiserum was used at a concentration of 0.6%, the mouse antiserum at a concentration of 0.4%. When von Willebrand plasma was subjected to Laurell immunoelectrophoresis using the mouse antiserum, no precipitin rockets developed. This indicates that the antiserum is monospecific.

The standard curve for the enzyme immunoassay (Fig. 14) represents the average of three separate assays run for each plasma dilution. The results for the enzyme immunoassay were comparable to those of Ness and Perkins (177), who used a commercial rabbit antifactor VIII/vWF antiserum. Using the same dilution of antiserum, the normal curve is similar to theirs with the exception that this curve occurs at lower plasma dilutions. The curve can be shifted to lower or higher plasma dilutions by changing the dilution of antiserum used in the assay.

In studying the inhibition of coagulant activity, it was found that the mouse antiserum contained 1-2 Bethesda inhibitor units. Thus, like rabbit antifactor VIII/vWF antisera, this mouse antiserum has a low anticoagulant activity (187-189). Antibodies to factor VIII/vWF produced in BALB/C mice (190) have been reported to have a very high anticoagulant activity. Variations in the inhibitory properties of antisera are not unusual, and occur due to the use of different strains of animals, different preparations of antigen, and varying methods of immunization.

Fluorescence Assay

N-(1-pyrene)maleimide is a non-fluorescent compound which becomes fluorescent after binding to free amino or sulfhydryl groups. In agreement with this, no fluorescence was observed at 390 nm (λex 345 nm) for free N-(1-pyrene)maleimide, however, after conjugation, there was fluorescence. The addition of purified F VIII/vWF or plasma to the

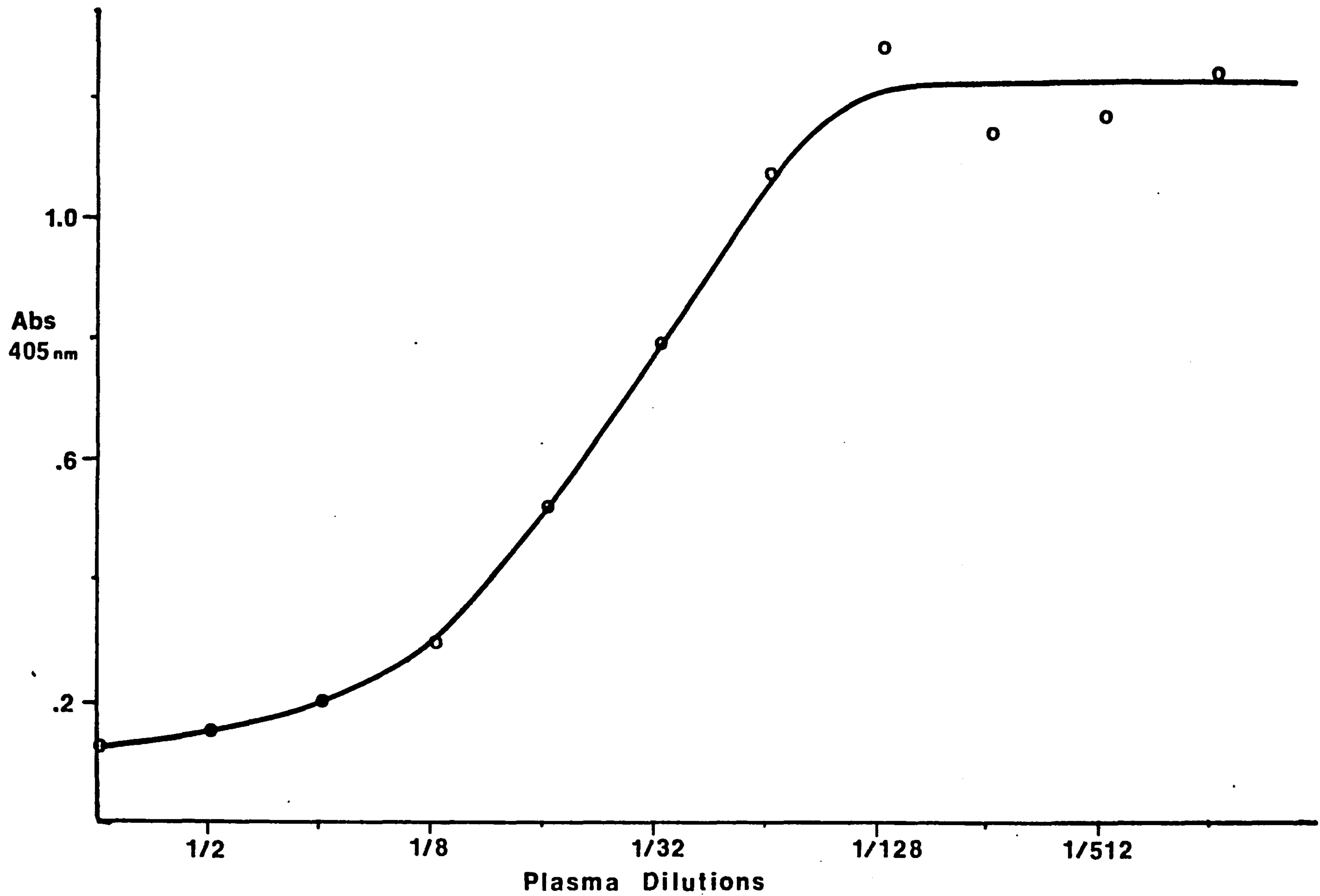


Figure 14 : Curve Derived from the Enzyme Immunoassay of F VIII/vWF Using Normal Plasma.

antisera conjugated to N-(1-pyrene)maleimide failed to produce a fluorescence enhancement. This is in constrast to the results reported by Liburdy (179) who was able to quantitate human IgG by measuring the fluorescence enhancement of the probe bound to antihuman IgG induced by the addition of human IgG. This discrepancy can be explained in several ways. Liburdy used concentrations of antibody and antigen which were one to two orders of magnitude higher than used in this report. It is not clear whether such large concentrations are needed to observe the effect. In a recent report (191), it has been shown that N-(1-pyrene)maleimide can be adsorbed and, upon doing so, it become fluorescent. Perhaps, a large background fluorescence,due to this non-specific adsorption, masked an observable fluorescence enhancement. The amount of anti F VIII/vWF antibody might be very small as compared to the total antibody content of the solution. In this case, a high background fluorescence may also mask the fluorescence enhancement. This assay might work using either a monoclonal antifactor VIII/vWF, or antibody from a hemophiliac who has developed inhibitors (specific for F VIII:C).

Summary

A method is described for the production of antibodies to human F VIII/vWF in the ascitic fluid of C57BL/6J mice. The method is simple, does not require large facilities for the housing of animals and, if one chooses to breed the mice, the cost of producing the antiserum is minimal. The mouse antiserum gave comparable results as compared to rabbit antiserum when used to quantitate factor VIII related antigen by Laurell immunoelectrophoresis and enzyme immunoassay. This method may also be applicable to other proteins available in submilligram quantitites.

III. CIRCULAR DICHROISM STUDIES OF HUMAN F VIII/vWF

A. Introduction

Electronic Transitions in the Peptide Bond

The major source of electronic transitions in proteins is the peptide bond. These transitions and the wavelengths at which they occur are listed in Table 4. The $\pi_2 \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ transitions occur in the vacuum ultraviolet range, and are not observable with conventional UV/visible instruments.

Due to the charge redistribution on the carbon, nitrogen, oxygen, and other atoms of the peptide bond, the group exists as a dipole. When electrons are promoted from the ground to excited states, redistrubition of charge occurs resulting in the change in the magnitude of the dipole. This change can be represented by a transition dipole that has direction. As a result of this change, an oscillating dipole is produced. The probability of transition between the ground and excited states, the oscillator strength, is related to the intensity of the absorption.

Besides considering electronic transitions in isolated peptide bonds, it is also necessary to consider the electronic interactions between adjacent peptide groups. These interactions are from dipole or exciton coupling (resonance coupling). The transition dipoles of adjacent residues can either reinforce or oppose each other, leading to exciton resonance splitting, a decreased or increased energy gap, and a shift in the absorption spectrum.

In the α helix, there are four combinations of the peptide transition dipoles, with the net transition dipoles being either perpendicular or parallel to the helix axis. This results in band splitting where the parallel orientation induces a red shift.

Table 4

Electronic Transitions of the Peptide Bond

And the Wavelengths At Which They Occur (192,193)

Transition	Wavelength (nm)
$\pi_1 \rightarrow \pi^*$	190
$n \rightarrow \pi^*$	220
$\pi_2 \rightarrow \pi^*$	165
$n \rightarrow \sigma^*$	150

Experimentally, a relationship between UV absorption and protein (polypeptide) conformation can be examined using polylysine. At high pH and at 25°C, polylysine is in an α-helical conformation. At neutral pH, it exists as a coil due to the repulsion of the ε-amino groups. At high temperature and at a high pH, polylysine is in an antiparallel β-sheet conformation. For the α-helical conformation, there are absorption maxima at 191 and 209 nm, which are from the $\pi \rightarrow \pi^*$ transitions for the electronic polarizations perpendicular to the helix axis and parallel to the helix axis respectively. For the β-sheet conformation, the maximum is at 196 nm and, for the coiled conformation, the maximum is at 191 nm. These are also due to the peptide bond $\pi \rightarrow \pi^*$ transitions. The very weak absorption bands at 221, 216, and 219 nm from the α-helix, coil, and β-sheet conformation respectively are due to the symmetry forbidden $n \rightarrow \pi^*$ transitions (192,193). A complete list of absorption maxima for the various conformations is given in Table 5.

Circular Dichroism

Circularly polarized light is produced by combining two waves of plane polarized light of equal amplitude and frequency that are one-quarter wavelength out of phase. The vector sum of right and left handed circularly polarized light is plane polarized light describing a sine wave motion along the axis of the light path.

Circularly polarized light is absorbed by optically active compounds in the regions of electronic transition. Passage of right and left handed circularly polarized light through a medium that selectively absorbs one of the components results in the emergence of elliptically polarized light. In this case, the vector sum of the emergent right and left handed circularly polarized light, being of

Table 5

Absorption Maxima for the α-helix, β-sheet, and Coil Conformations (192,193)

Conformation	UV max (nm)	C.D. max (nm)	Polarization	Transition
α-helix	155	-	$\parallel$ to helix axis	$\pi_2 \rightarrow \pi^*$
	191	191	$\perp$ to helix axis	$\pi_1 \rightarrow \pi^*$
	209	208	$\parallel$ to helix axis	$\pi_1 \rightarrow \pi^*$
	221	222	$\perp$ to helix axis	$n \rightarrow \pi^*$
β-sheet	171			$\pi_2 \rightarrow \pi^*$
	196	195		$\pi_1 \rightarrow \pi^*$
	219	217		$n \rightarrow \pi^*$
coil	191	197		$\pi_1 \rightarrow \pi^*$
	216	217		$n \rightarrow \pi^*$

different amplitudes, traces an ellipitical path. The ellipticity per unit length of sample, θ, is defined as $\tan \theta$ = minor axis of ellipse/major axis of ellipse. Circular dichroism, $\Delta\varepsilon$, is defined as $\varepsilon_L - \varepsilon_R$, where ε_L and ε_R are the extinction coefficients for left and right hand circularly polarized light. Circular dichroism measurements are expressed in terms of molar or mean residue ellipticity, $[\theta]$, where $[\theta] = 3300\ \Delta\varepsilon$.

Circular dichroism can be used to examine α-helix, β-sheet, and coil conformations using polylysine as a standard. The α-helix has absorption maxima at 191, 208, and 222 nm. The 191 and 208 nm maxima correspond to the $\pi \rightarrow \pi^*$ transitions for the perpendicular and parallel orientations respectively. The 222 nm band is large in magnitude, and is due to the $n \rightarrow \pi^*$ transition which is symmetry forbidden for linear dichroism but is allowed for circular dichroism.

