PURIFICATION OF BOVINE BLOOD CLOTTING FACTOR VIII (ANTIHEMOPHILIC GLOBULIN)

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
John Edward Mercer
1966

THESIS

LIBRARY
Michigan State
University

This is to certify that the

thesis entitled

PURIFICATION OF BOVINE BLOOD CLOTTING FACTOR VIII
(ANTIHEMOPHILIC GLOBULIN)

presented by

John Edward Mercer

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

E. P. Benedetti
Major professor

Date February 15, 1966

O-169

ABSTRACT

PURIFICATION OF BOVINE BLOOD CLOTTING FACTOR VIII
(ANTIHEMOPHILIC GLOBULIN)

by John Edward Mercer

The purpose of this project was to study methods for the purification of bovine blood clotting Factor VIII that could be applied to purification of this factor for clinical use in man.

The purification procedure required that conditions be established for the separation of Factor VIII from fibrinogen, resulting in minimal losses of Factor VIII activity. The Blomback procedure[1] was modified, based on the original observations of Simonetti[2], by the elimination of sodium citrate from the extraction buffers and remaining purification procedure and replacing it with sodium chloride of equal ionic strength to permit heat denaturation of fibrinogen at 56°C with minimal loss of Factor VIII activity. The modification of the procedure resulted not only in reduced heating losses of Factor VIII but also in changes in the protein distribution, causing further increases in purification.

Antisera prepared against partially purified Factor VIII showed that the fibrinogen-free Factor VIII preparations contained three immunologic components. Electrophoretic analysis on 7M urea-starch gel showed that three components were present in partially purified Factor VIII. Amino acid composition of several preparations showed

that preparations were quite uniform. Removal of a cryoglobulin fraction from the partially purified Factor VIII preparation resulted in minor losses of Factor VIII activity. Gel filtration of the preparation through G-200 Sephadex resulted in partial separation of two components, with the peak of Factor VIII activity coincident with the major (slow) component. Immunodiffusion studies confirmed the presence of single components in the isolated fractions representing the individual peaks and the presence of two components in the fractions representing the mixed peaks.

---

[1]Blomback, M. 1958. Purification of Antihemophilic Globulin. I. Some Studies on the Stability of Antihemophilic Globulin Activity in Fraction I-0 and a Method for its Separation from Fibrinogen. Arkiv. f. kemi, 12, 387.

[2]Simonetti, C., G. Casillas, and A. Pavlovsky. 1961. Purification du Facteur VIII Antihemophilique (FAH). Hemostase, 1, 57.

PURIFICATION OF BOVINE BLOOD CLOTTING FACTOR VIII

(ANTIHEMOPHILIC GLOBULIN)

By

John Edward Mercer

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Physiology

1966

to my wife, Marilyn,

for constant faith and encouragement

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. E. P. Reineke of the Department of Physiology and Dr. H. A. Lillevik of the Department of Biochemistry, co-chairmen of his guidance committee for their advice and guidance in research and preparation of this thesis. Sincere appreciation is also due Dr. L. F. Wolterink, Dr. W. D. Collings and Dr. P. O. Fromm for their guidance and encouragement throughout the course of his studies.

Acknowledgement is due the Michigan Department of Public Health for providing facilities, materials and technical assistance, and to Dr. L. A. Hyndman, Dr. H. Gallick, and other associates in the Division of Biologic Products for their helpful advice and encouragement. Special thanks is due Dr. B. H. Olson and Dr. G. L. Goerner of the Division of Antibiotic and Fermentation for performing the amino acid analysis.

This investigation was financially supported by funds from the Michigan Department of Public Health, and the American National Red Cross.

TABLE OF CONTENTS

		Page
I.	INTRODUCTION	1
II.	HISTORICAL	3
III.	MATERIALS AND METHODS	18
	A. Apparatus	18
	B. Materials and Reagents	19
	1. Chemical Reagents	19
	2. Clotting Reagents	22
	C. Methods	25
	1. Collection of Bovine Blood	25
	2. Fractionation of Clarified Plasma	26
	3. Assay of Factor VIII Activity	30
	4. Preparation of Antisera Against Human Plasma Bovine Plasma and Bovine Factor VIII	31
	5. Immunoelectrophoretic Analysis	34
	6. Immunodiffusion Techniques	36
	7. Starch Gel Electrophoresis	39
	8. Urea Gel Electrophoresis	39
	9. Tyrosine-Tryptophane Ratios	40
	10. Amino Acid Analysis	42
IV.	EXPERIMENTAL	44
	A. Modification of Blomback Procedure	44
	1. First Modification, A Comparative Study	44
	2. Complete Modification	50
	3. Second Comparative Study of Mercer Modification	50
	B. Antisera	55
	1. Preparation of Antigens	55
	2. Immunization and Testing	59
	3. Preparation of Anti-Bovine Factor VIII by Repository Immunization	60
	C. Immunoelectrophoretic Analysis of Bovine Factor VIII	63
	D. Gel Electrophoretic Studies	64
	E. Gel Filtration of Bovine Factor VIII	67
	F. Tyrosine-Tryptophane Ratios	73

TABLE OF CONTENTS - Continued

	Page
G. Amino Acid Analysis	73
1. Glycine Removal	73
2. Amino Acid Analysis	76
V. DISCUSSION	83
A. Modification of the Blomback Procedure	83
B. Preparation of Antisera	87
C. Immunoelectrophoretic Analysis	88
D. Gel Electrophoresis	89
E. Gel Filtration Studies	90
F. Cryoglobulin Studies	91
G. Amino Acid Analysis	93
H. Tyrosine-Tryptophane Ratio and Content	94
I. Significance	95
VI. SUMMARY	97
REFERENCES	100

LIST OF TABLES

TABLE		Page
I.	Plasma Clotting Factors Discovered Between the Years 1947 and 1955	6
II.	Summary of Data Obtained from Four Fractionation Runs with the Modified Procedure (Mercer)	52
III.	Comparison Between Two Comparative Studies (Runs 1 and 6)	57
IV.	Comparison of the Blomback Procedure and the Modified Procedure (Mercer)	58
V.	Antibody Titers from Injection of Adjuvant Enriched Bovine Factor VIII Six Weeks After Injection	62
VI.	Results of Immunoelectrophoretic Analysis of Plasma and Factor VIII Preparations at Various Stages of Purification	63
VII.	Comparison of Factor VIII Activity of Fraction I-1A Before and After Cryoglobulin Removal	70
VIII.	Results of Immunodiffusion Studies on Factor VIII Preparations After Heat Treatment, Cryoglobulin Removal, and Gel Filtration	72
IX.	Results of Tyrosine-Tryptophane Ratio and Content Determination by the Beavan and Holiday Method	74
X.	Results of Amino Acid Analysis of Three Lots of Bovine Factor VIII	77
XI.	Nitrogen Content of Three Lots of Factor VIII Calculated from Amino Acid Analysis Data	78
XII.	Comparison of Tyrosine and Tryptophane Content from Amino Acid Analysis and the Beavan and Holiday Technique	78
XIII.	Calculation of Tyrosine-Tryptophane Molar Ratios and Concentrations	82

LIST OF TABLES - Continued

TABLE		Page
XIV.	Comparison of the Effects of Heat Treatment for Two Minutes at 56°C of Fraction I-0 and Fraction I-1A, Prepared by the Blomback Procedure and the Initial Modified Procedure (Mercer)	84
XV.	Comparison of the Effects of Heat Treatment for Five Minutes at 56°C of Fraction I-0 and Fraction I-1A Prepared by the Blomback Procedure and the Modified Procedure (Mercer)	85

LIST OF FIGURES

FIGURE		Page
1.	A tentative mechanism for the intrinsic conversion of prothrombin to thrombin in human plasma. Davie et al. (1964)	7
2.	The scheme for the preparation of Factor VIII by the Blomback procedure (Blomback, 1958)	29
3.	Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/ml. (First comparative study)	46
4.	Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/mg. protein. (First comparative study) . .	48
5.	Comparison of the Blomback and the modified procedure (Mercer) on the basis of protein mg/ml. (Fig. 5-A), Factor VIII activity units/ml. (Fig. 5-B), and Factor VIII activity units/mg protein. (Fig. 5-C) . . .	49
6.	The scheme for the preparation of Factor VIII by the modified procedure	51
7.	Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/ml. (Second comparative study)	53
8.	Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/mg protein. (Second comparative study) . .	54
9.	Comparison of the Blomback and the modified procedure (Mercer) on the basis of protein mg/ml. (Fig. 9-A), Factor VIII activity units/ml. (Fig. 9-B), and Factor VIII activity units/mg protein (Fig. 9-C)	56
10.	Variations in in vivo antibody titers over a fifteen-week period	61
11.	Starch gel electrophoresis patterns of bovine Factor VIII at various stages of purification	65

LIST OF FIGURES - Continued

FIGURE		Page
12.	Urea starch gel electrophoresis patterns of bovine Factor VIII at various stages of purification . .	66
13.	Elution patterns of protein and Factor VIII activity form gel filtration of Factor VIII (Fraction I-1A Δ 56°) on G-200 Sephadex.	71
14.	A typical elution pattern obtained from the separation of Factor VIII and free glycine by gel filtration through G-25 Sephadex.	75
15.	A portion of a typical absorption spectrum as used to calculate tyrosine-tryptophane ratios by the method of Bencze and Schmid (1957)	80

I. INTRODUCTION

Blood coagulation mechanisms and components have been under intensive investigation for the past two or three decades. These studies have, for the most part, involved the use of the various components in crude form in systems designed to determine (sometimes inaccurately) the action or activity of the relatively impure component. In coagulation research, as in most areas of scientific investigation, the research must be classified as either basic research, i.e., the study of coagulation mechanisms, or applied research, the preparation of products for clinical control of hemorrhage. It is in the second category that much of the study on classical hemophilia (Factor VIII deficiency) must be placed.

Classical hemophilia (Factor VIII deficiency) is inherited as a sex-linked recessive trait, and expresses itself as a mild to severe disorder. Clinically, bleeding episodes are treated by the administration of fresh whole blood, fresh frozen plasma, or one of the several types of Factor VIII concentrates available. The extent of the therapy is dependent on the severity of the episode. Plasma or whole blood therapy is necessarily limited to minor episodes, as the circulatory overloading attendant with administration of large volumes of these products could tend to increase, rather than improve the bleeding disorder.

The increasing demands for fresh banked blood for other uses has limited the supply available for preparation of clinically effective Factor VIII concentrates. Factor VIII concentrates of animal origin have been proven clinically effective, but have been limited in use due to their antigenicity. Purification studies on Factor VIII of animal origin can therefore have several advantages. Knowledge gained from studies of animal preparations, hopefully, can be applied to work with concentrates of human origin. Purified animal Factor VIII can also be used to study coagulation mechanisms. Purification of Factor VIII preparations of animal origin may lead to more extensive clinical application. Reducing the number and quantity of potential antigens present in highly purified preparations could tend to decrease the antigenicity of these preparations thus reducing the limitations of their clinical use.

The purpose of this project was to study the purification of bovine Factor VIII. It is hoped to apply the findings to the purification of Factor VIII of human origin, and possibly to the preparation of highly purified Factor VIII of animal origin for clinical use in man.

II. HISTORICAL

A. General Coagulation

From the publication of De Motu Cordis by Harvey in 1628 on the function of the heart and circulatory system, followed by observations of Malphigi in 1666 of fibrin strands from the washed blood clot, there had been sporadic, but continued interest in the phenomena of hemostasis. It remained for Petit in 1731 to make the first scientific approach to the physiology of hemostasis. He observed the vascular plugging effect of clots, and their adherence to the intima of the vessel. Morand (1736) postulated that arteries underwent longitudinal contraction and this decreased the lumen to effect hemostasis. This later concept was more widely accepted than the ideas of Petit by clinicians of that era.

It was not until 1805 that Jones presented a concept of hemostasis that combined the two previous theories of clotting and vascular contraction. It was about this time that Otto (1803) first clinically recognized hemophilia. The classical theories of Morawitz (1904) and Fuld and Spiro (1904) were the first significant attempts to correlate existing data and condense it into two definitive equations.

$$\text{Prothrombin} \xrightarrow[\text{Thrombokinase}]{Ca^{++}} \text{Thrombin}$$

$$\text{Fibrinogen} + \text{Thrombin} \longrightarrow \text{Fibrin}$$

The fundamental bases of these equations were that thrombokinase released from injured tissue cells in the presence of calcium and prothrombin ⟶ thrombin. This theory immediately met with objections in that platelets were not considered as part of the theory. Bizzozero (1882) had previously shown that platelets were somehow involved in coagulation.

It was later shown by Collingwood and McMahon (1912) that a tissue source of thromboplastin (thrombokinase) was not necessary for coagulation. These findings caused a revision of thinking about the proposed theory and gave rise to the distinction of two mechanisms that triggered coagulation. The extrinsic mechanism was based on release of tissue thromboplastin, and the intrinsic mechanism based on the formation of a thromboplastin from plasma components.

Brinkhous (1947) proposed that the source of intrinsic thromboplastin was platelets, and that its release was dependent on a lytic factor in plasma that he called thrombocytolysin. Quick (1947) showed that platelets *per se* contain only small amounts of thromboplastin, and proposed that intrinsic thromboplastin was formed by the reaction of a platelet factor and a plasma factor, which he called thromboplastinogen. The thromboplastinogen of Quick and thrombocytolysin of Brinkhous have since been shown to be the same factor, namely Factor VIII (antihemophilic globulin).

The literature on general coagulation is too vast for complete coverage in this writing. In synopsis, the most noteworthy was the discovery, from 1947 to 1955, of six new plasma factors involved in

the extrinsic and intrinsic coagulation mechanisms. Table I gives a list of these six factors, with reference to the original publication.

There have been many excellent reviews of the clotting literature recently published. For a concise review of the pertinent literature leading to latest theories of the intrinsic coagulation mechanisms proposed by MacFarlane et al. (1964) as an enzyme cascade, or by Davie et al. (1964) as a waterfall sequence shown in Figure 1, the reader is referred to the work of Gaston (1964) or Gallick (1965).

B. Hemophilia

In 1905, Sahli suggested that hemophilia was due to a deficiency of thrombokinase. This suggestion and the earlier suggestions of Bizzozero (1882) and Hayem (1898) that platelets were the source of thrombokinase, led to the conclusion that platelets were abnormal in hemophilia. The first suggestion that the defects in hemophilia were in the plasma, not the platelets, was from the work of Addis (1911). Addis concluded that prothrombin was the abnormality in hemophilia. Frank and Hartmann (1927) showed that small amounts of prothrombin-free normal plasma when added to hemophilic plasma corrected the deficiency. This finding was most significant in that it suggested that the deficiency was not due, as previously concluded, to platelets or prothrombin abnormalities, and thus marked the beginning of a new approach to the study of hemophilia.

A globulin fraction was later isolated from normal plasma by Patek et al. (1936 and 1937) that, when injected intravenously into a hemophiliac, normalized the clotting time of the subject. These

Table 1. Plasma Clotting Factors Discovered Between the Years 1947 and 1955

Table I. Plasma Clotting Factors Discovered Between the Years 1947 and 1955

Most Common Name	Official Name	First Publications Author	Year
Accelerator globulin (AcG)	Factor V	Owren	1947
		Quick	1943
		Nolf	1938
Serum prothrombin conversion accelerator (SPCA)	Factor VII	Owen et al.	1948
Plasma thromboplastin component (PTC)	Factor IX	Aggeler et al.	1952
		Biggs et al.	1952
Stuart factor	Factor X	Duckert et al.	1955
		Howie et al.	1957
Plasma thromboplastin antecedent (PTA)	Factor XI	Rosenthal et al.	1953
		" "	1955
Hageman factor	Factor XII	Ratnoff et al.	1955

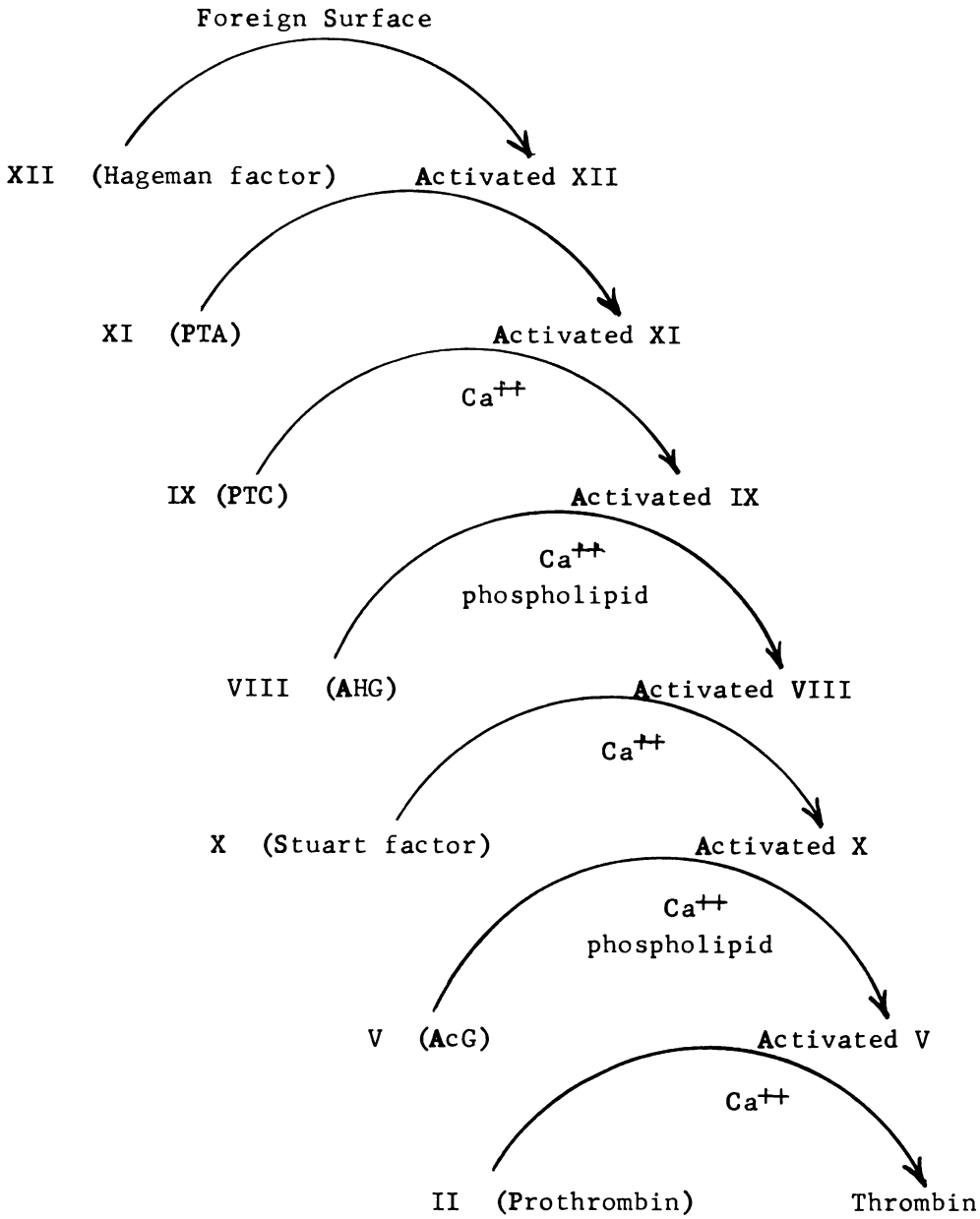

Fig. 1. A tentative mechanism for the intrinsic conversion of prothrombin to thrombin in human plasma. Davie et al. (1964).

results were confirmed later by Bendien et al. (1939) and Taylor et al. (1945). It was thus established that hemophilia was due to an abnormality or lack of a plasma constituent. With the discovery of other plasma components necessary for coagulation, the term hemophilia required further classification to avoid confusion with these related disorders. With the varied approaches to coagulation research during the ensuing years, there developed a multiplicity of terms to describe the clotting factors. An international committee on nomenclature of blood clotting factors was formed, and has assigned Roman numeral designations to the accepted blood clotting factors in an effort to alleviate this situation.

C. Purification of Factor VIII

In the past, several approaches have been used to isolate and further purify Factor VIII from human and various animal sources, including bovine, porcine, canine, ovine, and equine plasmas. The isolation techniques are based on precipitation of the factor from plasma by ethanol (Cohn et al., 1946), ether (Keckwick and Mackay, 1946, 1954), inorganic salts (Bidwell, 1955a, 1955b), and amino acids (Wagner et al., 1964), or by selective adsorption of Factor VIII from plasma or plasma fractions on kaolin (Seegers et al., 1957), tricalcium phosphate (Niemitz et al., 1961), and tricalcium citrate (Blomback et al., 1961).

Alexander and Landwehr (1948) demonstrated, that from their experience with Cohn Fraction I, between 10% and 35% of the plasma Factor VIII activity was recovered in this fraction. These values

cannot be taken as being absolute, in that the great variation in assay systems that have been used in the past and present are not always specific for the factor being assayed. Cohn's Fraction I has been the starting material for many and varied studies on the further purification of Factor VIII.

The isolation of Factor VIII from Fraction I first requires its separation from fibrinogen which is the major component of the fraction. Spaet and Kinsel (1953) accomplished the separation by heat denaturation of fibrinogen, followed by absorption of Factor VIII and reprecipitation by pH adjustment. Paper electrophoretic analysis of their purified Factor VIII preparation revealed that it contained a single component. However, it is possible that more refined techniques would have revealed the presence of additional components. Blomback (1958) developed methods for further purification of Fraction I, utilizing glycine ethanol buffers to extract the trace contaminants of the fraction, and a procedure for separation of Factor VIII activity and fibrinogen. The separation was only partial with 13% of the fibrinogen remaining in the Factor VIII.

Van Creveld et al. (1959, 1960, 1961), using ECTEOLA - cellulose chromatography were able to quantitatively separate fibrinogen and Factor VIII from Fraction I. A procedure using tricalcium phosphate to selectively adsorb Factor VIII from Fraction I was described by Niemetz et al. (1961). Complete separation was not obtained, as 20% of the fibrinogen remained with the Factor VIII. Janiak and Soulier (1962), co-workers of Niemetz, reported on an extension of this procedure involving subsequent washings of the tricalcium phosphate -

Factor VIII complex, followed by elution and stepwise ethanol fractionation of the eluate. The final product had 3 to 10 times the activity of an equal volume of plasma, representing a 30 to 50 fold purification. Tannic acid was used by Pavlovsky et al. (1961) to selectively precipitate fibrinogen from Fraction I-0 prepared by the Blomback procedure. Their data showed that complete separation was obtained with apparent full recovery of Factor VIII activity.

Blomback et al. (1961) used tricalcium citrate in an attempt to further purify Factor VIII from Fraction I-1A. They showed that Factor VIII was apparently adsorbed on tricalcium citrate. When the tricalcium citrate was eluted (dissolved) in 0.1M EDTA, a precipitate containing protein and lipid materials remained. When this precipitate was extracted with organic solvents, the lipid and the Factor VIII activity were found in the organic phase. This lipid extract contained low levels of Factor VIII activity as did the original supernatant from the adsorption step. When the supernatant and lipid extract were recombined, the resulting activity was greater than the sum of the separate activities. They also found that when supernate from hemophilic Fraction I-1A combined with normal lipid extract, there was little increase in activity. Lipid extracts from hemophilic Fractions I-1A were found to be inactive and devoid of phosphorous. These results suggested that Factor VIII is possibly a protein phospholipid complex.

Procedures not involving the initial precipitation of Factor VIII by the procedure of Cohn et al. (1946) have been described by several groups.

Keckwick and Mackay (1946, 1965) developed a procedure for the fractionation of plasma proteins using ether (1946), and devised methods for aseptic fractionation of plasma proteins with ether (1954). The original process of 1946 was developed using ether, because of the severe shortage of ethanol in England at that time. Keckwick and Wolf (1957) developed a method of further purification of Factor VIII based on ether fractionation, very similar to the Blomback procedure, involving two washings of the crude fraction before lyophilization.

Potassium phosphate and sodium citrate were used for isolation and purification of Factor VIII from animal plasma by Bidwell (1955a and b) resulting in 100 to 400 fold purification. The products were contaminated with fibrinogen that could not be removed by heating without complete loss of Factor VIII activity.

Michael and Tunnah (1963) studied the further purification of crude porcine Factor VIII, isolated by the Bidwell procedure, using ion exchange resins. The purified preparations obtained were at least 50% fibrinogen, and required stabilization.

Factor VIII has been isolated from plasma by adsorption on kaolin (Lorand and Laki, 1954). Seegers and his co-workers (1957, 1959) have utilized this technique followed by alcohol fractionation to extensively purify Factor VIII (platelet cofactor I). Ultracentrifugal analysis of their preparations revealed two to three components. With preparative ultracentrifugation, they were able to reduce the preparation to one component (S_{w20} = 7.1), but when subjected to Ouchterlony analysis, two components were revealed.

Amino acids -- glycine, β alanine, and γ aminobutyric acid were used by Wagner et al. (1964) as protein precipitants to purify Factor VIII from canine plasma. Their best preparations were purified 2000 times in terms of protein nitrogen.

With all of the varied methods used to purify Factor VIII from human and animal plasmas, there has as yet been no report of purification of this factor to a single component as measured by several parameters of purity.

D. Hemophilia Therapy

The first treatment of hemophilia reported by Lane in 1840 involved a direct transfusion to a bleeder with good clinical results.

The use of crude Factor VIII concentrates has been on the increase in the past decade. In Michigan, however, human Factor VIII concentrates have been produced for clinical trial by the Bureau of Laboratories, Michigan Department of Public Health since 1948 (Anderson, 1964), and until the mid 1950's, the Michigan Department of Public Health was the only producer of such concentrates in the United States. Many of the treatment failures obtained with these products have been due to improper use for treatment of hemorrhage not due to Factor VIII deficiency, or due to insufficient dosage.

Distribution studies show that the extravascular Factor VIII pool may be at least 1.5 times as great as the vascular pool (Adelson et al., 1963). It was further shown that the survival of injected Factor VIII was biphasic. The first phase, the intravascular -

extravascular equilibration phase, has a half life of 3.8 hours, and the utilization and destruction phase has a half life of 2.9 days in normal humans.

In a hemophiliac during hemorrhage, the utilization would be expected to be much more rapid, causing an apparent shortening of the equilibration phase. This has been confirmed by Brinkhous (1964) in studies with Irish setter dogs suffering from hemophilia. After an initial priming dose, the equilibration phase was rapid and thought to be a combination of equilibration and utilization. Subsequent administration showed that there was slower utilization. With the cessation of bleeding, utilization approached a normal rate with an apparent half life of 18-24 hours. It was also noted that Factor VIII, when administered as concentrates, seemed to have a longer circulating half life than when administered as plasma (Brinkhous, 1964).

Douglas (1958) studied the effects of massive plasma infusion. He found that one liter of plasma raised the circulating Factor VIII level to 14% of normal, and similar infusions were required every 6 to 12 hours to maintain this level. He determined that the circulating half life of Factor VIII from plasma infusion was nine hours.

Replacement therapy should be based on replenishing both the vascular and extravascular pools at the onset of therapy in order to maintain therapeutic levels of Factor VIII in the vascular pool.

McMillan et al. (1961) found that preparation of patients for surgery required raising plasma Factor VIII levels 30% to 60% of

normal initially, and maintaining plasma levels at 15% to 30% for a minimum of 10 days.

There have been several reports on the use of human Factor VIII concentrates in the treatment of hemorrhage and preparation of hemophiliacs for elective surgery. (Gugler, 1961; Pavlovsky et al., 1961; McMillan et al., 1961; Maycock et al., 1963; and Field et al., 1963). These five reports entail 104 patients, many of whom were treated for more than one hemorrhagic episode. In the report by Maycock et al. (1963), six individuals were treated for a total of 84 individual bleeding episodes over a three-year period. In all cases reported, there was only one case that developed refractoriness to replacement therapy with Factor VIII concentrates. Many and varied reactions were noted during infusion of Factor VIII preparations that were readily controlled by administration of antihistamines or corticosteroids, or by reduction of the infusion rate.