The β-sheet spectrum has a maximum at 195 nm from the $\pi \rightarrow \pi^*$ transition and a maximum at 217 nm from the $n \rightarrow \pi^*$ transition. The spectrum of the coil form has a maximum at 197 nm from the $\pi \rightarrow \pi^*$ transition and a maximum at 217 nm from the $n \rightarrow \pi^*$ transitions.

Estimation of Protein Structure from Circular Dichroism Spectroscopy

Since each type of protein structure has a distinct CD spectrum, the spectrum for a given protein can be envisioned as being a linear combination of its component spectra. For example, if a protein was composed of 1/3 α-helix, 1/3 β-sheet, and 1/3 coil, its spectrum would be the mathematical average of the three component spectra.

This approach has been used by Greenfield and Fasman (194), who assumed that polylysine is 100% α-helix, 100% β-sheet, or 100% coil under the appropriate conditions. The standard reference ellipticities for each conformation, based upon this assumption, are given in Table 6.

Table 6

Reference Ellipticities for the α-helix, β-sheet, and Random Coil Conformations Using a Polylysine Standard (194)

Ellipticities in deg·cm^3/decimole

Wavelength (nm)	α-helix	β-sheet	Random Coil
190	74,000	22,400	-32,200
191	76,900 ±8,400	25,300	-34,700
192.5	73,300	30,000	-37,500
195	64,300	31,900 ±5,000	-41,000
197	44,300	30,000	-41,900 ±4,000
200	14,300	24,300	-36,400
202	0	19,300	-25,600
205	-25,000	5,700	-14,500
208	-32,600 ±4,000	4,700	-3,400
210	-32,400	-10,800	-1,400
211	-32,100	-12,100	0
214	-31,000	-16,400	3,500
215	-31,400	-17,900	4,100
217	-33,100	-18,400 ±1,800	4,600 ±500
220	-35,300	-15,700	4,400
222	-35,700 ±2,800	-13,800	3,400
225	-32,400	-11,400	2,700
230	-21,900	-6,400	800
234	-11,400	-3,600	0
238	-4,300	-1,400	-140
240	-3,300	700	-150
250	0	0	0

One may compute a CD spectrum for any combination of the three conformations by multiplying the percent conformation by the standard reference ellipticity for each conformation and adding these up for each wavelength. The curve is derived by plotting the computed ellipticities vs. wavelength.

It is a bit more difficult to calculate the fractional content of α-helix, β-sheet, and coil from a given CD spectrum. Greenfield and Fasman have found that the fraction of α-helix can be calculated from the ellipticity at 208 nm. At this wavelength (Table 6), the α-helix has a large ellipticity (-33,000), while the ellipticities of the β-sheet and coil conformation are small and nearly equal (-4,000). If the ellipticity of the protein at 208 nm ($[\theta]_{208}$) equals (F_α x -33,000) + [(F_β + F_c) x -4,000] and $F_\beta + F_c = 1 - F_\alpha$, then it follows that $[\theta]_{208}$ = (F_α x -33,000) + [(1 - F_α) x -4,000) and

$$F_\alpha = \frac{[\theta]_{208} + 4{,}000}{-29{,}000}.$$

Once the fraction of α-helix is estimated, an estimate of the fractional content of β-sheet and coil structure is made by comparing the spectrum under study with calculated CD spectra of varying structural content.

Although the results obtained using this method generally agree with those from x-ray analysis, there are some inherent problems in the use of polylysine as a model. Most important is that the conformations exhibited by polylysine are not exactly representative of those found in proteins. Also, proteins contain other, non-polylysine-like conformations, such as the β-bend.

An alternate method of analysis has been applied by Chen, Yang, and Martinez (195) and revised by Chen, Yang, and Chau (196). This is based upon the concept that the CD of a protein at any given wavelength can be expressed as

$$X = F_{\alpha}X_{\alpha} + F_{\beta}X_{\beta} + F_{R}X_{R}$$

where X is the ellipticity at the given wavelength, the F's are the fractional content of α-helix, β-sheet, and unordered structure, and X_{α}, X_{β}, and X_{R} are the reference ellipticities for each conformation at the given wavelength. Using the CD spectra of five proteins (sperm whale myoglobin, egg white lysozyme, dogfish lactate dehydrogenase, papain from papaya, and bovine pancreatic ribonuclease) with known fractional content of α-helix and β-sheet ($R = 1 - \alpha - \beta$), they were able to calculate the values of X_{α}, X_{β}, and X_{R} at various wavelengths (Table 7). The fractional content of α-helix and β-sheet of a protein of unknown conformation can be calculated by substituting into the equation the experimentally determined ellipticity and the values of X_{α}, X_{β}, and X_{R}. Then, by setting up a series of simultaneous equations (one equation for each wavelength) one may solve for F_{α} and F_{β} ($F_{R} = 1 - F_{\alpha} + F_{\beta}$). The results obtained using this method are usually closer to the x-ray analysis than when polylysine is used as the reference compound.

Table 7

Reference Ellipticities for α-helix, β-sheet, and Random Coil Using 5 Proteins as Standards (196)

Ellipticities in deg•cm^2/decimole

Wavelength (nm)	α-helix	β-sheet	Random Coil
190	70,000	-2,880	-20,300
193	77,000	12,900	-36,000
196	68,200	14,000	-37,900
194	37,200	6,050	-23,200
201	16,800	8,810	-23,300
204	-9,120	300	-11,300
207	-22,300	-4,320	-5,770
210	-26,400	-8,190	-2,200
213	-24,800	-8,680	-850
216	-26,600	-9,210	1,230
219	-28,900	-6,890	1,720
222	-30,000	-3,360	1,580
225	-28,700	1,540	260
228	-24,000	4,390	-480
231	-17,300	4,580	-770
234	-11,300	3,540	160
237	-6,250	2,410	-90
240	-2,950	3,370	-1,040
243	-1,230	2,040	-930

B. Experimental Methods

Factor VIII/vWF was purified as previously described, with the exception that the eluting buffer was 1 mM Tris, 0.15 M NaCl, 0.0025% NaN_3, pH 7.35.

Protein concentration was measured by the microbiuret method as previously described.

Circular dichroism spectra were measured with a Jasco J-20 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at room temperature (25°C). The spectra between 200-250 nm were recorded with protein concentrations between 100-200 μg/ml using a 0.1 cm pathlength cell. Baseline deviations due to buffer were subtracted from all spectra. Spectra were measured at least two times, and the averaged values are reported.

All spectra are reported in terms of mean residue ellipticity $[\theta]$:

$$[\theta] = \frac{\theta_{observed} \times MRW}{10 \times d \times c}$$

where $\theta_{observed}$ is the observed ellipticity in degrees; MRW is the mean residue molecular weight which was determined to be 127 from the amino acid analysis of F VIII/vWF; d is the pathlength in centimeters; and c is the protein concentration in grams per milliliter.

To study the effect of NaCl on the CD changes, solid NaCl was added to the F VIII/vWF solution to reach a final concentration of 0.6 M or 1.0 M. No corrections were made due to dilution (approximately 1.8% at 1.0 M NaCl) because the observed changes were much greater than those due to dilution.

The effect of $CaCl_2$ was studied by adding solid $CaCl_2$ (Sigma, Grade I) to reach a final concentration of 0.1 M or 0.25 M. As with the NaCl, no corrections were made due to dilution (approximately 0.6% at 0.25 M $CaCl_2$).

In the ethylenediaminetetraacetate (EDTA) study, the F VIII/vWF solution was dialyzed against a 100-fold volume of the tris buffer containing a 0.02 M EDTA for two hours at 25°C. The EDTA was removed by dialysis against a 100-fold volume of the tris buffer, changing the buffer three times over a 24-48 hours period.

To study the effect of dithiothreitol (DTT), solid DTT was added to reach final concentration of 3 mM and 5 mM.

The effect of heat on F VIII/vWF was studied by immersing the F VIII/vWF solution in a boiling water bath for 10 minutes.

To see if heparin would change the CD spectrum of F VIII/vWF, appropriate volumes of a 1 mg/ml stock solution of heparin (Sigma, sodium salt, Grade I) were added to yield a final heparin concentration of 1 μg/ml or 5 μg/ml. Since the dilution was between 0.1 to 0.5%, no corrections were made.

To study the effect of phospholipid on F VIII/vWF, rabbit brain cephalin (Sigma) was added in an amount equal to that used in the F VIII procoagulant assay. In this case, the F VIII/vWF solution was diluted by a significant amount (approximately 16%), and corrections were made due to the dilution.

The effect of pH on the CD spectrum of F VIII/vWF was studied by dialyzing the F VIII/vWF solution against Tris buffers of pH 5, 6, 8, and 9.

The secondary structure composition for native F VIII/vWF was determined by the method of Greenfield and Fasman (194). Secondary structure compositions were calculated for each CD spectrum by the method

of Chen, Yang, and Chau (196). The constraint was made that $F_R = 1 - F_\alpha - F_\beta$, and the series of simultaneous equations were solved using a Texas Instruments Model 59 calculator which was programmed as described in the Appendix.

C. Results and Discussion

The CD spectra of native F VIII/vWF and F VIII/vWF in the presence of 0.6 M and 1.0 M NaCl are given in Figure 15. F VIII/vWF exhibits a very shallow spectrum which is indicative of a protein with a small percentage of ordered structure. For reasons of clarity, in these and in all subsequent spectra, the ellipticity axis has been expanded. The error in the ellipticity is ±10%.

Using the Greenfield and Fasman method (194), the α-helix and β-sheet content of native F VIII/vWF was determined to be 5% and 50% respectively. Using the method of Chen, et. al. (196), the α-helix content was calculated to be 12% and the β-sheet content was calculated to be 28% (Table 8). The low helical content is expected from the overall shallowness of the spectrum, and the absence of maxima at 208 nm and 222 nm. The difference in the values for β-sheet content are due to the different standards used and the different methods of determination. In the Greefield and Fasman method, β-sheet content is determined by comparing the spectrum under study to calculated spectra. From this, the β-sheet content can be estimated to the nearest 10%. The large amount of unordered structure or random coil (45-60%) is also expected from the shallowness of the spectrum.

The addition of NaCl (Fig. 15) or $CaCl_2$ (Fig. 16) to F VIII/vWF causes a change in its CD spectrum. This is evident by a reduced ellipticity which is indicative of a decrease in ordered structure. In agreement with this, the calculated α-helix and β-sheet content is reduced in the presence of NaCl and $CaCl_2$ (Table 8).

The conformational changes induced by NaCl and $CaCl_2$ have significance in that, at 1 M NaCl and 0.25 M $CaCl_2$, the F VIII:C dissociates

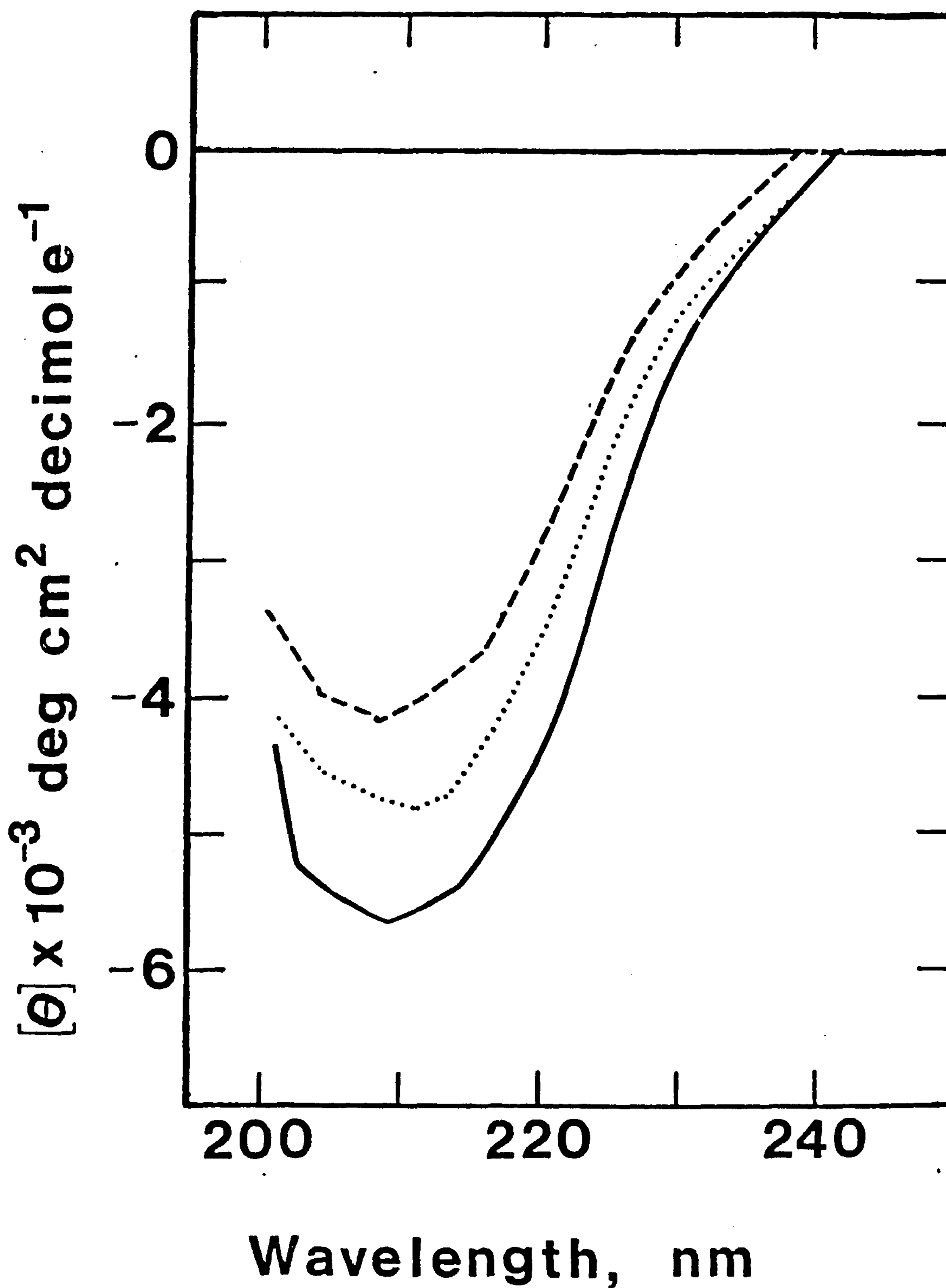


Figure 15 : The CD Spectrum of F VIII/vWF alone ———, and in the Presence of 0.6M NaCl ·····, and 1M NaCl -----.

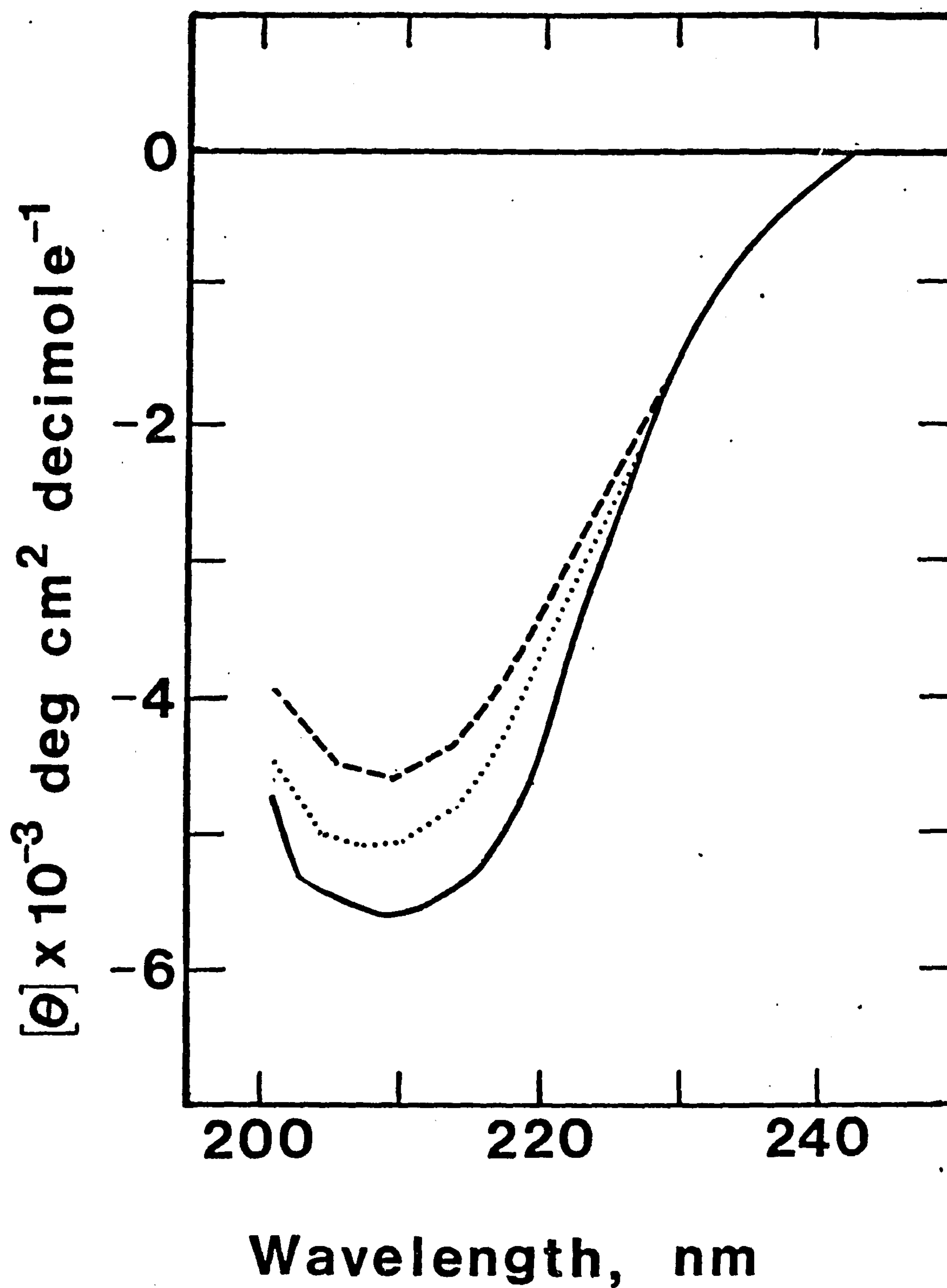


Figure 16 : The CD Spectrum of F VIII/vWF alone ———, and in the Presence of 0.1M $CaCl_2$ ·····, and 0.25M $CaCl_2$ -----.

Table 8

Calculated Secondary Structure Composition for F VIII/vWF

	% α-helix	% β-sheet
native	12	28
.6 M NaCl	9	26
1 M NaCl	7	26
.1 M $CaCl_2$	11	25
.25 M $CaCl_2$	10	23
EDTA	9	23
3 mM DTT	7	20
5 mM DTT	5	15
Boiled	7	27
1 μg/ml Heparin	11	28
5 μg/ml Heparin	10	26

from the vWF. It is not certain whether the conformational change (which may be envisioned as an unfolding) is a prerequisite for the dissociation or whether the dissociation induces the conformation change.

There is some evidence to indicate that calcium is involved in the structure and/or function of F VIII/vWF. EDTA destroys F VIII:C activity, and ^{45}Ca has been shown to bind to F VIII:C. The CD spectrum of EDTA treated F VIII/vWF is markedly different than that of native F VIII/vWF (Fig. 17). This would indicate that calcium is involved in the structural integrity of the molecule. The change is irreversible since the addition of $CaCl_2$ did not alter the spectrum. The retention of vWF activity is dependent upon the ability of the molecule to assemble to a large molecular weight species. Since vWF activity is retained, it is apparent that the conformational change induced by EDTA does not prevent the molecular assembly of vWF.

From the amino acid analysis of F VIII/vWF (134,135), it is evident that there are approximately 120 half-cystine residues per 200,000 subunit. Since there are no free sulfhydryl groups, it can be concluded that there are approximately 60 cystine residues per 200,000 subunit. The change in molecular weight following reduction indicates that there are interchain disulfide bonds. The effect of dithiothreitol (DTT) on the CD spectrum of F VIII/vWF is given in Figure 18. In addition to the decrease in ellipticity, as the concentration of DTT is increased, there is a change in the ellipticity maximum. Sinc the concentration of DTT exceeds that which would be required to reduce the interchain disulfide bonds, the conformational changes observed are likely due to reduction of intrachain disulfide bonds.

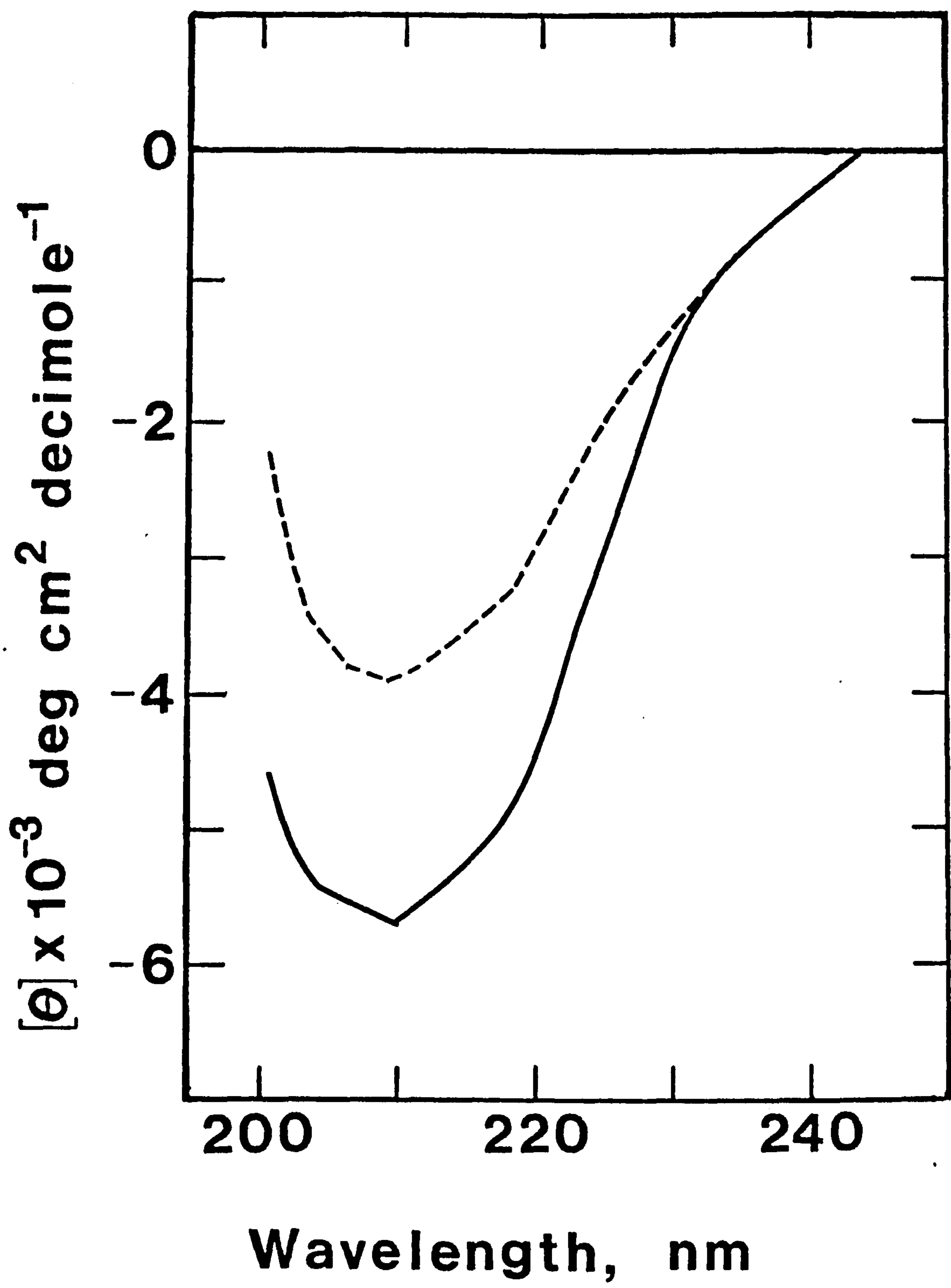


Figure 17 : The CD Spectrum of Native F VIII/vWF ———, and EDTA Treated F VIII/vWF -----.