Recently, Pool et al. (1964) have developed a simple new approach to the concentration of Factor VIII from plasma, involving a cryoglobulin precipitate obtained from fresh plasma. The procedure achieves a 50 fold concentration of Factor VIII. A single dose of this preparation containing 750 mg protein raised the circulating Factor VIII level in a patient with severe hemophilia to 40% of normal. There has been too limited clinical experience with this type of preparation to evaluate its potential worth in prolonged therapy.

There have been many reports by investigators in England concerning the use of Factor VIII concentrates of animal origin in major

surgery, treatment of extensive injury, and bleeding episodes of hemophiliacs (MacFarlane et al., 1954; Frankel and Honey, 1955; Biggs, 1960; Handly et al., 1961; Ingram, 1962; and Smith, 1965).

Treatment of patients (with Factor VIII of animal origin) has been limited to a single regimen not exceeding 14 days. This limitation was imposed because of the possible sensitization to the animal source material, because of decreasing response to Factor VIII present, and because of evidence of platelet agglutinins in the animal products. The concentrates of animal Factor VIII used to date have been crude, thus increasing the chances of sensitization to the several antigenic (protein) components.

Follow-up studies on several patients treated with animal Factor VIII preparations over a period of weeks or months, failed to show demonstrable sensitization to animal Factor VIII. In most cases treated, the hemostatic effect of the animal Factor VIII preparations was excellent. Reactions encountered during transfusion were manageable by reduction of the infusion rate or administration of antihistaminic drugs.

Boudreaux and Frampton (1960) reported on an apparently peroral acting peanut factor that produced hemostasis in hemophilia. Boudreaux, himself a hemophiliac, noticed that ingestion of peanuts seemed to improve his condition. They suggested that the genetic block to Factor VIII synthesis could have been partially overcome by the peanut factor in the diet. Astrup et al. (1960) suggested that the explanation of the phenomenon was based on an upset of the dynamic equilibrium of fibrin formation and fibrin resolution by a factor in peanuts that

delayed fibrin resolution. Brakman et al. (1962) showed that ingestion of peanuts was regularly followed by a decrease in the fibrinolytic activity of the blood, and suggested that the hemostatic effect was an indirect effect of an antiprotease present in peanuts. Astrup et al. (1962) isolated a protease inhibitor from peanut flour that contained nearly all of the antiprotease activity of the original flour as assayed by the fibrin plate method against a plasminogen activator. When the extract as administered to a patient, the results were similar to those obtained with the crude flour. Strauss et al. (1965) studied the effect of epsilon amino caproic acid (EACA), itself a potent antifibrinolytic agent, on severe hemophilia, and found that it had no effect.

E. Inhibitors of Factor VIII

The existence of inhibitors or anticoagulants directed against Factor VIII has been recognized for several decades. (Joules et al., 1938; Lozner et al., 1940; Munro et al., 1943; Verstraete, 1957; Breckenridge et al., 1962; Leitner et al., 1963).

The presence of these anticoagulants against Factor VIII in hemophiliacs has raised the question of whether hemophilia is due to a deficiency of Factor VIII synthesis, or whether it is primarily due to the presence of abnormally high concentrations of inhibitors specific for Factor VIII. In the past, several investigators have supported the latter hypothesis.

Verstraete (1957) studied an anticoagulant from a hemophiliac subject that was found to be identical with one isolated from a

normal subject. He also studied hemophiliacs in which anticoagulants could not be demonstrated. Leitner et al. (1963) studied a purified inhibitor of Factor VIII, and found that dissociation of the Factor VIII inhibitor complex could not be demonstrated.

Extensive studies on the inhibitor hypothesis of hemophilia have been made by Mammen (1963). He has been able to demonstrate that an inhibitor present in hemophilic plasma can be denatured by freeze drying, or ether extraction, that is labile to acid and to storage at room temperature. Denaturation of the inhibitor in hemophilia plasma with ether enabled the isolation of the normal complement of Factor VIII from the plasma. He concluded that hemophiliacs have normal amounts of Factor VIII.

The inhibitor described by Mammen is evidently not similar to that described by Leitner et al. (1963) in that their purified inhibitor was very stable to changes in temperature and pH, and the Factor VIII inhibition with their inhibitor was irreversible.

Mammen (1964) has also reported the isolation of Factor VIII from bovine serum.

McLester et al. (1965) have presented data that they feel further substantiate the hypothesis that hemophilia is due to a deficiency of Factor VIII.

Regardless of the mechanisms of the pathologic origin of hemophilia, the fact remains that the most successful therapy of the disorder has been the administration of high potency Factor VIII concentrates.

III. MATERIALS AND METHODS

The methods herein described are standard laboratory procedures. Any modification of their effectiveness in the purification of bovine Factor VIII preparations will be described and documented in the Experimental section of this thesis.

A. Apparatus

Following is a list of the standard laboratory equipment and apparatus used in this project, listed according to function.

Temperature Control--Two Precision-Freas Model 160 constant temperature water baths, one maintained at 37°C ± 0.1°C, and one maintained at 56°C ± 0.5°C. One Aminco Model 4-8615 50 gallon constant temperature bath filled with 40% (v/v) ethanol, maintained at -4°C ± 0.1°C. A constant temperature cold room was maintained at 0 to +4°C.

Centrifugation--International refrigerated centrifuges, one Model REF and one Model PR-2.

pH Measurement--One Beckman Model G pH meter with glass electrode.

Absorbance Measurement--One Beckman Model DU spectrophotometer with quartz cells. One Coleman Jr. spectrophotometer with pyrex cuvettes.

Fraction Collection--One Research Specialties Co. Model 1205 fraction collector with volumetric measuring siphons of 1 ml and 10 ml capacity.

Electrophoretic Equipment--One Beckman Spinco Durrum Cell used for immunoelectrophoresis on 25 x 75 mm microscope slides. Buchler Model 3-1072 vertical gel mold used for starch gel and urea starch gel electrophoresis. Power supplies for electrophoresis, Heathkit Models PS-3 and IP 32 variable voltage power supplies.

Time Measurement--Gallet one-fifth second stop watch. General Electric 20-second interval timer. Mechrolab Model 202 clot timer.

Freeze Drying Equipment--One Virtis lucite drying chamber with the following accessory equipment: Virtis temperature controller, Virtis cold finger condenser, Virtis McLeod gauge, and Welch Duoseal vacuum pump.

B. Materials and Reagents

1. Chemical Reagents

Chemicals--All inorganic and organic chemicals were of reagent grade unless otherwise specified.

pH 6.8 Glycine-Citrate-Ethanol Buffer (Ionic Strength 0.33): 75 gm (1M) of glycine and 16.2 gm (0.055M) trisodium citrate were dissolved in 800 ml of distilled water. The pH was adjusted to 6.8 with concentrated hydrochloric acid. 68.5 ml of 95% ethanol was added and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Glycine-Citrate-Saline-Ethanol Buffer (Ionic Strength 0.33): 75 gm (1M) of glycine, 1.62 gm (.0055M) trisodium citrate, 17.55 gm (.3M) of sodium chloride were dissolved in 800 ml of distilled water. The pH was adjusted to 6.8 with concentrated hydrochloric acid, 68.5 ml of 95% ethanol was added and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Glycine-Saline-Ethanol Buffer (Ionic Strength 0.33): 75 gm (1M) of glycine and 19.3 gm (.33M) sodium chloride were dissolved in 800 ml of distilled water. The pH was adjusted to 6.8 with dilute (approximately 1M) hydrochloric acid, 68.5 ml of 95% ethanol was added, and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Citrate Buffer 0.055M (Ionic Strength 0.33): 16.2 gm of trisodium citrate were dissolved in 900 ml of distilled water. The pH was adjusted to 6.8 with concentrated hydrochloric acid and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Sodium Chloride 0.33M (Ionic Strength 0.33): 19.3 gm of sodium chloride were dissolved in 900 ml of distilled water. The pH was adjusted to 6.8 and the total volume was adjusted to 1 liter with distilled water.

pH 6.8 Chloride-Citrate-Dextrose Buffer (CCD) (Ionic Strength 0.175): 1.46 gm sodium chloride, 7.35 gm trisodium citrate and 30 gm dextrose were dissolved in 900 ml of distilled water. The pH was adjusted to 6.8 and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Chloride-Dextrose Buffer (Ionic Strength 0.175): 10.22 gm of sodium chloride and 30 gm of dextrose were dissolved in 900 ml of distilled water. The pH was adjusted to 6.8 and the total volume adjusted to 1 liter with distilled water.

pH 8.2 Barbital Buffer (Ionic Strength 0.1): 15.85 gm of sodium diethylbarbiturate were dissolved in 600 ml of distilled water 0.1N HCl is added until the desired pH is reached (≃230 ml) and the total volume is adjusted to 1 liter with distilled water. This stock buffer solution is diluted 1:2 with distilled water for use.

pH 7.3 Imidazole-Saline Buffer (Ionic Strength 0.8): 3.4 gm of imidazole and 3.85 gm of sodium chloride were dissolved in distilled water. 0.1N HCl was added until the desired pH was reached (≃183 ml) and the total volume adjusted to 1 liter with distilled water.

pH 6.8 Imidazole-Saline Buffer (Ionic Strength 0.33): 3.4 gm of imidazole and 17.54 gm of sodium chloride were dissolved in 500 ml of distilled water. The pH was adjusted by adding 0.1N hydrochloric acid, requiring approximately 300 ml. The volume was then adjusted to 1 liter with distilled water.

pH 8.6 EDTA-Borate-Tris Buffer--This was prepared according to the procedure of Boyer et al. (1963) with one modification. Disodium EDTA was substituted for EDTA free acid on an equimolar basis and the final pH was adjusted to 8.6 with concentrated hydrochloric acid. The buffer was prepared by dissolving 7.44 gm of disodium-ethylene diamine tetraacetic acid, 30.92 gm of boric acid and 109 gm of tris-hydroxymethyl-aminomethane in 800 ml of distilled water, the pH

adjusted to 8.6, and the volume adjusted to 1 liter. This buffer is a concentrated stock and is diluted for use as specified elsewhere in this thesis.

53.3% (v/v) Acetate Buffered Ethanol--560 ml of 95% ethanol and 5.62 ml 80 x concentrated acetate buffer was diluted to 1 liter with distilled water.

pH 8.5 Borate-Saline Buffer--6.184 gm of boric acid, 9.536 gm of sodium tetraborate $.10H_2O$ and 4.384 gm of sodium chloride were dissolved in 800 ml of distilled water. The pH was checked and adjusted if necessary to 8.5 with dilute HCl or NaOH. The volume was then adjusted to 1 liter with distilled water.

Phosphate-Saline Buffer (Immunodiffusion)--Phosphate-saline buffer was prepared as two solutions A and B. Solution A was prepared by dissolving 4.5 gm of sodium chloride in 250 ml of distilled water. Solution B was prepared by dissolving 4.1 gm of disodium monohydrogen phosphate and 1.18 gm of potassium dihydrogen phosphate in 250 ml of distilled water. Equal volumes of solutions A and B were mixed for use in immunodiffusion studies on cellulose acetate membranes as described elsewhere in this thesis.

2. Clotting Reagents

a. Calcium Chloride

A 1M stock solution of calcium chloride was prepared by dissolving 110.99 gm of anhydrous $CaCl_2$ in 1 liter of double distilled water. This stock solution was used to prepare 0.025M, 0.03M and 0.06M solutions of calcium chloride by pipetting the appropriate

amount of 1M stock solution into volumetric flasks and diluting to the mark with distilled water.

b. Hemophilic Substrate Plasma

Blood 500 ml was drawn from a subject (Ritchie) with a mild deficiency of antihemophilic globulin into a double pak plastic blood donor bag (Fenwal), containing 75 ml of NIH solution A anti-coagulant. The blood was centrifuged, the plasma expressed into the satellite bag, and the cellular components returned to the donor. The plasma was clarified by centrifugation at 4000 x G to remove cells and dispensed into small aliquots in silicone-coated vials, quick-frozen in a dry ice-ethanol bath and stored frozen at -30°C.

c. Kaolin Suspension

5 mg/ml (Mallinckrodt N.F. colloidal) was prepared by suspending 1 gm of kaolin in a ≃200 ml of 0.9% saline. The suspension was centrifuged for 20 minutes at 2000 RPM and the supernatant decanted and discarded. This washing procedure was repeated three additional times. The washed kaolin was suspended volumetrically to a total volume of 200 ml in 0.9% saline and stored at room temperature.

d. Inosithin (Soya Bean Phospholipid) (Associated Concentrates) 0.2 mg/ml

1 gm of dried inosithin was suspended in 400 ml of 0.9% saline. The suspension was allowed to stand overnight at room temperature to insure complete solution. The volume was adjusted to 500 ml with 0.9% saline mixed well and dispensed into a 2 ml aliquot in small (3 ml) serum bottles, stoppered and stored at -20°C.

e. Saline Solution 0.9% (w/v)

9 gm of NaCl was dissolved volumetrically in 1 liter of distilled water.

f. 3.8% (w/v) Trisodium Citrate (Bakers Analyzed Reagent Grade)

38 gm of trisodium citrate was dissolved volumetrically in 1 liter of distilled water.

g. Standard Factor VIII

The standard Factor VIII preparation used in this study was a lyophilized Cohn Fraction I with an established potency of 1.52 Surgenor units/mg protein.

The working standard was prepared by dissolving 100 mg of the dried Fraction I in 10 ml of 0.9% saline. The potency of the redissolved standard was determined by running micro Kjeldahl protein determination on the standard and multiplying the protein content (mg/ml) by the potency (Surgenor units/mg protein) to determine the potency on a unit/ml basis.

The potency of this standard was originally established by many comparisons with a preparation that was originally standardized by Dr. D. M. Surgenor and found to contain 11.25 Surgenor units/10 mg dry weight.

Definition of Unitage - The Surgenor Unit

The Surgenor unit was established by assigning a potency of 1 unit of Factor VIII activity to a specific Cohn Fraction I then in use in Dr. D. M. Surgenor's laboratory. Based on this assigned

potency, Dr. Surgenor established that average normal plasma contained 46 Surgenor units per milliliter.

The "D.B.S." Unit

Dr. D. Aronson of the Division of Biologics Standards, National Institutes of Health, has defined a unit of Factor VIII activity as that amount of activity (Factor VIII) found in one milliliter of average normal plasma.

Enzyme activity is normally measured by the amount of a specific substrate converted in a given time. In the case of Factor VIII this is difficult in that its specific substrate has not as yet been definitely established.

C. Methods

1. Collection of Bovine Blood

Nine liters of bovine blood were collected during the bleed-out procedure attendant to slaughter of one cow[1] into a large stainless steel vessel containing 1 liter of 3.8% sodium citrate or 10% EDTA. The blood was mixed rapidly and thoroughly with the anti-coagulant and strained through several layers of surgical gauze to remove any foreign materials that might have fallen in during the bleeding procedure. The foam was also removed by the gauze.

The blood was transported to the laboratory and cooled to 0 to +4°C during the centrifugation procedure. Plasma was separated by

---

[1]Due to the nature of blood source (slaughter house) it was not possible to control the breed of animal used in this study.

centrifugation in 1 liter plastic bottles at 1340 x G for 60 minutes followed by aspiration of the plasma from above the cell pack. Pooled plasma was clarified by centrifugation at 4000 x G for 30 minutes to remove platelets and extraneous cellular components carried over during the pooling procedure, and the clarified plasma removed by decantation. The clarified plasma was either fractionated immediately, or quick-frozen in a dry ice-ethanol bath and held at -40°C until fractionated.

2. Fractionation of Clarified Plasma

a. Precipitation of Fraction I (containing AHG, fibrinogen and traces of other plasma proteins)

Plasma Fraction I was isolated from fresh bovine plasma according to the method of Cohn et al. (1946). The plasma was cooled to 0°C. 177 ml of 53.3% ethanol (-10°C), mixed with 1 ml of 80 x concentrated acetate buffer pH 4.0, was slowly added to each liter of plasma with constant stirring. During the ethanol addition, the plasma was further cooled to -3°C and stirred for 30 minutes after the completion of the ethanol addition step, followed by a standing time of 45 minutes at -3°C to -4°C. The precipitate was sedimented by centrifugation at 1340 x G for 15 minutes. Following centrifugation, the supernatant plasma-ethanol mixture termed Super I was decanted, measured, and sampled for protein and potency assay, and the bulk of the supernatant was discarded. The precipitated wet paste, termed Fraction I was further purified by the procedures described in the Experimental section of this thesis.

b. Preparation of Fraction I-0

Fraction I-0 was prepared according to the procedure of Blomback (1958) as follows: Fraction I paste was resuspended to one-fourth of the original plasma volume in glycine-citrate-ethanol buffer pH 6.8 at -3°C, and stirred for 30 minutes. The suspended paste was sedimented by centrifugation at 1340 x G at -3°C for 15 minutes, the supernatant liquid termed Extract 1 (E-1) was decanted, measured, sampled for protein determination and the bulk of the E-1 discarded.

A second extraction of the Fraction I paste was carried out by the same procedure. The supernatant termed Extract 2 (E-2) was measured, sampled for protein determination and the bulk of the E-2 discarded. The wet paste was redissolved in 0.055M citrate buffer pH 6.8 at 30°C to one-eighth of the original plasma volume. The resulting solution termed Fraction I-0 was sampled for protein and potency determination and submitted to further purification to Fraction I-1A as described below.

c. Preparation of Fraction I-1A

During this purification procedure there was a partial separation of antihemophilic globulin (Factor VIII) and fibrinogen (Factor I).

Fraction I-0 solution was diluted to a protein concentration of 0.75% (7.5 mg/ml) with .055M citrate buffer, based on biuret protein determination. The dilute Fraction I-0 was further diluted with two volumes of cold 0.45M glycine and Fraction I-1A was precipitated by dropwise addition of 10% (v/v) ethanol to a final concentration

of 0.5% (v/v) with constant stirring at 0°C. The suspension was stirred for 15 minutes after completion of the ethanol addition and Fraction I-1A was sedimented by centrifugation at 1340 x G for 15 minutes at 0°C. The supernatant (Super I-1A) was decanted, sampled, and the bulk of the supernatant discarded. The precipitate was redissolved in one-eighth of the original plasma volume of CCD buffer at 30°C. The fraction was sampled for potency and protein determinations, and the bulk of the fraction was lyophilized pending further purification. Figure 2 gives a schematic presentation of the Blomback procedure.

d. Heat Denaturation of Fibrinogen

Fibrinogen was removed from partially purified Factor VIII by heat denaturation at 56°C for a period of 2 to 5 minutes as follows: The fraction to be heat treated was placed in an erlenmeyer flask with a capacity of at least 2½ times the volume of the sample. The flask was immersed in a constant temperature bath at 56°C and agitated constantly during the heating procedure. The heat denaturation procedure was timed from the time that the sample reached 56°C. After heating, the sample was rapidly cooled to 0 to +4°C. The soluble material was decanted from the precipitated fibrinogen into 50 ml conical centrifuge tubes and clarified by centrifugation for 10 minutes at 1100 x G to remove insoluble materials that were carried over during decantation.

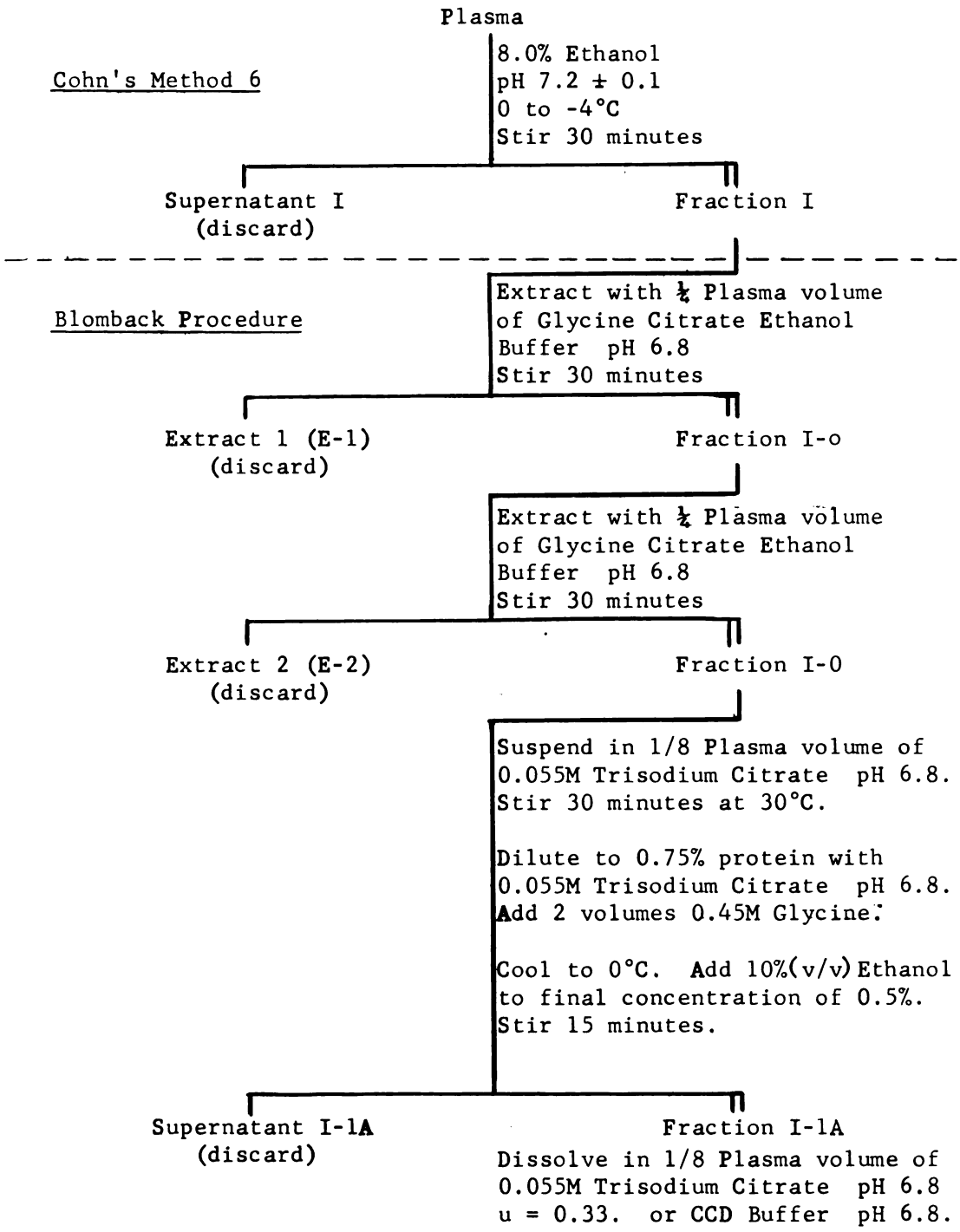

Fig. 2. The scheme for the preparation of Factor VIII by the Blomback procedure (Blomback, 1958).

3. Assay of Factor VIII Activity

Factor VIII activity was assayed by the method of Hardisty and MacPherson (1962). This is a modification of the one stage partial thromboplastin assay of Langdell et al. (1952). The assay consists of a system utilizing hemophilic plasma as the clotting substrate which should supply all of the necessary coagulation factors except Factor VIII which is supplied in variable amounts by the test material. To insure complete surface activation, an optimal concentration of kaolin (5 mg/ml) is supplied to the reaction mixture, along with a partial thromboplastin soya bean phospholipid (Inositihin 0.2 mg/ml).

The procedure consisted of preincubation of a mixture of equal volumes (0.1 ml) of hemophilic plasma, kaolin, inositihin, and a dilution of the test material (Factor VIII source) for 10 minutes at 37°C. This incubation period was required to insure complete activation of the coagulation components. The incubated mixture was recalcified by the addition of 0.1 ml of .06M calcium chloride and the coagulation time following recalcification was recorded in seconds. Throughout the course of this study, all clotting assays were performed on a Mechrolab model 202clot timer. The procedure was repeated for four dilutions of the test material ranging from 1:10 to 1:100. A standard curve was prepared, using a standard Factor VIII preparation with a potency of 1.52 units/mg protein. The clotting times obtained were plotted on a log scale setting the clotting time in seconds on the ordinate and dilution on the abscissa. The

concentration of Factor VIII in the test sample was determined according to the following calculation by selecting a clotting time common to the standard and all unknown samples.

$$\frac{\text{units/ml of Std}}{\text{dilution of Std}} = \frac{\text{units/ml of Unknown}}{\text{dilution of Unknown}}$$

The activity of the reference standard was based on Kjeldahl protein determination on each standard preparation (Ma et al., 1942). The activity of the individual standard was determined as activity units/ml by multiplying the protein (mg/ml) by specific activity (units/mg protein).

4. Preparation of Antisera Against Human Plasma Bovine Plasma and Bovine Factor VIII

a. Antisera Against Soluble Antigens

Antisera were prepared in rabbits against 1.0% (w/v) bovine plasma and 1.0% (w/v) human plasma and 0.1% (w/v) bovine Factor VIII preparations by repeated intravenous injection of 1 ml of aqueous antigen per injection for a total of nine injections over an 18 day period. The immunized rabbits were held for one week before testing.

b. Antisera Against Bovine Factor VIII - Freunds Complete Adjuvant Emulsions

i. Preparation of Antigen Emulsion--Fraction I-1AΔ 56°C was concentrated about eight fold by dialysis against a concentrated solution of Carbowax. This was accomplished by placing moistened ground Carbowax in a visking dialysis tubing and immersing the tubing into the solution to be concentrated. The solution was held at 0 to +4°C during the concentration procedure. The concentrated

fraction was emulsified with an equal volume of Freund's complete adjuvant. Care was taken to emulsify small quantities at a time with the total desired volume of adjuvant until the total antigen volume was used. This initial emulsification was accomplished using a syringe with a 15 gauge needle. After the initial emulsification step was completed, the crude emulsion was passed several times through a small bore (20 - 24 gauge) needle to produce a stable emulsion suitable for injection. Elimination of the initial step made it impossible to produce emulsions of suitable consistency for injection.

ii. Immunization--Normal rabbits were injected by various routes (subcutaneous, deep intramuscular, foot pads) with 1.0 ml of emulsified antigen at five injection sites (0.2 ml/site). The immunized animals were held for 40 days without further injection before testing.

iii. Test Bleeding--All rabbits were bled by heart puncture, removing between 5 and 10 ml of blood. The blood was allowed to clot at room temperature and incubated for one hour at 37°C to assure complete clot retraction. The clots were removed and the serum clarified by centrifugation at 1100 x G for 10 minutes to remove red cells. The clarified serum was carefully aspirated from the cell pack and transferred into clean vials. The clarified sera were heat treated for one hour at 56°C in a constant temperature bath to denature nonspecific reactants.

iv. Titration of Antisera--The sera were titered using the interfacial ring precipitation test described by Campbell et al. (1963). This test was carried out on a micro scale using 4 mm OD x 45 mm precipitin tubes and capillary pipettes drawn from 8 mm OD glass tubing.

The test antigens were diluted (based on protein) over a range of 1:1000 to 1:128,000 by serial two fold dilutions of the soluble injection antigen in borate-saline buffer pH 8.5.

Sera were used full strength or at a 1:4 dilution in borate buffer depending on the expected titer. For each titration, eight 4 x 45 mm precipitation tubes were set up in a labeled test tube rack that indicated the dilution range. Small tubes made individual labeling difficult. About 0.025 ml of the serum to be tested was pipetted into each of the tubes carefully to avoid inclusion of air bubbles at the meniscus. Starting with the highest antigen dilution, using a micro pipette, the tube of antiserum corresponding to the dilution was carefully overlaid with an equal volume of the antigen dilution. Extreme care was taken to avoid mixing at the interface of the antiserum and antigen dilution. Using the same pipette, rinsed well with the next lower dilution, the process was repeated, overlaying each tube of serum with its corresponding antigen dilution until all dilutions were used.

The tubes were allowed to stand for 20 minutes and observed under indirect light against a black background. A positive test was observed as a sharp zone of precipitate formed at the

interface of the serum and antigen. Variations in the thickness and sharpness of the precipitation ring were caused by relative mixing at the interface and titer of serum.