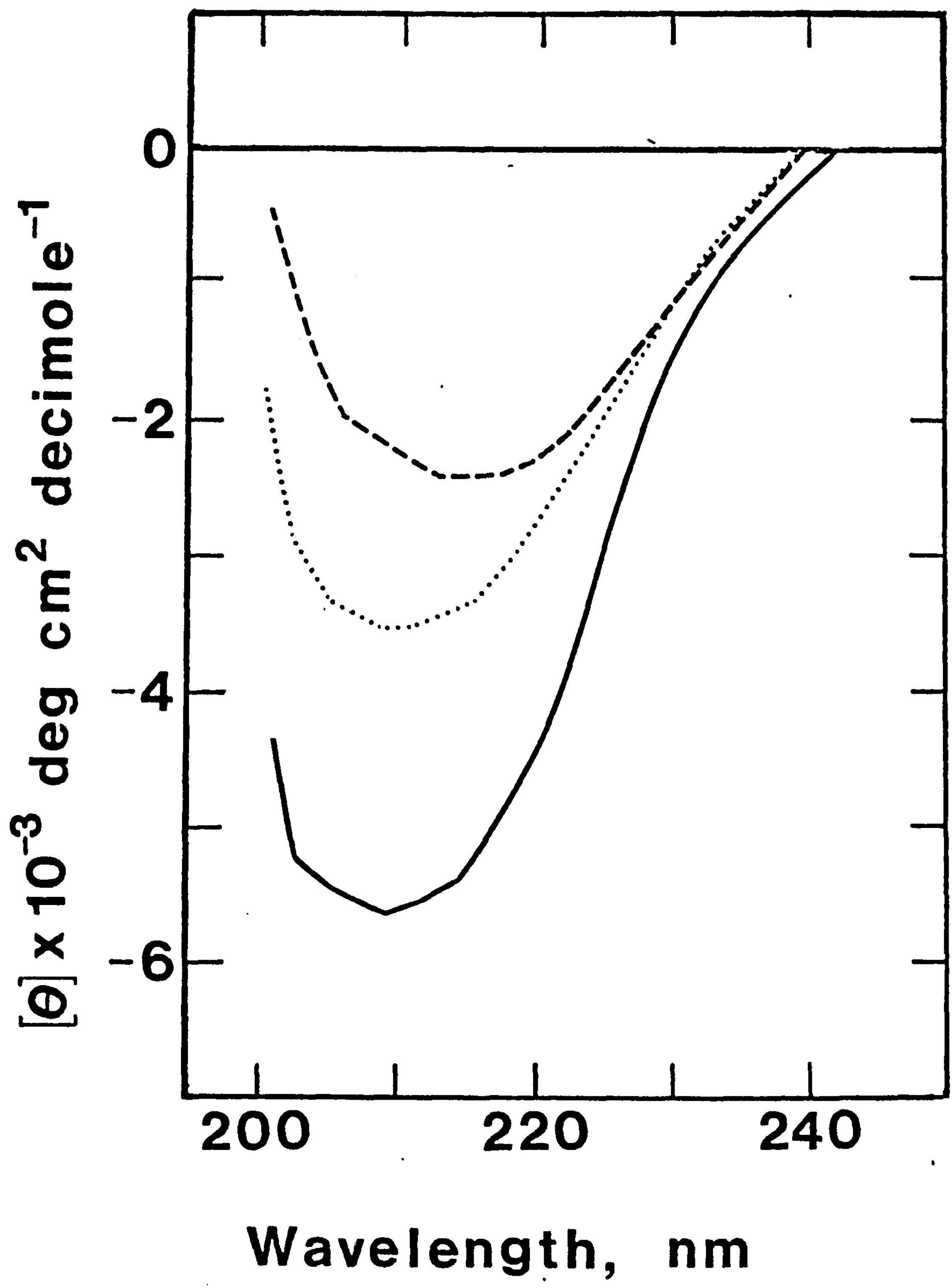


Figure 18 : The CD Spectrum of Native F VIII/vWF ———, and Reduced with 3mM DTT ·····, and 5mM DTT -----.

In an attempt to denature F VIII/vWF, the protein solution was immersed in a boiling water bath for 10 minutes. The CD spectrum of boiled F VIII/vWF is given in Figure 19. Although there is a change in the CD spectrum, the magnitude of the change is small as compared to the changes induced by EDTA and DTT. Although there is a loss of procoagulant activity when boiled, there is almost no change in the factor VIII related antigen. In contrast to this, EDTA causes a larger change (decrease) in the factor VIII related antigen, and factor VIII related antigen is undetectable after the addition of DTT to F VIII/vWF.

The CD spectrum of F VIII/vWF in the presence of heparin is given in Figure 20. Heparin causes a reduced ellipticity, indicative of a conformation change in which there is an increase in unordered structure or an unfolding. Since both heparin and membrane surfaces are polyanionic in nature, the interaction between F VIII/vWF and heparin may be similar to that of heparin and membrane surfaces.

There was no change in the CD spectrum of F VIII/vWF in the pH range of 5-9, nor was there any change in the presence of phospholipids.

Summary

Circular dichroism analysis of F VIII/vWF indicates that it is composed of a small amount of α-helix and a large amount of unordered structure. The structure of the protein is extremely stable as it is resistant to changes in pH and boiling. There are a large number of disulfide bonds which are essential for the structural integrity of F VIII/vWF. The large number of disulfide bonds are very likely responsible for the stability of the protein to pH and boiling. Treatment of F VIII/vWF with EDTA causes an increase in unordered structure, indicating that there may be a structural calcium. The addition of NaCl, $CaCl_2$, and heparin appears to induce an unfolding in F VIII/vWF.

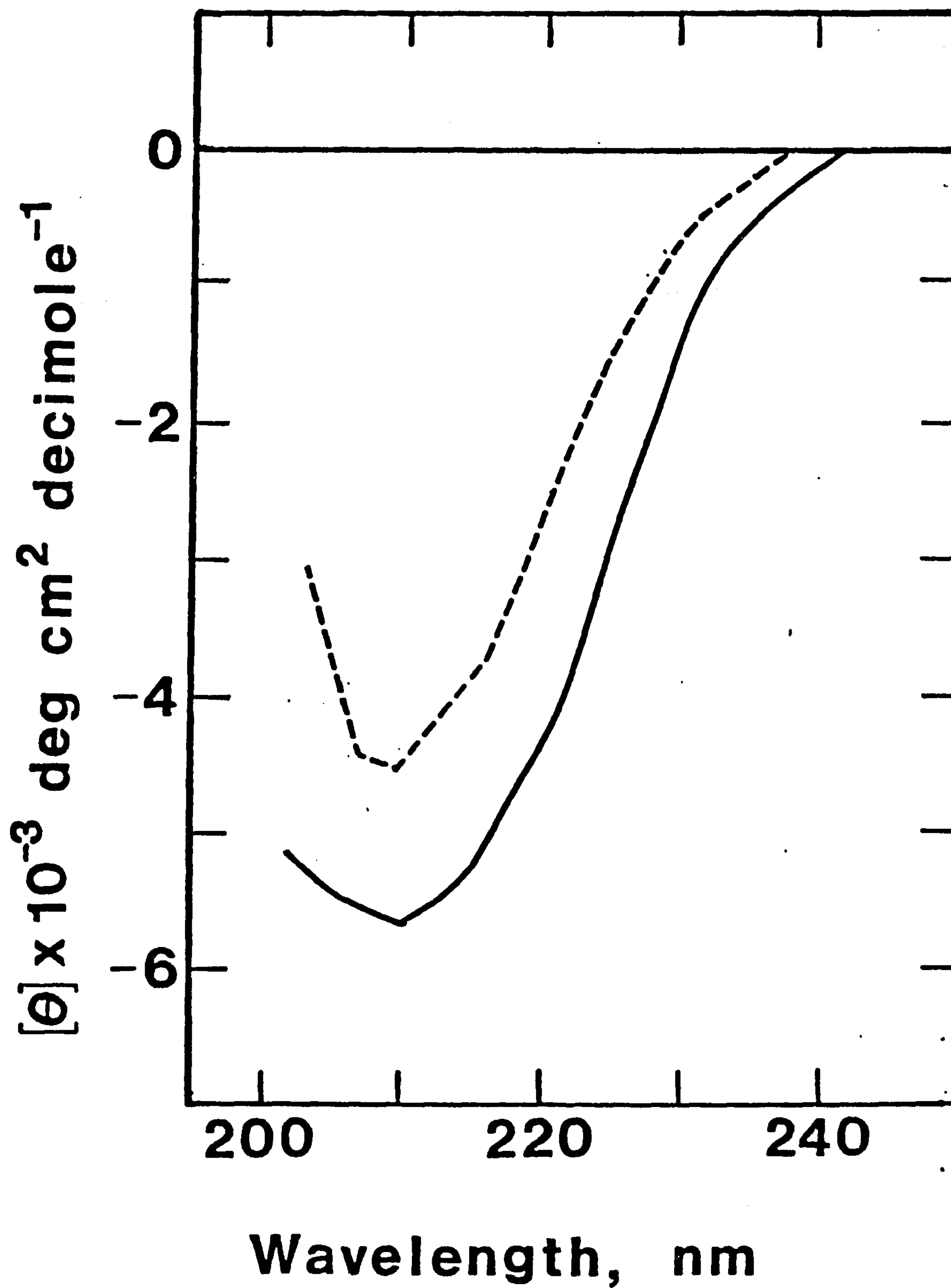


Figure 19 : The CD Spectrum of Native F VIII/vWF ———, and Boiled F VIII/vWF -----.

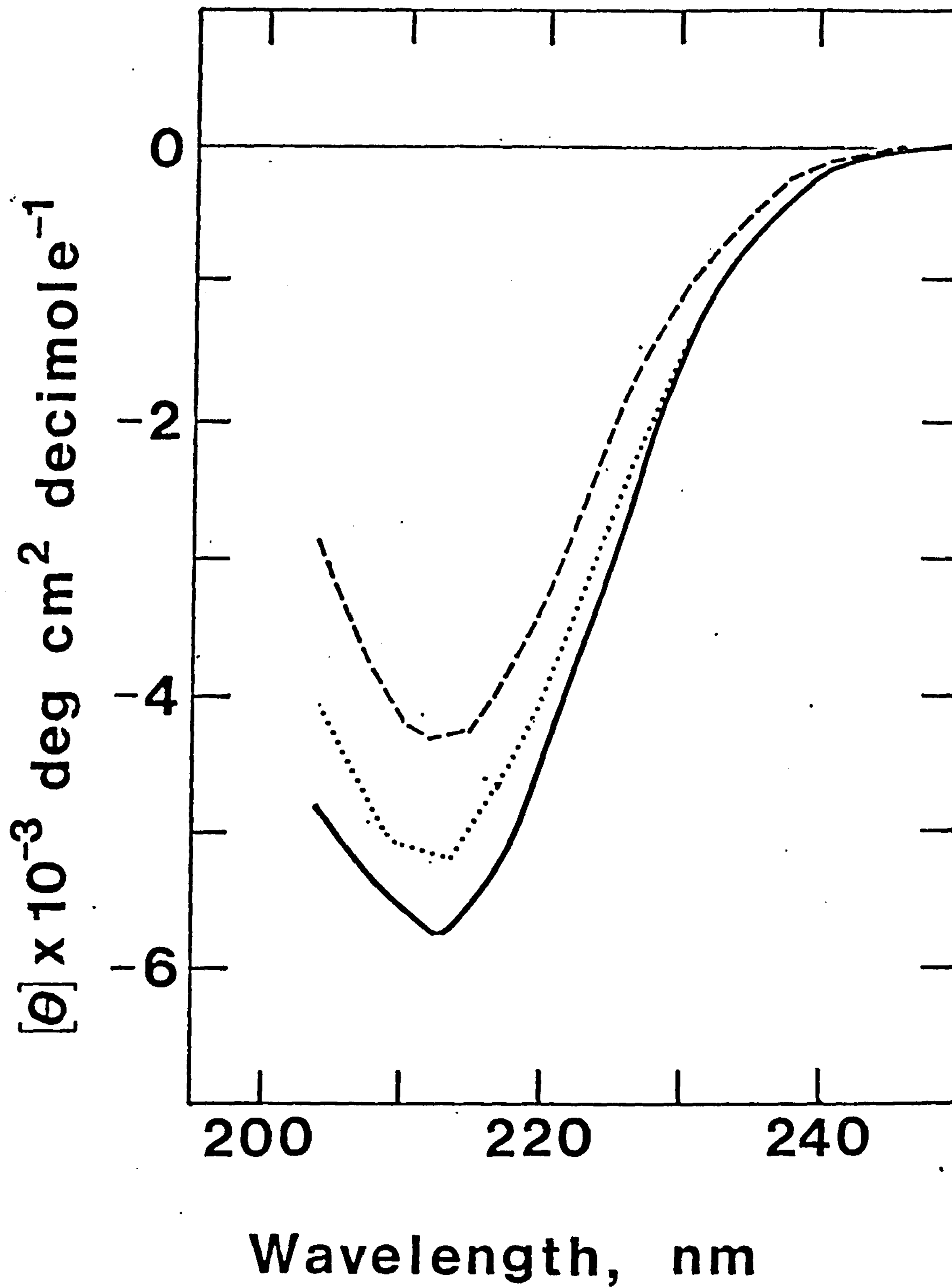


Figure 20 : The CD Spectrum of F VIII/vWF alone ———, and in the Presehce of 1μg/ml Heparin ·····, and 5μg/ml Heparin.---.