Each antiserum tested was tested against its homologous antigen and a heterologous antigen, i.e., antibovine plasma was tested against bovine plasma and human plasma. The results of the titration were reported as the highest dilution of antigen that gave a positive reaction with the antiserum. In cases where the antiserum had been diluted before testing, the final titer of the antiserum was obtained by multiplying the apparent titer (highest positive antigen dilution) by the dilution factor of the antiserum.

5. Immunoelectrophoretic Analysis

Immunoelectrophoresis was performed by a modification of the procedure of Grabar and Williams (1953) in 1% (w/v) buffered agar on 25 x 75 mm microscope slides in the Spinco Durrum cell. Molten 1% buffered agar (Oxoid Ionagar) was layered evenly on pre-coated microscope slides at 2 ml agar per slide. The agar was allowed to cool for five minutes and transferred to a humid chamber. Using one of two templates, (two spot - one slit, or one spot - two slit) sample wells and antiserum slits were cut in the agar. The agar was removed from the sample wells by gentle suction with a Pasteur pipette. The slides were placed in the Spinco cell, the buffer (Barbital pH 8.2 ionic strength 0.5) was poured into the chambers and the filter paper wicks placed in position. Contact between the

slides and wicks was made by pipetting on molten buffered agar at the juncture between the wicks and slides.

The sample wells of the slides were filled with the desired antigens to be tested using melting point capillaries opened at both ends fitted with a small rubber bulb. The cover was placed on the cell and a current of 40 ma. was applied across the cell for a period of 1.5 hours.

At the completion of the electrophoresis the current was turned off and the power source disconnected from the cell, the cover was removed and the contact between slides and wicks was broken. The first slide was removed and the cover replaced on the cell. The agar in the precut antiserum slit(s) was removed by gentle suction with a disposable Pasteur pipette, and the slide returned to the Spinco cell. Each slide was thus treated individually, keeping the remaining slides in the humid atmosphere of the cell until all slits had been prepared.

The slits were then filled rapidly with the desired antisera, with the aid of a Pasteur pipette drawn to a fine tip. Double diffusion of the antigen and antiserum was allowed to develop for 24 hours at 0 to +4°C in the closed Spinco cell.

At the termination of the diffusion period, the precipitin lines were readily visible.

The developed slides were dialyzed against several changes of 0.9% saline over a 24-hour period, followed by several changes of distilled water for a further period of 24 hours. The agar layer was allowed to air dry. This was expedited by flooding the slide

with distilled water and overlaying the agar with a 25 x 75 mm strip of Whatman #1 filter paper. Then the slides were stained for two minutes in triple stain and differentiated with several changes of 2% (v/v) acetic acid, the final acid bath containing 2% (v/v) glycerol in addition to the acid. The slides were air dried in a dust-free chamber. A staining procedure helped visualize and differentiate minor lines not readily seen in the unstained preparation. The stained precipitin lines were readily observed under low power magnification and permanent photographic records could be made using the slides as a negative in a photographic enlarger.

6. Immunodiffusion Techniques

During the course of this study, two different micro-immunodiffusion techniques varying only in the support medium were used. The first procedure was a modification of the procedure of Beale and Mason (1962) using agar as the support medium. The second was according to the procedure of Johnson et al. (1964) using cellulose acetate as the support medium. The buffer system used in both procedures was based on the work of Thorne and Belton (1957) and was a phosphate-saline buffer made as two solutions (Solutions A and B).

a. Agar Gel Procedures

0.5 gm of Oxoid Ionagar was dissolved in 25 ml of Solution A, and to the dissolved agar solution was added 25 ml of Solution B prewarmed to 56°C. The agar was mixed by swirling and kept molten in a hot water bath c.a. 60°C. On a clean glass microscope slide, 0.7 ml of molten buffered agar was distributed evenly over an area 2.5 x 4.5 cm

and allowed to cool for two minutes. A perspex micro immunodiffusion template was gently placed on the surface of the agar, avoiding air bubbles between the template and agar. In any case where agar was seen rising in the wells of the template, the template was removed and the slide discarded. The slide with template in place was immediately placed in a humid chamber until all necessary slides were prepared.

The desired antisera were placed in the center well of each template and the peripheral wells filled with the antigens to be tested. The slides were incubated for 40-48 hours in a sealed humid chamber. After incubation, the templates were removed. At this point, the precipitin lines were viewed under low magnification. The slides were made permanent by dialysis, drying and staining as described for immunoelectrophoresis.

b. Cellulose Acetate Procedure

The method for immunodiffusion on cellulose acetate strips was performed as follows: Strips of cellulose acetate 22 x 45 mm (Oxoid or Millipore cellotate) were soaked in phosphate-saline buffer according to manufacturer's suggested procedure and laid centrally on the surface of a clean glass slide, one strip per slide. A clean perspex microdiffusion template was placed on the surface of the cellulose acetate membrane. By placing the template in the buffer layer at one end of the membrane and sliding it to the center of the membrane, air bubbles were avoided at the interface of the membrane and template. The protruding ends of the membrane were blotted with filter paper to remove excess buffer until the membrane at the bottom

of the wells changed from a shiny to matte appearance. The slide was immediately placed in a humid chamber and the sample and antisera wells were charged without further delay. Leakage, if any, between the adjacent wells was detected immediately by changes in the appearance of the membrane in the unfilled wells. If leaks were detected, the preparation was discarded.

When all slides were thus prepared and filled, the humid chamber was sealed and left undisturbed for a period of 40-48 hours. At the end of the incubation period, the slides were removed from the chamber and the templates washed from the slides in flowing tap water. The membranes were removed and washed by immersion in several changes of 0.9% saline over a period of 4-8 hours followed by a two-hour wash in several changes of distilled water. The washed membranes were stained by immersion in 0.1% (w/v) Thiazine Red-R in 1% (v/v) acetic acid, for 2-10 minutes. The stained membranes were blotted to remove excess dye and differentiated in several changes of 4% (v/v) acetic acid. The stained strips were dried by pressing between several layers of paper towels.

Clearing of the stained preparations was effected by soaking in a commercial preparation of cottonseed oil or suitable organic solvent clearing agent. The cleared strips were blotted to remove excess oil and placed on clean microscope slides. The reaction area was covered by a clean overslip with gentle pressure to express entrapped air bubbles. The exposed ends of the membrane were trimmed flush with the edges of the coverslip. The oil was wiped off the slide and the coverslip sealed with a suitable sealant (Canada balsam

or permount). This gave a permanent preparation of the immunodiffusion pattern that could be viewed with low power magnification or photographed. Membranes cleared with organic solvents were dried on the slide and covered with a coverslip sealed to the membrane with permount.

7. Starch Gel Electrophoresis

Starch gel electrophoresis was carried out by the method of Smithies (1959). Starch was prepared in 0.026M pH 8.6 borate buffer and molded in a vertical gel mold. Electrophoresis was carried on for 16 hours at room temperature at 16 milliamperes. The gel was sliced and stained in Amido black 10B 0.1% (w/v) in 10% (v/v) acetic acid 50% (v/v) methanol and 40% (v/v) water. The stained gel was differentiated in 50% (v/v) methanol, 40% (v/v) water, 10% (v/v) acetic acid and sealed in Saran Wrap.

8. Urea Gel Electrophoresis

Urea gel electrophoresis was carried out by a modification of the procedure of Wake and Baldwin (1961), using an EDTA-Tris-Borate buffer system introduced by Boyer et al. (1963). The modifications were necessary to effect the conversion of the urea gel from a horizontal to a vertical electrophoresis system. The discontinuous buffer system described for horizontal urea gel electrophoresis was found unsuitable in that during electrophoresis, there was localized shrinkage of the gel along the front of buffer migration, resulting in decreased mechanical strength of the gel, causing fracture of the gel at the point of sample insertion. The change

to the mixed buffer described by Boyer (1963), which was more definitive in alleviation of the shrinkage problem, eliminated the problem of gel fracture.

The urea starch gel prepared in a 1:20 dilution of the stock mixed buffer, contained 60 gm of starch and 147 gm of urea in 360 ml of buffer. The molten gel was degassed under vacuum, and poured into the gel mold. The gel was allowed to harden overnight at 0 to +4°C. Electrophoresis was carried out for 16-24 hours at 16 m amps. The gel was sliced and stained in Amido black 10B for 15 minutes and destained electrolytically in 7% acetic acid overnight (Ferris et al., 1963). The stained gel was wrapped and sealed in Saran Wrap and photographed.

9. Tyrosine-Tryptophane Ratios

Tyrosine-tryptophane ratios were determined on bovine Factor VIII preparations at various stages of purification according to the methods of Beavan and Holiday (1952). Samples were prepared by dilution in 1.0M NaOH to give a final concentration of 0.1M NaOH. Absorbancy of the samples was determined in quartz cells in the Beckman DU at 280 and 294.4 mu against a blank containing the original solution buffer made 0.1M in NaOH.

Ratios were calculated from the data using the following formula:

$$\frac{\text{M tyrosine}}{\text{M tryptophane}} = \frac{0.592\ D_{294.4} - 0.263\ D_{280}}{0.263\ D_{280} - 0.170\ D_{294.4}}$$

where: M tyrosine = moles of tyrosine

M tryptophane = moles of tryptophane

$D_{294.4}$ = absorbancy of sample at 294.4 mu

D_{280} = absorbancy of sample at 280 mu

Calculations of molar absorptivity "a" were made according to the formula:

$$\frac{I}{I_o} = 10^{-abc}$$ where: I = Transmitted light

I_o = Incident light = 100

a = absorptivity

b = optical path = 1 cm

c = concentration of protein

As rephrased: $\log \frac{I}{I_o} = -ac \quad b = 1$

or:

$$\frac{\log \frac{I}{I_o}}{c} = -a$$

Molar absorption constant K was derived from "a" as 2.303a = K. The K's for each sample were determined at both wave lengths and used to calculate molar concentration of trysine and tryptophane/mg protein as follows:

M tyrosine = $(0.592\ K_{294.4} - 0.263\ K_{280}) \times 10^{-3}$

M tryptophane = $(0.263\ K_{280} - 0.170\ K_{294.4}) \times 10^{-3}$

10. Amino Acid Analysis

a. Preparation of Samples

Due to the concentration of free glycine carried over during the purification procedure, methods for removal of free glycine were developed. The first method used was a TCA precipitation and washing method that turned out to be laborious and ineffective resulting in mechanical loss of Factor VIII protein, and incomplete removal of glycine. Glycine was readily removed by gel filtration of Factor VIII preparations through a G25 Sephadex column equilibrated with 0.033M NaCl. The gel filtration procedure accomplished a two-fold purpose of removing free glycine and reducing the salt concentration 10 fold, the salt concentration of the original samples being 0.33M NaCl. The protein peak in the column eluates was detected by absorbancy measurements on selected eluate fractions at 280 mu. Glycine was determined by a spot test method on filter paper using 0.1% Ninhydrin in pH 5 citrate buffer. The protein containing glycine-free fractions were pooled. The protein was concentrated by precipitation in 5% (w/v) final concentrations of trichloroacetic acid. The precipitation step was carried out on three aliquots of equal volume. Two were used for protein determination, and the third submitted for amino acid analysis.

b. Hydrolysis

The samples submitted for analysis were hydrolyzed with 6M HCl, dried and redissolved in buffer for analysis in the Beckman 120B amino acid analyzer. Amino acid identification was based on

the "standard peak position" of the individual amino acid established for the particular conditions by Dr. B. H. Olson (Michigan Department of Public Health).

Individual amino acid content was determined from the chromatograms using the half height method described in Instruction Manual AIM 2 for the Beckman 120B amino acid analyzer. The C_{HW} constants used were determined by Dr. Olson for this particular instrument. Tryptophane was estimated by dividing the tyrosine content derived from the chromatographic analysis by the tyrosine-tryptophane ratios determined for the particular sample.

IV. EXPERIMENTAL

Introduction: The most direct method for the separation of the closely associated proteins fibrinogen (Factor I) and antihemophilic globulin (Factor VIII), is the denaturation (precipitation) of fibrinogen from a mixed solution of the two proteins by heating at 56°C for a period of three to five minutes. This is a simple, rapid method, but it has the disadvantage of variable losses of Factor VIII activity ranging from 50% - 90% incurred during the heating process. Simonetti et al. (1961) observed that in the absence of citrate, these heating losses are minimized. The first experiment conducted in this work involved a comparative study of this citrate effect.

A. Modification of Blomback Procedure

1. First Modification, A Comparative Study

During the fractionation and partial purification procedures described by Blomback, citrate containing buffers are used, and the final product, Fraction I-1A is redissolved in a citrate buffer. The first modification of the Blomback procedure involved the 10-fold reduction of citrate in the buffers used, and replacement of citrate with saline of equivalent ionic strength. At the Fraction I-0 stage of the modification, citrate was completely replaced by saline of equivalent ionic strength.

Bovine blood was collected, using a 3.8% solution of trisodium citrate as anticoagulant, 9 volumes of blood, and 1 volume of 3.8% trisodium citrate. The resulting plasma obtained was fractionated to Fraction I by Cohn's method 6. (Cohn et al., 1946). The Fraction I paste obtained was divided into two equal portions. One-half was carried through the standard Blomback procedure to Fraction I-1A, and the other half carried through the modified procedure (10-fold reduced citrate) to Fraction I-0', and further to Fraction I-1A' in the absence of citrate. To avoid confusion, the materials obtained from the modified procedure were designated by the use of the prime designation (i.e., Fraction I-0', Fraction I-1A', etc.)

All fractions obtained from this experiment were assayed in the Blomback assay system which consisted of a recalcified clotting time of Factor VIII deficient plasma using the test material as the source of Factor VIII. The test material was diluted 1:10, 1:20, 1:50, and 1:100. Equal volumes of diluted test material and Factor VIII deficient plasma were mixed, recalcified and the clotting time determined. The unknown samples were quantitated from a standard curve prepared in the same way with a reference standard Factor VIII preparation of known potency. This assay system was replaced with the Hardisty assay system as described earlier for all subsequent experiments.

Figure 3 gives a comparison of the two procedures on the basis of Factor VIII activity (units/ml). The greatest variations appeared in Fraction I-0 and Fraction I-0'. These data confirm the work of

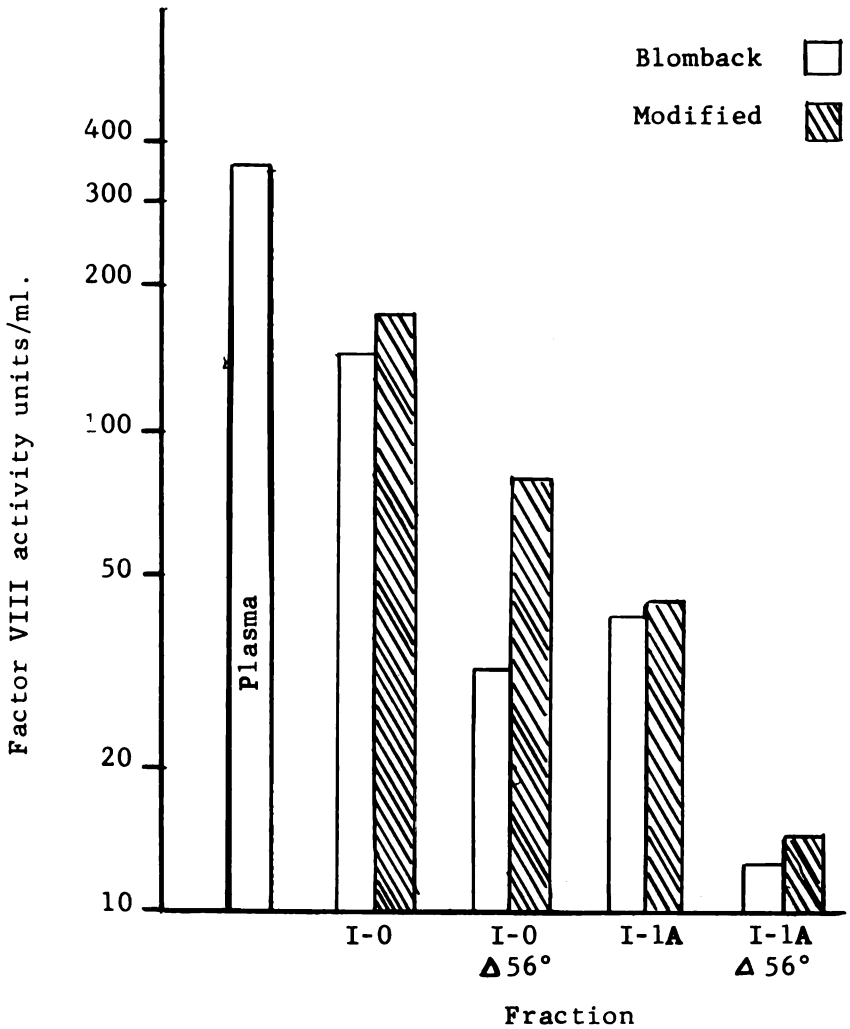

Fig. 3. Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/ml. (First comparative study).

Simonetti et al. (1961) on heat stability of Fraction I-0 in the absence of citrate, as seen by the greater than two-fold difference in activity of the heat-treated materials. Minimal differences were observed during subsequent purification to Fraction I-1A.

Examination of the data comparing the specific activity (Factor VIII units/mg protein) in Figure 4 showed the true significance of the modified procedure. The differences are due mainly to the variations in protein content of the fractions, and not in the activity on a unit/ml basis as seen from Figure 3. Thus, in the actual purification, the cardinal feature on the modification was based on altered protein content of the purified fractions Fraction I-1A and Fraction I-1A' before and after heat treatment. There was a 4.7-fold difference after heat denaturation.

Figure 5 summarizes the data of protein mg/ml (Figure 5A), activity units/ml (Figure 5B), and activity units/mg (Figure 5C) of the fractions showing the interrelationship of these three variables. Starch gel electrophoretic analysis of the fractions before heating showed that two components, one major assumed to be fibrinogen, and one minor component assumed to be the Factor VIII containing component, were visualized. After heat treatment at 56°C, the major component of the Fraction I-0 56°C was diminished in size. In the Fraction I-A 56°C, the major component was absent, leaving essentially one component.

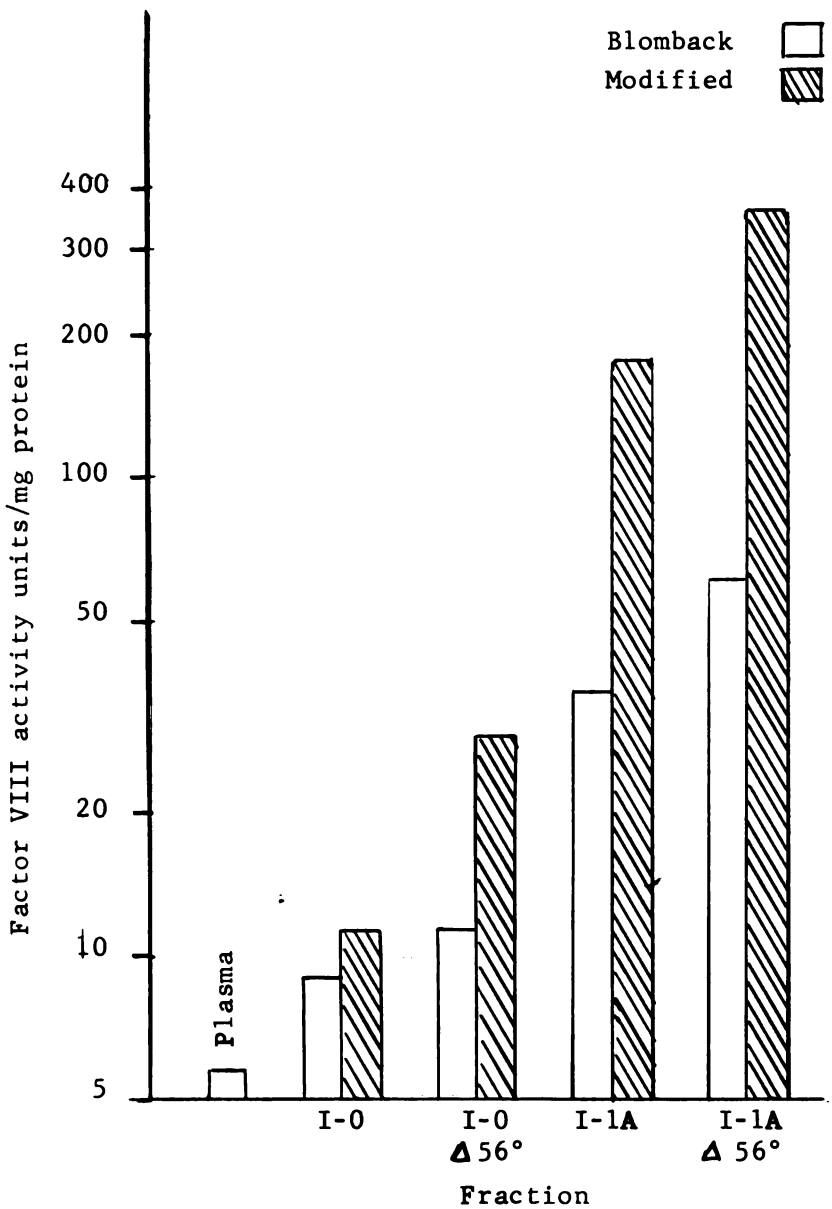

Fig. 4. Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/mg protein. (First comparative study).

Fig. 5-A

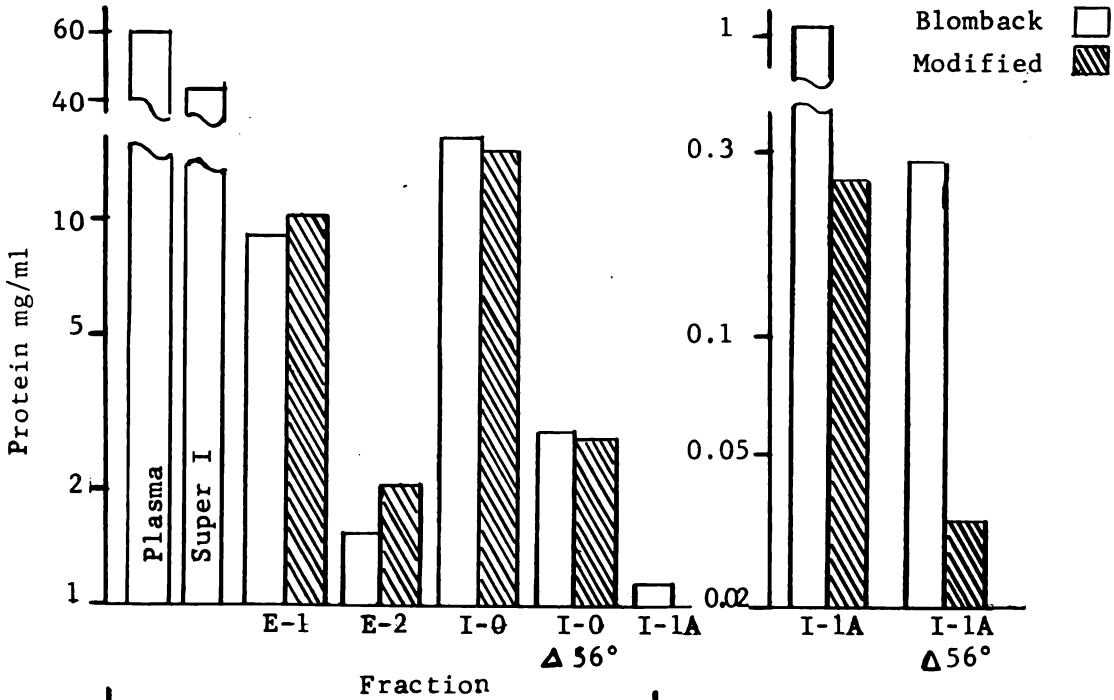

Fig. 5-B

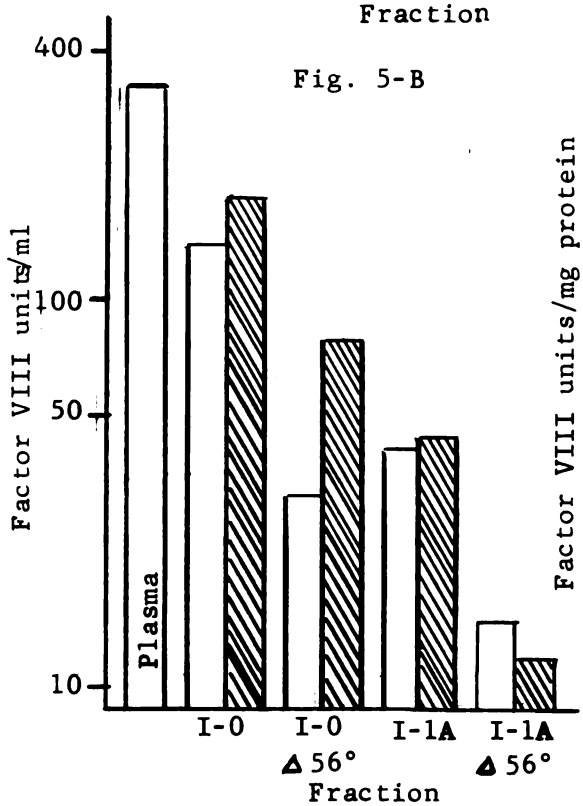

Fig. 5-C

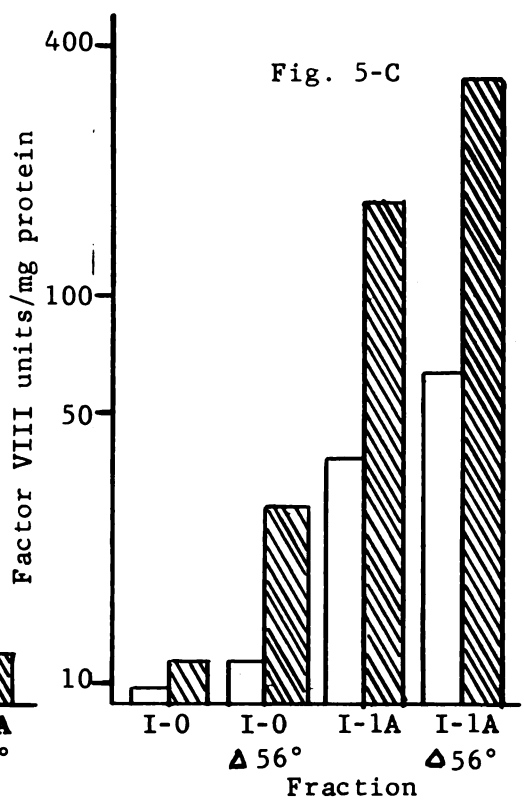

Fig. 5. Comparison of the Blomback and the modified procedure (Mercer) on the basis of protein mg/ml (Fig. 5-A), Factor VIII activity units/ml (Fig. 5-B), and Factor VIII activity units/mg protein (Fig. 5-C)

2. Complete Modification

Based on these findings, the Blomback procedure was further modified by complete elimination of citrate from the extraction buffers in the stage leading to Fraction I-0', resulting in a modified Blomback procedure devoid of citrate, save that carried over from the anticoagulant of the starting plasma. Figure 6 gives a schematic presentation of the modified procedure.

Four fractionation runs were carried out by this modified procedure to the Fraction I-1A' stage. The fractions obtained were lyophilized and stored at -20°C pending further studies. Table II summarizes the data obtained from these four runs, giving the average and range of the results.

3. Second Comparative Study of Mercer Modification

A second run was performed comparing the standard Blomback procedure and the modified procedure (Mercer). The Fraction I obtained from 3.9 liters of bovine plasma was divided into two equal portions. One was carried through the standard Blomback procedure and the other through the modified procedure. All fractions obtained were sampled for potency and protein determinations, and the remainder lyophilized and stored at -20°C pending further studies. Potency determinations were made using the Hardisty assay system as previously described.