IV. THE INTERACTION OF HUMAN F VIII/vWF WITH HEPARIN

A. Introduction

Heparin is a naturally occurring, highly sulfated glycosaminoglycan consisting of repeating disaccharide sequences of α-L-iduronic acid 2-sulfate, and 2-deoxy-2-sulfamino-α-D-glucose 6-sulfate. Other sugars, such as D-glucuronic acid, L-iduronic acid, and 2-acetamido-2-deoxy-glucose, are also present. Due to its polyanionic nature, heparin interacts with cationic molecules. In addition, it has been demonstrated that heparin interacts with some anionic molecules.

The ability of human F VIII/vWF to aggregate platelets in the presence of ristocetin is inhibited by polyanions such as heparin and dextran sulfate (197,198). F VIII/vWF in plasma has been shown to have an affinity for these immobilized polyanions. The degree of affinity and inhibition is directly dependent upon their sulfate content. It seems, therefore, that the interaction is partly electrostatic in nature.

F VIII/vWF is anionic at physiological pH. As evidence for this, F VIII/vWF has been shown to bind to an anionic exchange resin at pH 6.0 and 0.15 M NaCl (199). The mechanism by which F VIII/vWF interacts with heparin is uncertain.

Glycosaminoglycans are a ubiquitous constituent of mammalian cell surfaces (200). In light of this and considering the interaction of F VIII/vWF with heparin (197,198), analysis of the F VIII/vWF-heparin interaction may lead to a better understanding of how F VIII/vWF interacts with cell surfaces.

B. Experimental Methods

F VIII/vWF was purified as described previously with the eluting buffer being 25 mM imidizole, 0.15 M NaCl, 0.025% NaN_3, pH 7.35.

Preparation of Heparin-Agarose Conjugate

3 g of CNBr (Eastman) was added to 30 ml of Sepharose 4B (Pharmacia) in 30 ml of water. The pH was maintained at 11 with 4 M NaOH, and the temperature was kept at 20°C by adding ice. After 12 minutes, a large amount of ice was added, and the suspension was transferred to a sintered glass funnel. It was then washed with 500 ml of cold bicarbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5). After washing, the activated Sepharose was transferred to a beaker containing 150 mg of heparin (Sigma, Grade I) in 30 ml of bicarbonate buffer. Coupling proceded for 16 hours at 4°C with constant stirring. After coupling, an aliquot of the supernatant was removed for analysis, and the resin was washed with 500 ml of bicarbonate buffer. Residual active sites were blocked by adding 30 ml of 1 M ethanolamine pH 9 for two hours at 25°C. The resin was then washed with 500 ml of imidazole buffered saline (IBS) (Imidazole, 0.15 M NaCl, 0.025% NaN_3, pH 7.35) and kept at 25°C. For production of antisera, the 30 ml of resin was packed into a 1.5 x 30 cm column (Pharmacia). For the recovery studies, 10 ml of the resin was packed into a 0.9 x 30 cm column (Pharmacia). For all subsequent studies, 5 ml of the resin was packed into a 0.9 x 15 cm column (Pharmacia).

Assay of Heparin Bound to Agarose

The heparin content of the resin was determined as described by Iverius (201). The aliquot removed from the supernatant after coupling as well as one from a control experiment in which nonactivated Sepharose was incubated with heparin were analyzed for heparin

content (202). The ratio of the absorbances at 520 nm of the supernatant from the activated Sepharose and the control represents the percent of heparin added which remained unbound. In addition, a standard curve using heparin was prepared by which the quantity of unbound heparin was directly determined. The quantity of bound heparin is determined from the total amount of heparin added (150 mg), and the concentration of unbound heparin.

Heparin-Agarose Chromatography of F VIII/vWF

For the production of antisera, a sample of pooled, purified F VIII/vWF (containing 30-50 U of factor VIII procoagulant activity) in IBS was applied to the heparin-agarose column and eluted with IBS until the eluate had no absorbance at 280 nm or until 60 ml had passed through the column. The NaCl concentration of the eluting buffer was increased to 0.3 M and the column was eluted with this buffer until there was no absorbance at 280 nm of the eluate.

For the recovery studies, pooled purified F VIII/vWF (containing approximately 10 U F VIII:C) was applied to the column and eluted with IBS until 20 ml had passed through the column. The NaCl concentration was increased to 0.3 M, and the column was eluted with this buffer until 20 ml had passed through it.

To study the effect of pH, a volume of F VIII/vWF containing approximately 5 U F VIII:C was applied to the column. It was then eluted with either IBS pH 6.35 or IBS pH 8.35 (the columns were equilibrated with the eluting buffer) until 15 ml had passed through the column. The NaCl concentration of the buffer was increased to 0.3 M and the column was eluted with this buffer until 15 ml had passed through it.

The effect of citrate and $CaCl_2$ was determined in the same way as the effect of pH, with the exception that the buffers used were: IBS pH 6.35 + 10 mM citrate, IBS pH 6.35 + 5 mM $CaCl_2$, IBS pH 7.35 + 10 mM citrate, and IBS pH 7.35 + 5 mM $CaCl_2$.

Production of Antisera

The factor VIII which eluted from the heparin-agarose column was pooled, dialyzed against 0.15 M NaCl overnight at 25°C and stored at -20°C until used. Antisera to this material was produced in mice as previously described. All clotting, protein, and immunoassays were performed as previously described.

C. Results and Discussion

The heparin content of the resin was determined to be 1-2 mg of heparin per ml gel. This is approximately 2 to 4 times the amount in commerically available heparin-agarose gels.

When F VIII concentrate is subjected to immunoelectrophoresis using an unadsorbed antiserum, two precipitin lines are evident (Fig. 12). This indicates that the protein solution used to produce the antiserum is not homogeneous. If the heparin-agarose resin would effect a further purification of F VIII/vWF, then an antiserum produced against the heparin-agarose eluate would yield only one precipitin line. In order to recover enough protein needed to produce an antiserum, a large (30 ml) column was needed. The elution profile of this column is given in Figure 21. The F VIII/vWF which was applied to the column, bound in 0.15 M NaCl and was eluted in 0.3 M NaCl. The first protein peak appears to be due to overloading of the column.

Antisera raised against the F VIII/vWF which was eluted from the heparin-agarose column was identical to the antisera raised against the material which was applied to the column (Fig. 22A). The circular dichroism spectrum of the eluted F VIII/vWF was also identical to the F VIII/vWF which was applied to the heparin column (not shown). This indicates that the eluted F VIII/vWF retained its native structure.

Immunoelectrophoresis of F VIII concentrate using the unadsorbed antisera raised against F VIII/vWF before and after the heparin-agarose column (Fig. 22B) shows two precipitin lines. This indicates that no purification was achieved using the heparin column.

To study the recovery of F VIII/vWF activities, a 10 ml column was used. The elution profile of this column is given in Figure 23. When the NaCl concentration is increased to 0.3 M, F VIII:C and F VIII

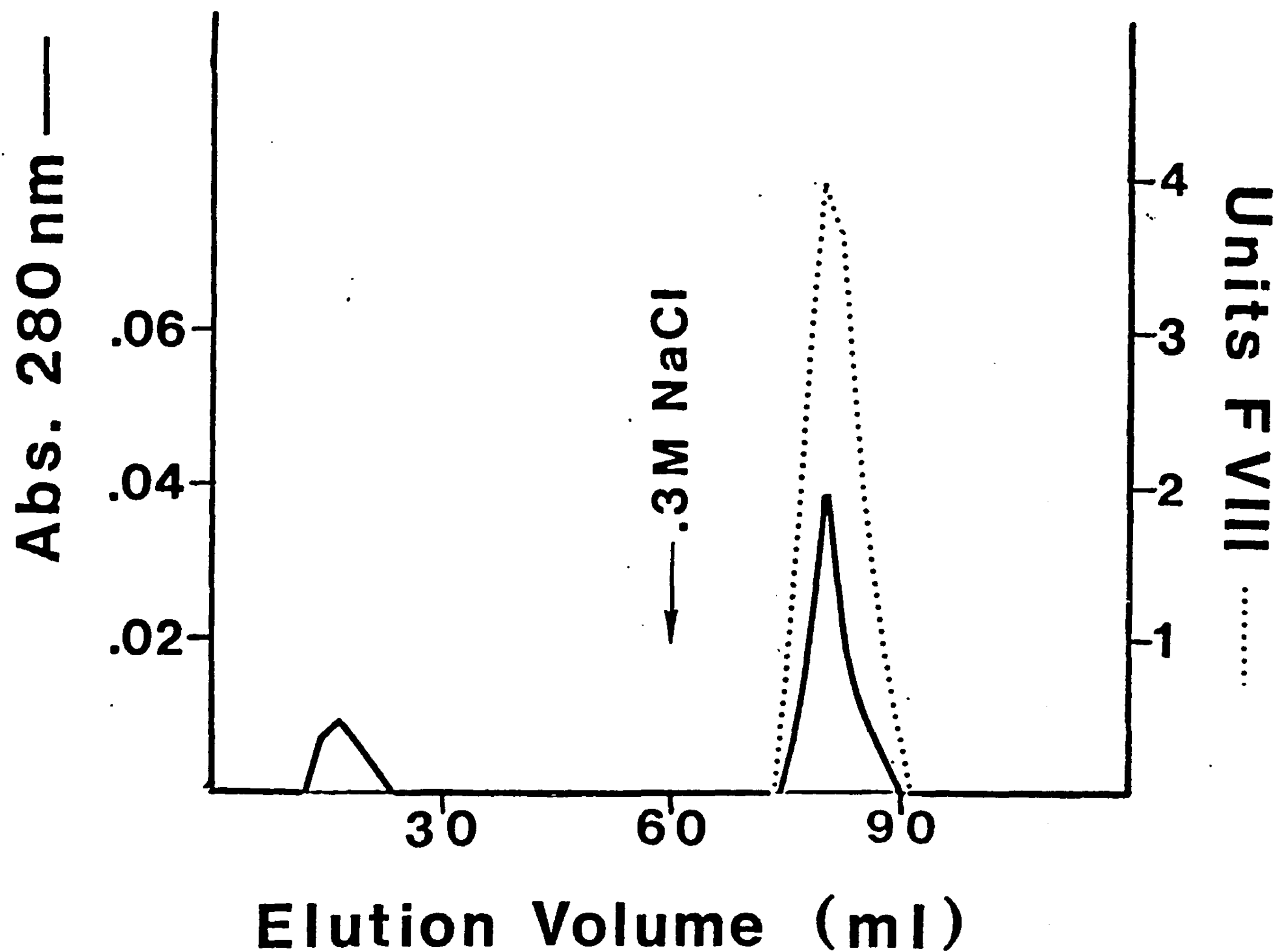


Figure 21 : Elution Profile of F VIII/vWF which was Applied to the Heparin-Agarose Gel. The Eluted Protein was Used to Produce Antiserum. Units of Factor VIII Related Antigen are Shown.

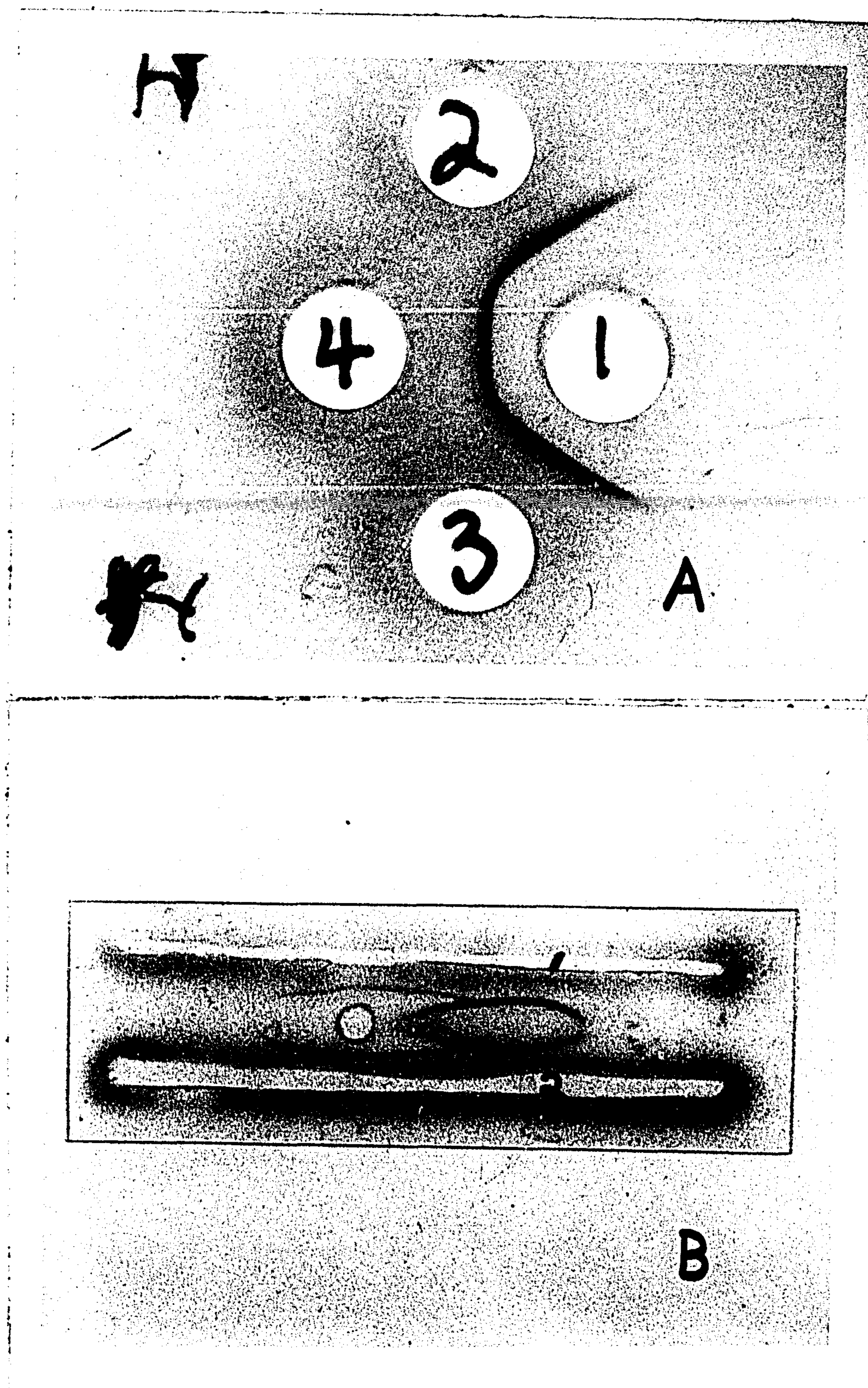


Figure 22 : Comparison of Antisera Produced Against Purified F VIII/vWF and F VIII/vWF which Eluted from the Heparin-Agarose Column. A) Immunodiffusion. Well 1 : Hemofil. Wells 2 and 3 : Antiserum Against Heparin-Agarose Eluate. Well 4 : Antiserum Against Purified F VIII/vWF. B) Immunoelectrophoresis. Well : Hemofil. Troughs Contain Anti F VIII/vWF 1) Against Purified F VIII/vWF, 2) Against Heparin-Agarose Eluate.

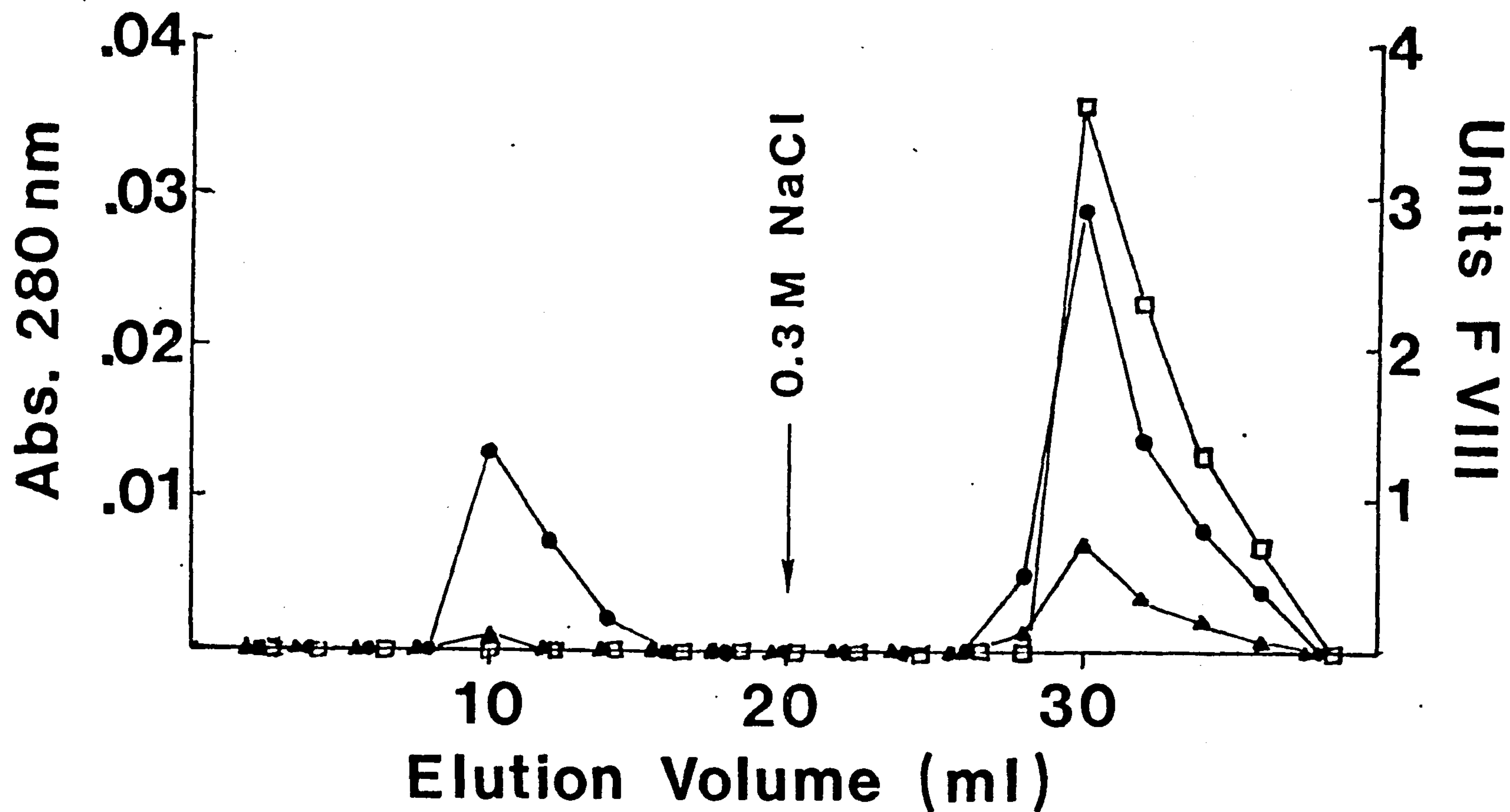


Figure 23 : Elution Profile of F VIII/vWF which was Applied to a 10 ml Heparin-Agarose Column for the Recovery Studies. ● Absorbance at 280 nm. ◘ F VIII Related Antigen. ▲ F VIII Procoagulant Activity.

related antigen coeluted. vWF activity also coeluted, but was not quantitated. Averaged over four runs, 50% of the protein, 50% of the factor VIII related antigen, and 35% of F VIII:C were recovered.

The effect of pH, citrate, and $CaCl_2$ on binding are listed in Table 9. At pH 6.35, binding was the same as that at pH 7.35 and the bound protein was eluted after increasing the NaCl concentration to 0.3 M. In contrast to this, at pH 8.3, most of the F VIII/vWF eluted in 0.15 M NaCl and, when the NaCl concentration was increased to 0.3 M, only a small amount of protein eluted.

At pH 7.35, in the presence of 5 mM $CaCl_2$, all the F VIII/vWF passes through without being retained on the column at 0.15 M NaCl. When the NaCl concentration is increased to 0.3 M, no protein elutes from the column. In the presence of 10 mM citrate, most of the F VIII/vWF passes through at 0.15 M NaCl. At 0.3 M NaCl, the small amount of protein that bound elutes off the column.

At pH 6.35, the addition of 10 mM citrate does not affect binding at 0.15 M NaCl. At 0.3 M NaCl, all the bound F VIII/vWF elutes. In the presence of 5 mM $CaCl_2$, a small amount of F VIII/vWF passes through the column at 0.15 M NaCl, most of it being bound. At 0.3 M NaCl, the bound F VIII/vWF elutes.

The effect of pH on binding indicates that the interaction may be through a postively charged residue on F VIII/vWF. However, it cannot rule out the possibility that the interaction occurs through a calcium linkage (at pH 8.35, hydroxide complexes could form).

If the interaction occurs through a postively charged residue, then this doesn't explain the inhibition of binding by $CaCl_2$ and citrate at pH 7.35. If the interaction occurs through a calcium linkage, then there should be inhibition of binding at pH 6.35 with citrate and $CaCl_2$.

Table 9

The Effect of pH, NaCl, Citrate, and $CaCl_2$ on Binding of F VIII/vWF to Immobilized Heparin

pH	NaCl	Citrate and $CaCl_2$	Binding
6.35	.15 M		+
6.35	.30 M		-
7.35	.15 M		+
7.35	.30 M		-
8.35	.15 M		-
6.35	.15 M	10 mM citrate	+
6.35	.30 M	10 mM citrate	-
6.35	.15 M	5 mM $CaCl_2$	+
6.35	.30 M	5 mM $CaCl_2$	-
7.35	.15 M	10 mM citrate	-
7.35	.15 M	5 mM $CaCl_2$	-

An explanation of the data would be that there is both an interaction through a positively charged residue and a calcium linkage. At pH 7.35, the interaction occurs predominantly via the calcium linkage and, therefore, there is inhibition by citrate and calcium. At pH 6.35, the interaction occurs predominantly through the positively charged residue and, therefore, there is no inhibition by citrate and calcium.

In addition, it is likely that the two types of interactions occur at different sites on the heparin. Inhibition of binding by exogenous calcium is due to the exogenous calcium occupying a "calcium binding site" on heparin. To add credence to this, in a recent report (203), it was found that calcium binding to heparin was in excess of theoretical predictions, suggestion a localized or specific interaction.