The results of this and the preceding study are comparable with some minor variations. Figure 7 gives comparison of activities on a unit/ml basis. Figure 8 compares specific activities, and

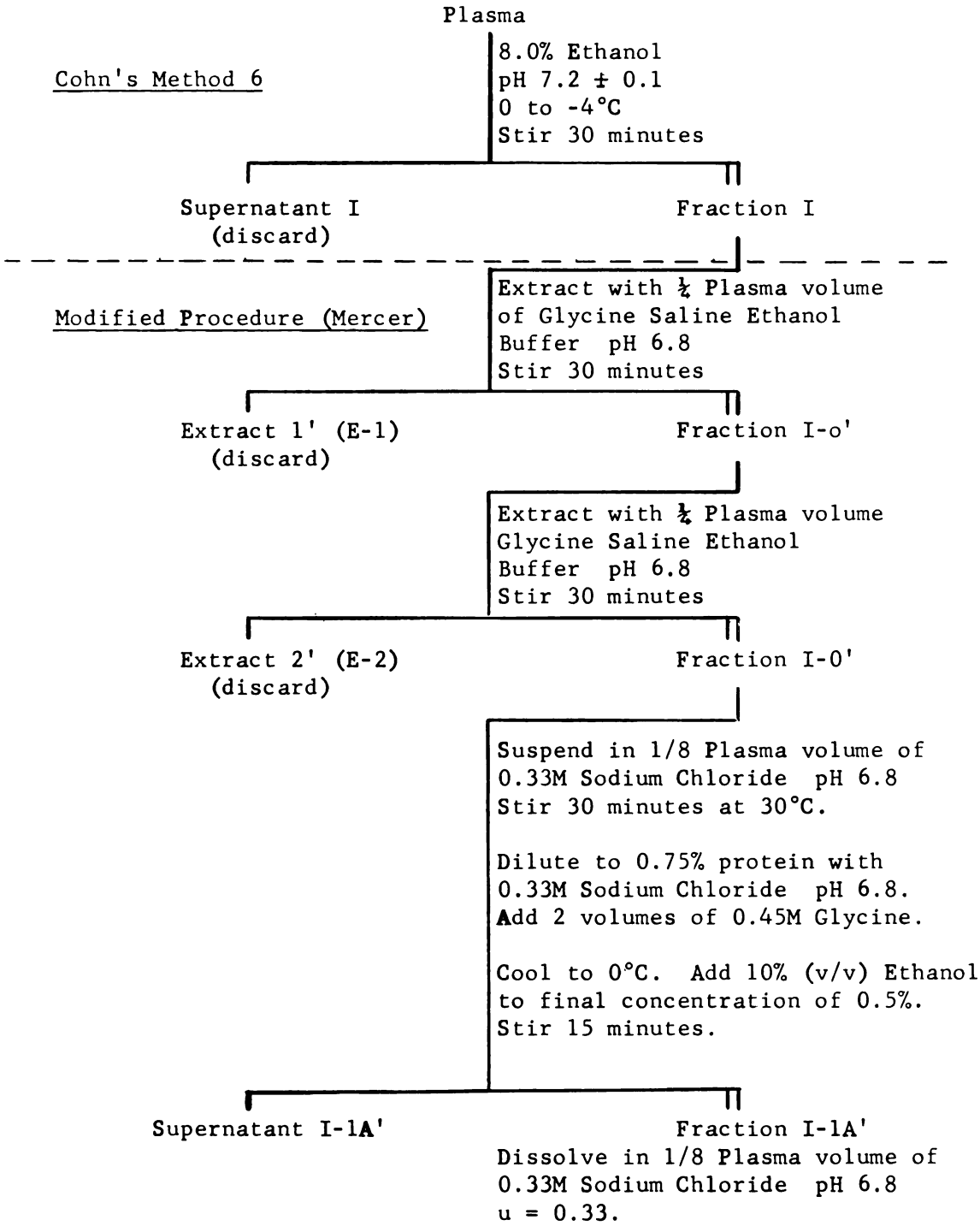

Fig. 6. The scheme for the preparation of Factor VIII by the modified procedure.

Table II. Summary of Data Obtained from Four Fractionation Runs with the Modified Procedure (Mercer)

	Protein			Factor VIII Activity			
	mg/ml	Recovery % from Plasma	From Fraction I-0	Units/ml	Units/mg	Recovery % from Plasma	From Fraction I-0
Plasma	58 (55.2-61.7)	100		99.7 (52.6-194)	1.72 (0.85-3.32)	100	-
Super I	45.2 (42.3-48.3)	87.1 (85.1-88.25)		6.53 (4.1-8.3)	0.146 (0.09-0.185)	9.1 (4.9-13.7)	-
Extract 1 (E-1)	11.75 (10.3-15.1)	4.37 (4.28-4.7)					-
Extract 2 (E-2)	2.09 (1.57-3.03)	0.83 (0.78-0.9)					-
Fr I-0	14.4 (13.6-15.7)	6.5 (5.5-7.0)	100	148 (106-221)	10.22 (7.8-15.2)	44.9 (32.4-68)	100
Fr I-1A	1.77 (1.54-2.01)	0.354 (0.244-0.50)	5.6 (4.44-8.25)	62.4 (43.1-100)	34.8 (28.0-52.1)	9.6 (2.3-15.1)	21 (6.47-40.2)

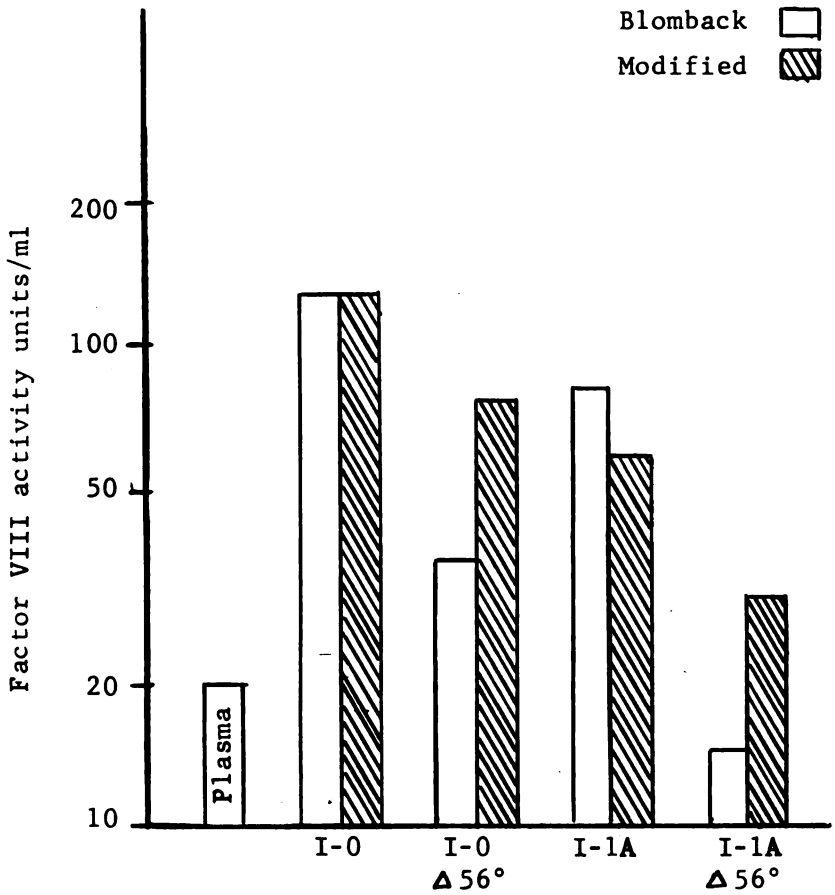

Fig. 7. Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/ml. (Second comparative study).

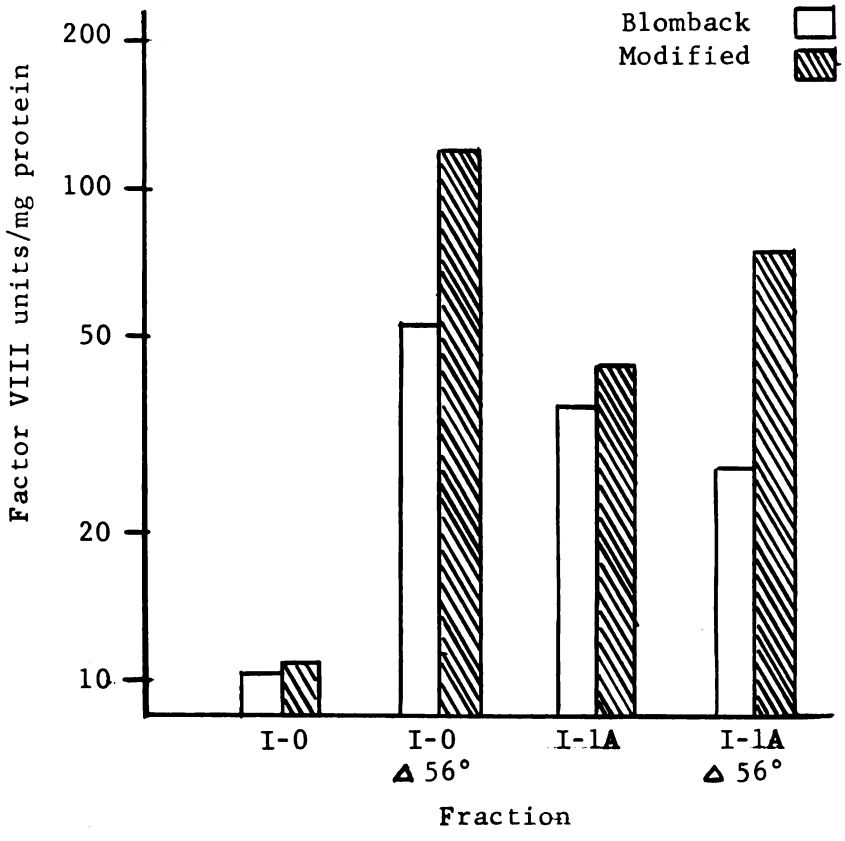

Fig. 8. Comparison of Blomback procedure and modified procedure (Mercer) on the basis of Factor VIII activity units/mg protein. (Second comparative study).

Figure 9a, b, and c summarize the three variables, protein content, activity, and specific activity of the various fractions. With this, as in the first run, the results showed that the differences in activity are significant, but not as striking, as in the first comparative study.

Table III compares the data of the two runs according to variations between individual comparable fractions from each run. There does not seem to be any set pattern to the variations between the individual materials. It must be remembered that strict comparisons of the two experiments were not possible due to the different assay systems used. The Blomback assay system was affected more by factors other than Factor VIII.

Table IV compares the two procedures from each study. Such comparison is not affected by variation in assay systems. The general trend is the same in both studies, showing that the material obtained by the modified procedure was superior to that obtained by the standard Blomback procedure, both on the basis of heat stability and specific activity of the partially purified Factor VIII preparation.

B. Antisera

1. Preparation of Antigens

Bovine and human plasma were diluted to a concentration of 1% protein with sterile 0.9% saline, and used for intravenous injection at this concentration.