Summary

F VIII/vWF binds to immobilized heparin at physiological pH and ionic strength. When the ionic strength is increased, F VIII/vWF is eluted retaining all its biological activities.

The interaction between F VIII/vWF and heparin is supported by the change in the CD spectrum of F VIII/vWF in the presence of heparin (Fig. 20).

The effect of pH, citrate, and calcium on the binding suggests that it occurs by two independent mechanisms: Through a positively charged residue on F VIII/vWF and through a calcium linkage.

V. THE USE OF TERBIUM EMISSION AS A PROBE OF CALCIUM BINDING SITES IN F VIII/vWF

A. Introduction

If a protein contains calcium as a structural or functional entity, the investigation of the calcium is difficult due to a lack of suitable physical techniques. Calcium cannot be studied by conventional optical absorption and emission spectroscopy or magnetic resonance techniques (204,205).

Lanthanide ions compete with calcium ions for binding sites on proteins. Lanthanide ions have the same coordination numbers as calcium ions, and their ionic radii are similar to that of calcium. Therefore, if the ion size is of significant importance in determining binding, then lanthanide ions should be able to substitute for calcium ions.

Most of the lanthanide ions contain unpaired f electrons enabling the use of nuclear magnetic resonance and electron spin resonance methods to probe their environment. The use of lanthanide ion emission spectra as a probe is limited due to the weak absorptivities of lanthanide ion transitions in the visible and the near UV regions. Excitation of lanthanide ions requires either a laser or concentrations in excess of 10^{-2} M.

When bound to proteins, terbium ion luminescence may be excited by energy transfer from an aromatic residue. This indirect method of excitation and subsequent emission involves the absorption by an aromatic residue, a non-radiative transfer of energy to a nearby terbium ion, and an enhanced (10^5) terbium ion phosphorescence in the 535-555 nm region. The energy transfer probably occurs by a Förster dipole-dipole resonance transfer mechanism with a $1/r^6$

dependence on the distance between the donor and acceptor.

If a protein solution containing Tb^{+3} is excited at 285 nM and there is emission at 545 nm, then this indicates that the Tb^{+3} is bound to the protein in close proximity to a tryptophan residue. Scatchard analysis of the data will yield the number of bound Tb^{+3} and the binding constant.

B. Experimental Methods

F VIII/vWF was purified as previously described with the exception that the eluting buffer in the Bio Gel A-15M step was 25 mM imidazole, 0.15 M NaCl, pH 7.0 (containing no NaN_3).

Fluorescence measurements were made with a Perkin-Elmer MPF-2A spectrofluorometer at 25°C. Emission spectra were recorded with an excitation wavelength of 285 nm and 10 nm slit widths.

Solutions of terbium(III) chloride hexahydrate (Aldrich) in imidazole buffer were made just before use in the concentration range 10^{-4} to 10^{-2} M. A volume of the $TbCl_3$ solution was added to the F VIII/vWF solution to reach a final concentration of 10^{-7} to 10^{-5} M $TbCl_3$ (a 1-to-100 fold molar excess of $TbCl_3$). Alternately, the F VIII/vWF solution was dialyzed against the imidazole buffer containing 20 mM EDTA and 10 mM $TbCl_3$ (a 10^5 M excess) overnight and then against the imidazole buffer for 48 hours.

C. Results and Discussion

Fig. 24 is the emission spectrum of F VIII/vWF in the 260-600 nm region. The peak at 285 nm is the scattering peak and, at 570 nm, is the second order scattering peak. The intrinsic tryptophan fluorescence has a peak at 335 nm and a shoulder at 365 nm.

The addition of Tb^{+3} at a concentration of 10^{-5} M causes no change in the emission spectrum in the 530-560 nm region. The only noticeable changes in the emission spectrum are an increase in the scattering and second order scattering peaks. Dialysis of F VIII/vWF against 20 mM EDTA and 10 mM $TbCl_3$ also resulted in no change in the 530-560 nm region in the spectrum, but an increase in the scattering peaks was observed.

It can be concluded that, if there are calcium binding sites on F VIII/vWF, either Tb^{+3} cannot replace the Ca^{+2} or, if it does, the sites are not in close proximity to a tryptophan residue and the presence of the Tb^{+3} is not detectable. The increase in the intensity of the scattering peaks can be attributed to protein aggregation. Turbidity due to terbium can be ruled out because the increase is not seen when $TbCl_3$ is added to buffer alone.

This is the first report of an intrinsic fluorescence spectrum for F VIII/vWF. Since the intrinsic fluorescence is dependent upon the environment of the tryptophan residues, this can be used to study conformational changes in the protein.

The intrinsic fluorescence peak is actually a peak and a shoulder. The two maxima are shifted above and below the wavelength at which free tryptophan fluoresces (around 350 nm). The λ_{max} of the tryptophan fluorescence spectrum shifts to shorter wavelengths and the intensity of λ_{max} increases as the polarity of the tryptophan environment

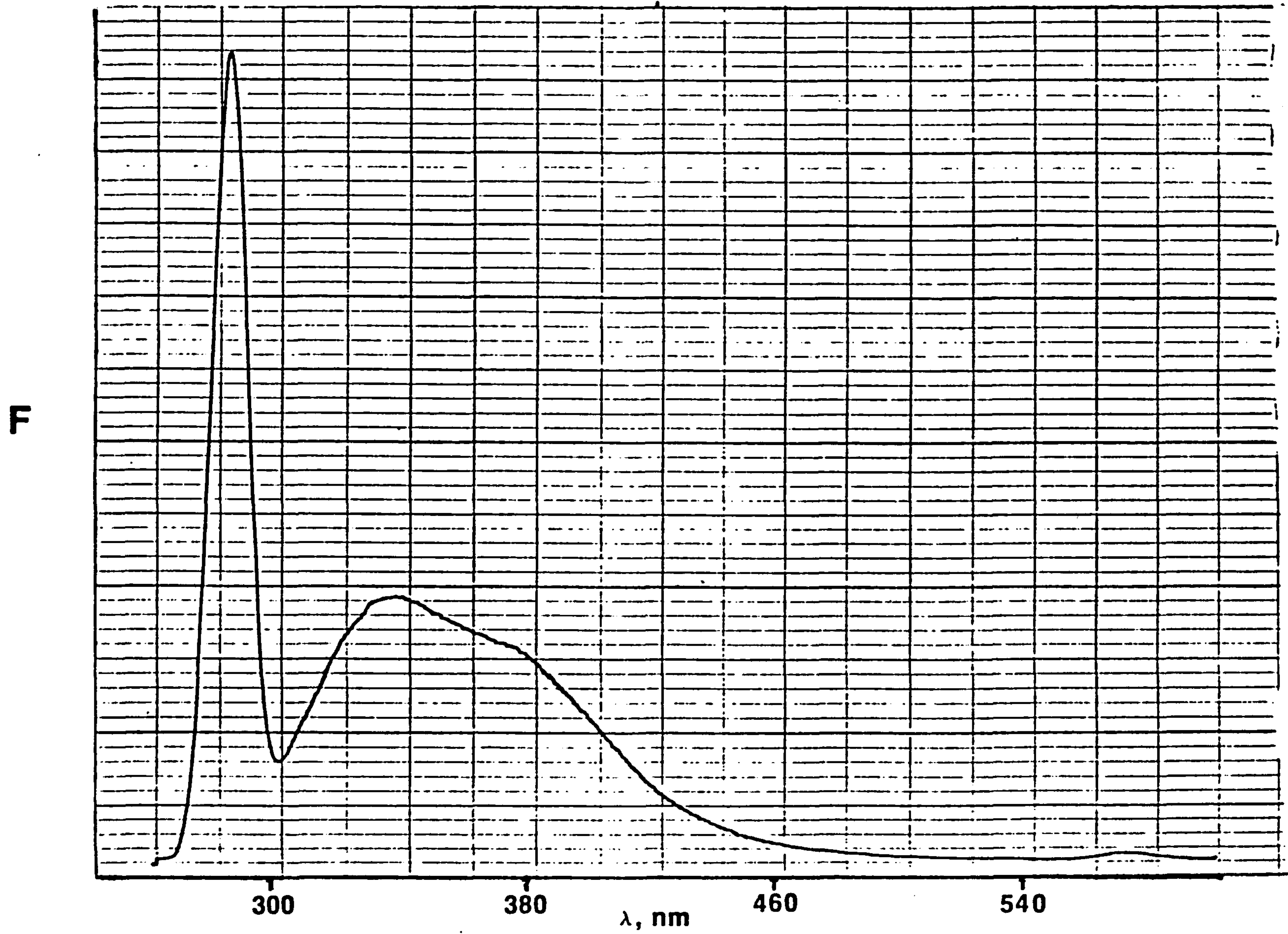


Figure 24 : The Fluorescence Emission Spectrum of Human F VIII/vWF when Excited at 285 nm.

decreases (206). One interpretation of the intrinsic fluorescence spectrum is that the tryptophan residues are in two types of environments, one being less polar than the other. One environment could be on the surface of the protein, the other in a hydrophobic area in the protein.

Summary

The use of terbium emission is not an effective method to probe possible calcium binding sites in F VIII/vWF. If there are calcium binding sites, either terbium cannot replace calcium or, if it does, the bound terbium is not detectable by fluorescence.

Also reported here is the intrinsic fluorescence of F VIII/vWF. This may prove to be a useful property for studying conformation changes in F VIII/vWF.

VI. CONCLUSIONS

Although much work has been done on the characterization of F VIII/vWF, the biochemical nature of the F VIII:C and the vWF has yet to be determined. Furthermore, little is understood about the nature of the interaction between the F VIII:C and the vWF.

The vWF appears to be a stable entity containing many disulfide bonds. The CD data supports this, with the protein's structure being resistant to changes in pH and temperature, and susceptible to reduction with DTT. Since the F VIII:C dissociates from the vWF in 1M NaCl and 0.25M $CaCl_2$, the interaction is most likely electrostatic in nature. The CD results indicate that with the dissociation, there is a concomitant structural change in F VIII/vWF.

The possible role of calcium as a structural or functional entity is evident in the loss of activity of the F VIII:C when exposed to EDTA, and the binding of ^{45}Ca to the F VIII:C. The change in the CD spectrum of EDTA treated F VIII/vWF and the effect of citrate on the binding of F VIII/vWF to heparin (at pH 7.35) supports the notion that calcium plays a structural or functional role in F VIII/vWF. However, the loss of activity with EDTA and the binding of ^{45}Ca involve the F VIII:C, whereas the CD changes and the interaction with heparin most likely involve the vWF.

A structural or functional role for calcium in the vWF is in contrast to the findings that EDTA does not inhibit platelet aggregation (121), nor does it alter the binding of ^{125}I F VIII/vWF to platelets (149). EGTA did inhibit binding, however, this inhibition

was thought to be due to some property of the molecule other than its chelating ability. It seems likely then, that the interaction between F VIII/vWF and the platelet receptor does not involve calcium, but the calcium may be involved in the non-specific interaction of F VIII/vWF with negatively charged surfaces. When a suitable method is developed to separate the F VIII:C from the vWF in large quantities, then one will be able to measure the change in the CD spectrum of each component in the presence of perturbating agents. Then one will know whether the changes observed for the F VIII/vWF are due to changes in the F VIII:C, the vWF, or both.

Since the existence of calcium on the vWF is questionable, the calcium dependent interaction between F VIII/vWF and heparin may occur via the F VIII:C. If this is the case, it may prove to be useful in the separation of the F VIII:C from the vWF.

From the pH dependence on the interaction, it would appear that the positively charged residue involved in the interaction is a histidine residue. Identification of this residue requires modification of specific residues with the concomitant loss in binding to heparin.

That there is an interaction between F VIII/vWF and heparin has its own significance due to the ubiquitous use of heparin as an anticoagulant. The secondary effects of heparin (binding to F VIII/vWF and inhibiting platelet aggregation) can cause undue bleeding.

The antibodies produced in mice were used to quantitate F VIII related antigen in all subsequent work. The antibodies also proved to be useful for qualitative purposes (determining which fractions of

column eluates contain F VIII related antigen; and the extent of purity achieved by the heparin-agarose column).

Approximately 20% of all hemophiliacs receiving factor VIII concentrates develop antibodies to F VIII:C. Using the method to produce antibodies in mice, one may be able to develop a large colony of mice producing antibodies to F VIII:C or F VIII/vWF. This then may be used as a model in an attempt to specifically inhibit the antibody production.

Appendix

TITLE C.D. Analysis PAGE 1 OF 7

PROGRAMMER Mark Bobrow DATE 12/4/81

Partitioning (Op 17) 5.5.9 4.9 Library Module Master Printer ____ Cards ____

PROGRAM DESCRIPTION

To determine the fractional content of α helix, β sheet, and random coil of proteins from their far ultraviolet circular dichroism spectra (200-250 nm).

USER INSTRUCTIONS

STEP	PROCEDURE	ENTER	PRESS	DISPLAY
1	Reset	RST		
2	Enter λ_1	λ_1	R/S	λ_1
	207-231, 207-222, 216-231			
	Enter λ_2	λ_2	R/S	1
3	Enter $[\theta]_1$ as Σ 207, 210, 213	$[\theta]_1$	R/S	$[\theta]_1$
	$[\theta]_2$ as Σ 216, 219, 222	$[\theta]_2$	R/S	$[\theta]_2$
	$[\theta]_3$ as Σ 225, 228, 231	$[\theta]_3$	R/S	$[\theta]_3$
	or (207,210; 213,216; 219,222)			
	or (216,219; 222,225; 228,231)			
4	For α helix		A	Fraction α
	For β sheet		B	Fraction β
	For random coil		C	Fraction R

USER DEFINED KEYS

- A For α helix
- B For β sheet
- C For Rndm Coil
- D R=1-α-β
- E Other matrices
- A'
- B'
- C'
- D'
- E' Combined D & E

DATA REGISTERS

REGISTERS 01 - 22 FOR MATRIX LIBRARY PROGRAM

LABELS (Op 08)

FLAGS 0: X, 1: X, 2: X

TITLE C.D. Analysis PAGE 2 OF 7

PROGRAMMER ______ DATE ______

Partitioning (Op 17) ______ Library Module ______ Printer ______ Cards ______

PROGRAM DESCRIPTION

USER INSTRUCTIONS

STEP	PROCEDURE	ENTER	PRESS	DISPLAY
5	To set the constraint $R=1-\alpha-\beta$			
	Reset		RST	
	To set		D	2
	Go to steps 2-4			
6	To enter any other λ		RST	
	To set		E	3
	Set up as :			
	[A] a d g	a	R/S	a
	[B] b e h	b	R/S	b
	[C] c f i	↓		
		i	R/S	1
		[A]	R/S	[A]
		[B]	R/S	[B]
		[C]	R/S	[C]
	Go to step 4			

USER DEFINED KEYS: A, B, C, D, E, A', B', C', D', E'

DATA REGISTERS

Register	Value	Register	Value
23	-22300.	30	-24000.
24	[illegible]	31	-17300.
25	[illegible]	32	-2320.
26	[illegible]	33	-2150.
27	-38??0.	34	[illegible]
28	-30010.	35	-4210.
29	-29750.	36	[illegible]
		37	-3350.
		38	1540.
		39	4390.

LABELS (Op 08)

FLAGS 0 1 2 3 4 5 6 7 8 9

TITLE C.D. Analysis PAGE 3 OF 7

PROGRAMMER ______ DATE ______

Partitioning (Op 17) ______ Library Module ______ Printer ______ Cards ______

PROGRAM DESCRIPTION

USER INSTRUCTIONS

STEP	PROCEDURE	ENTER	PRESS			DISPLAY
7	To enter any other λ and use the constraint $R = 1 - \alpha - \beta$ Enter [A], [B], [C] and a – i as in step 6		RST E′			 2

USER DEFINED KEYS	DATA REGISTERS	LABELS (Op 08)
A	0	
B	1	
C	2	
D	3	
E	4	
A′	5	
B′	6	
C′	7	
D′	8	
E′	9	

FLAGS	0	1	2	3	4	5	6	7	8	9

TITLE C.D. Analysis PAGE 4 OF 7

PROGRAMMER ______ DATE ______

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005	[illegible]	-	
006	[illegible]	RCL	
007	[illegible]	[illegible]	
008	95	=	
009	[illegible]	[illegible]	
010	[illegible]	[illegible]	
011	[illegible]	[illegible]	
012	[illegible]	[illegible]	
013	[illegible]	[illegible]	
014	[illegible]	[illegible]	
015	42	STO	
016	07	[illegible]	
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018	[illegible]	[illegible]	
019	[illegible]	[illegible]	
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023	[illegible]	[illegible]	
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027	[illegible]	RCL	
028	[illegible]	[illegible]	
029	[illegible]	-	
030	[illegible]	RCL	
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036	[illegible]	[illegible]	
037	[illegible]	+	
038	[illegible]	RCL	
039	[illegible]	[illegible]	
040	[illegible]	+	
041	[illegible]	RCL	
042	[illegible]	[illegible]	
043	[illegible]	=	
044	[illegible]	STO	
045	[illegible]	[illegible]	
046	[illegible]	RCL	
047	[illegible]	[illegible]	
048	[illegible]	+	
049	[illegible]	RCL	
050	[illegible]	[illegible]	
051	[illegible]	-	
052	[illegible]	RCL	
053	[illegible]	[illegible]	
054	[illegible]	=	
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059	85	+	
060	43	RCL	
061	33	33	
062	85	+	
063	43	RCL	
064	34	34	
065	95	=	
066	42	STO	
067	11	11	
068	43	RCL	
069	35	35	
070	85	+	
071	43	RCL	
072	36	36	
073	85	+	
074	43	RCL	
075	37	37	
076	95	=	
077	42	STO	
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081	85	+	
082	43	RCL	
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085	43	RCL	
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087	95	=	
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093	43	RCL	
094	42	42	
095	85	+	
096	43	RCL	
097	43	43	
098	95	=	
099	42	STO	
100	14	14	
101	43	RCL	
102	44	44	
103	85	+	
104	43	RCL	
105	45	45	
106	85	+	
107	43	RCL	
108	46	46	
109	95	=	
110	42	STO	
111	15	15	
112	43	RCL	
113	47	47	
114	85	+	
115	43	RCL	
116	48	48	
117	85	+	
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120	95	=	
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141	43	RCL	
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143	95	=	
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145	08	08	
146	43	RCL	
147	25	25	
148	85	+	
149	43	RCL	
150	26	26	
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152	42	STO	
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154	43	RCL	
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159	95	=	

MERGED CODES

62 63 64 72 73 74 83 84 92

TITLE C.D. Analysis PAGE 5 OF 7

PROGRAMMER DATE

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172	85	+	
173	43	RCL	
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175	95	=	
176	42	STO	
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180	85	+	
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213	87	IFF	
214	01	01	
215	39	COS	
216	61	GTO	
217	33	X²	
218	76	LBL	
219	34	√X	
220	43	RCL	
221	26	26	
222	85	+	
223	43	RCL	
224	27	27	
225	95	=	
226	42	STO	
227	08	08	
228	43	RCL	
229	28	28	
230	85	+	
231	43	RCL	
232	29	29	
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234	42	STO	
235	09	09	
236	43	RCL	
237	30	30	
238	85	+	
239	43	RCL	
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241	95	=	
242	42	STO	
243	10	10	
244	43	RCL	
245	35	35	
246	85	+	
247	43	RCL	
248	36	36	
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271	43	RCL	
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276	43	RCL	
277	46	46	
278	85	+	
279	43	RCL	
280	47	47	
281	95	=	
282	42	STO	
283	15	15	
284	43	RCL	
285	48	48	
286	85	+	
287	43	RCL	
288	49	49	
289	95	=	
290	42	STO	
291	16	16	
292	87	IFF	
293	00	00	
294	38	SIN	
295	87	IFF	
296	02	02	
297	39	COS	
298	61	GTO	
299	33	X²	
300	76	LBL	
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302	02	2	
303	42	STO	
304	07	07	
305	86	STF	
306	00	00	
307	91	R/S	
308	61	GTO	
309	00	00	
310	00	00	
311	76	LBL	
312	38	SIN	
313	43	RCL	
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315	75	-	
316	43	RCL	
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318	95	=	
319	42	STO	

MERGED CODES

62 63 64 72 73 74 83 84 92

TITLE C.D. Analysis PAGE 6 OF 7

PROGRAMMER ________ DATE ________

LOC	CODE	KEY	COMMENTS
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326	75	-	
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330	85	+	
331	43	RCL	
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334	95	=	
335	42	STO	
336	09	09	
337	43	RCL	
338	11	11	
339	75	-	
340	43	RCL	
341	14	14	
342	95	=	
343	42	STO	
344	10	10	
345	43	RCL	
346	12	12	
347	85	+	
348	43	RCL	
349	13	13	
350	75	-	
351	53	(	
352	43	RCL	
353	15	15	
354	85	+	
355	43	RCL	
356	16	16	
357	54	)	
358	95	=	
359	42	STO	
360	11	11	
361	61	GTO	
362	[illegible]	[illegible]	
363	76	LBL	
364	15	E	
365	76	LBL	
366	35	[illegible]	
367	86	STF	
368	01	01	
369	61	GTO	
370	00	00	
371	10	10	
372	76	LBL	
373	29	CP	
374	91	R/S	
375	42	STO	
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383	91	R/S	
384	42	STO	
385	11	11	
386	91	R/S	
387	42	STO	
388	12	12	
389	91	R/S	
390	42	STO	
391	13	13	
392	91	R/S	
393	42	STO	
394	14	14	
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396	42	STO	
397	15	15	
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403	38	SIN	
404	61	GTO	
405	33	X2	
406	76	LBL	
407	10	E'	
408	02	2	
409	42	STO	
410	07	07	
411	86	STF	
412	02	02	
413	86	STF	
414	00	00	
415	61	GTO	
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417	76	LBL	
418	45	[illegible]	
419	43	RCL	
420	14	14	
421	42	STO	
422	30	30	
423	43	RCL	
424	15	15	
425	42	STO	
426	31	31	
427	61	GTO	
428	05	05	
429	37	37	
430	76	LBL	
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432	36	PGM	
433	02	02	
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436	36	PGM	
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438	14	D	
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445	43	RCL	
446	07	07	
447	33	X2	
448	85	+	
449	[illegible]	[illegible]	
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453	43	RCL	
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457	07	07	
458	22	INV	
459	77	GE	
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465	73	RC*	
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467	22	INV	
468	67	EQ	
469	04	04	
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471	43	RCL	
472	07	07	
473	44	SUM	
474	01	01	
475	01	1	
476	44	SUM	
477	05	05	
478	43	RCL	
479	03	03	

MERGED CODES

62	72 STO	83 GTO
63	73 RCL	84
64	74 SUM	92 INV SBR

TITLE C.D. Analysis PAGE 7 OF 7

PROGRAMMER ______ DATE ______

LOC	CODE	KEY	COMMENTS
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488	43	43	
489	76	LBL	
490	53	(	
491	42	STO	
492	20	20	
493	43	RCL	
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498	95	=	
499	42	STO	
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505	14	14	
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507	61	GTO	
508	04	04	
509	43	43	
510	76	LBL	
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519	43	RCL	
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521	95	=	
522	61	GTO	
523	04	04	
524	43	43	
525	76	LBL	
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528	36	PGM	
529	02	02	
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532	00	00	
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534	87	IFF	
535	02	02	
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538	20	20	
539	91	R/S	
540	76	LBL	
541	13	E	
542	43	RCL	
543	21	21	
544	91	R/S	
545	76	LBL	
546	13	C	
547	43	RCL	
548	22	22	
549	91	R/S	

MERGED CODES

62, 63, 64, 72 STO, 73 RCL, 74 SUM, 83 GTO, 84, 92 INV SBR

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