Fig. 9-A

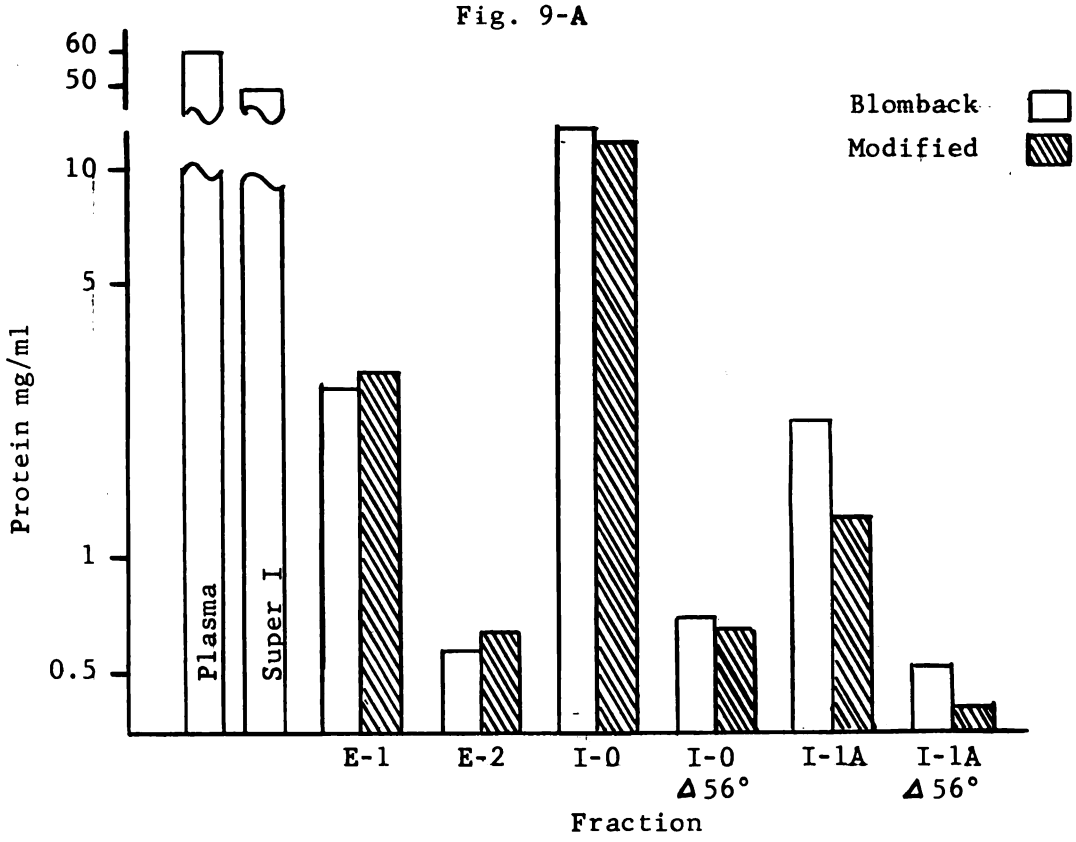

Fig. 9-B

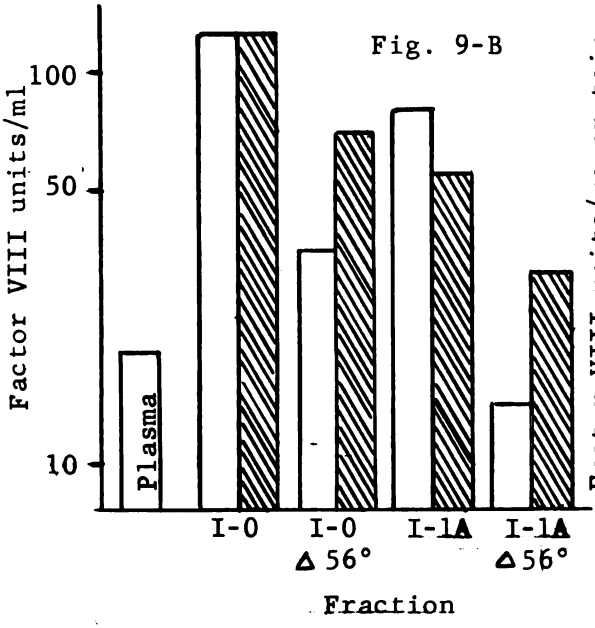

Fig. 9-C

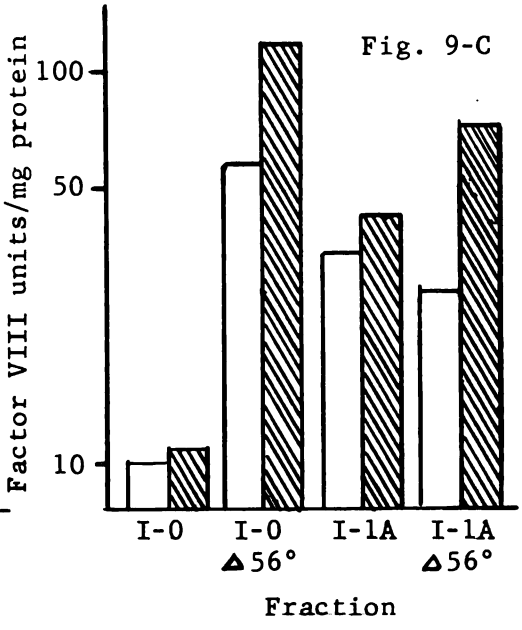

Fig. 9. Comparison of the Blomback and the modified procedure (Mercer) on the basis of protein mg/ml (Fig. 9-A), Factor VIII activity units/ml (Fig. 9-B), and Factor VIII activity units/mg protein (Fig. 9-C).

Table III. Comparison Between Two Comparative Studies (Runs 1 and 6)

Material	Run	Activity u/ml	% diff.	Spec. Activity u/mg	% diff.	Protein mg/ml	% diff.
Blomback Procedure							
Plasma	1	358	94.5	5.9	94.5	60.6	0.5
	6	19.6		0.326		60.3	
Fraction I-0	1	145	11	9.24	10	15.7	20
	6	129		10.3		12.55	
Fraction I-0 Δ 56°C	1	31.9	13	11.55	78	2.76	75
	6	36.7		52.2		0.69	
Fraction I-1A	1	41.5	49	35.8	21	1.15	48.5
	6	81.5		45.5		2.23	
Fraction I-1A Δ 56°C	1	14.6	2	62.4	53	0.234	54
	6	14.9		29.1		0.512	
Modified Procedure (Mercer)							
Plasma	1	358	94.5	5.9	94.5	60.6	0.5
	6	19.6		0.326		60.31	
Fraction I-0′	1	171	24.5	11.25	5	15.2	20.5
	6	129		10.63		12.1	
Fraction I-0′ Δ 56°C	1	79	0.5	28.8	76	2.74	76
	6	78.6		120		0.655	
Fraction I-1A′	1	42.8	21	173	76	0.247	80
	6	54		43.2		1.245	
Fraction I-1A′ Δ 56°C	1	12.28	59.5	361	79.5	0.033	92
	6	30.3		74		0.409	

Table IV. Comparison of the Blomback Procedure and the Modified Procedure (Mercer)

		Run 1						Run 6					
		Activity		Spec. Activity		Protein		Activity		Spec. Activity		Protein	
Material		u/ml	% diff.	u/mg	% diff.	mg/ml	% diff.	u/ml	% diff.	u/mg	% diff.	mg/ml	% diff.
Fr I-0	B	145	15	9.24	18	15.7	3	129	0	10.3	3	12.55	3.5
	M	171		11.25		15.2		129		10.63		12.1	
Fr I-0 Δ56°C	B	31.9	59.5	11.55	61	2.76	0.5	36.7	53	52.2	56.5	0.69	5
	M	79		28.8		2.74		78.6		120		0.655	
Fr I-1A	B	41.5	3	35.8	79.5	1.15	53.5	81.5	34	45.5	5	2.23	44
	M	42.8		173		0.247		54		43.2		1.245	
Fr I-1A Δ56°C	B	14.6	16	62.4	83	0.234	86	14.9	51	29.1	61	0.512	20
	M	12.28		361		0.033		30.3		74		0.409	

Bovine Factor VIII was prepared by methods described, and consisted of heat-treated Fraction I-1A'. Due to the low protein concentration of the Factor VIII preparation, it was concentrated two fold by dialysis against Carbowax, giving a final protein concentration of .09%.

2. Immunization and Testing

Twenty-one normal rabbits (7 rabbits per antigen) were each injected with 2 ml of soluble antigen in the marginal ear vein every 48 hours for a total of nine injections. One week after the last injection, the rabbits were test bled by heart puncture, removing 5 to 10 ml of blood. The sera obtained were clarified by centrifugation and heat treated for one hour at 56°C. The heat-treated sera were titered using the interfacial precipitin test, using the corresponding antigen and the heterologous antigen to check for cross reactions. All rabbits showing the highest antibody titers were bled by heart puncture, removing 40 to 50 ml of blood. The sera obtained were processed and stored frozen at -40°C in small aliquots for testing in immunoelectrophoretic procedures.

At the end of the seventh week, the high titer rabbits were again test bled by heart puncture, and the sera obtained processed and titered. Due to the significant drop in antibody titer, a series of five booster injections (2 ml of antigen per injection) was given over a 10-day period.

One week after the last injection, the rabbits were test bled and the resulting sera processed and titered. There was a significant rise in antibody titer after the booster injections.

At the end of the thirteenth week, a booster injection series was initiated (2 ml of antigen per injection). Four injections were given over a period of eight days. One week after the last injection, the rabbits were test bled and titered. Rabbits having the highest titers were bled on the same day by heart puncture, removing 40 to 50 ml of blood. The sera obtained were clarified, heat treated at 56°C for one hour, and stored frozen at -40°C. Figure 10 summarizes the variations in the highest antibody titers obtained over the 15-week period.

3. Preparation of Anti-Bovine Factor VIII by Repository Immunization

The relatively low anti-Factor VIII titers obtained from the injection of soluble antigens were probably due to the low circulating level of antigen, and required the use of more effective methods.

a. Preparation of Antigens

Two-hundred fifty ml of bovine Factor VIII (Fraction I-1A Δ 56°C) were concentrated 8.4 fold by dialysis against Carbowax. The concentrated Factor VIII was emulsified with an equal volume of Freund's complete adjuvant and injected immediately after emulsification.

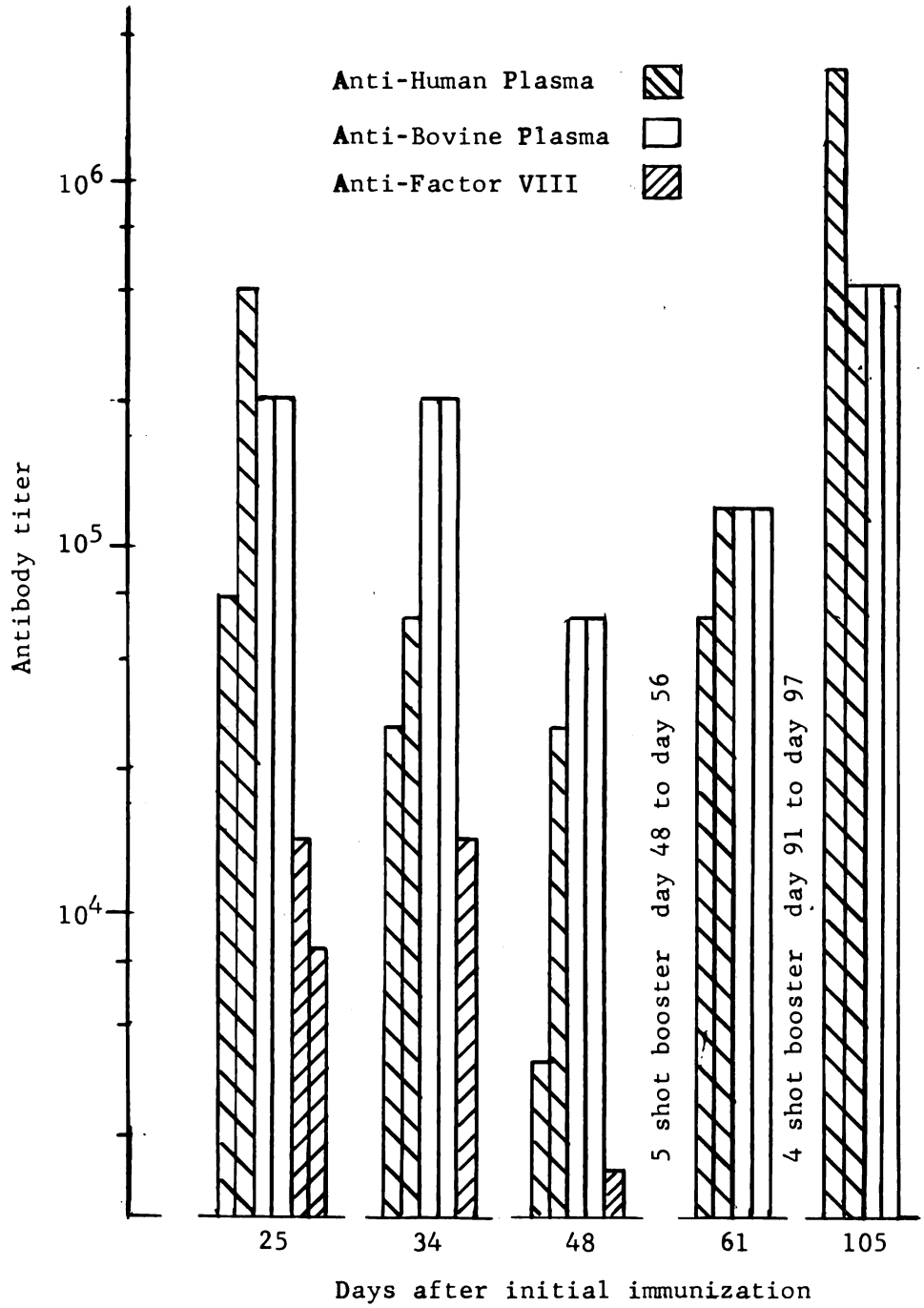

Fig. 10. Variations in <u>in vivo</u> antibody titers over a fifteen-week period.

b. Immunization and Testing

Five normal rabbits were each injected once with 1 ml of antigen-adjuvant emulsion by various routes. These routes were subcutaneous neck and ears, subcutaneous foot pads and ears, and deep intramuscular. The injections were made at five sites, with 0.2 ml of emulsion per injection site.

Six weeks after injection, the rabbits were bled by heart puncture, removing 5 to 10 ml of blood. The sera obtained were clarified and heat treated at 56°C for one hour. Antibody titers were determined against bovine Factor VIII, bovine plasma and human plasma, using the interfacial precipitin test. The titers obtained were somewhat better than those obtained by immunizations with soluble antigen preparations. There was no observable cross immunization to human material detected. Table V summarizes the titers obtained using bovine Factor VIII and bovine plasma as the test antigens.

Table V. Antibody Titers from Injection of Adjuvant Enriched Bovine Factor VIII Six Weeks After Injection

Injection Route	Titers	
	vs. Bovine Plasma	vs. Bovine Factor VIII
Subcutaneous	1:3000	1:20,000
Foot Pads	1:64,000	1:80,000
Deep Intramuscular	1:16,000 1:32,000	1:20,000 1:80,000

C. Immunoelectrophoretic Analysis of Bovine Factor VIII

Immunoelectrophoresis in agar was performed on several of the partially purified fractions of bovine Factor VIII. Due to the low concentration of the original material and the dilution suffered during electrophoresis, the precipitin lines formed were quite faint, but still visible. The best patterns obtained were those developed with antibovine plasma antisera.

Studies were made on fractions at various stages of purification to demonstrate the effect of the purification steps on the immunologically identifiable components present at the various stages of the purification procedures. Table VI summarizes the results of the immunoelectrophoretic analysis on the various fractions tested.

Table VI. Results of Immunoelectrophoretic Analysis of Plasma and Factor VIII Preparations at Various Stages of Purification

Antigen	Antisera Patterns	
	vs. Anti-bovine Plasma	vs. Anti-bovine Factor VIII
Plasma	Plasma Pattern	2 lines
Fraction I-0'	3 lines α, β, γ	2 lines γ absent
Fraction I-1A'	2 lines α, β	2 lines α, β
Fraction I-1A 56°	2 lines α, β	2 lines α, β
Supernatant I-1A	1 line	1 line

D. Gel Electrophoretic Studies

Starch gel electrophoresis was performed on Fraction I-0' and Fraction I-1A' before and after heat denaturation of fibrinogen. The stained gel showed the presence of two components in the materials before heat denaturation. The major component (probably fibrinogen) was present in both fractions before heat denaturation. After denaturation, the major component was diminished, but not absent from Fraction I-0', but was completely removed from Fraction I-1A'. Figure 11 is a graphic representation of starch gel electrophoresis patterns.

The inclusion of urea in starch gels used for electrophoresis was reported to improve the resolution of protein components during electrophoresis (Wake and Baldwin, 1961). Starch gels were prepared to contain a final concentration of 7M urea, and used to determine the number of protein components present in purified bovine Factor VIII preparations in various stages of purification. There were many mechanical difficulties encountered in the urea gel electrophoresis procedure, some caused by improper design of the gel mold. The major problem involved the cross contamination of samples in adjacent sample wells. These difficulties rendered the results of some analyses useless.

The important finding from these determinations was that Fraction I-1A' $\triangle$ 56°C was resolved into three electrophoretic components of approximately equal concentrations. Figure 12 is a graphic representation of urea starch gel electrophoresis patterns.

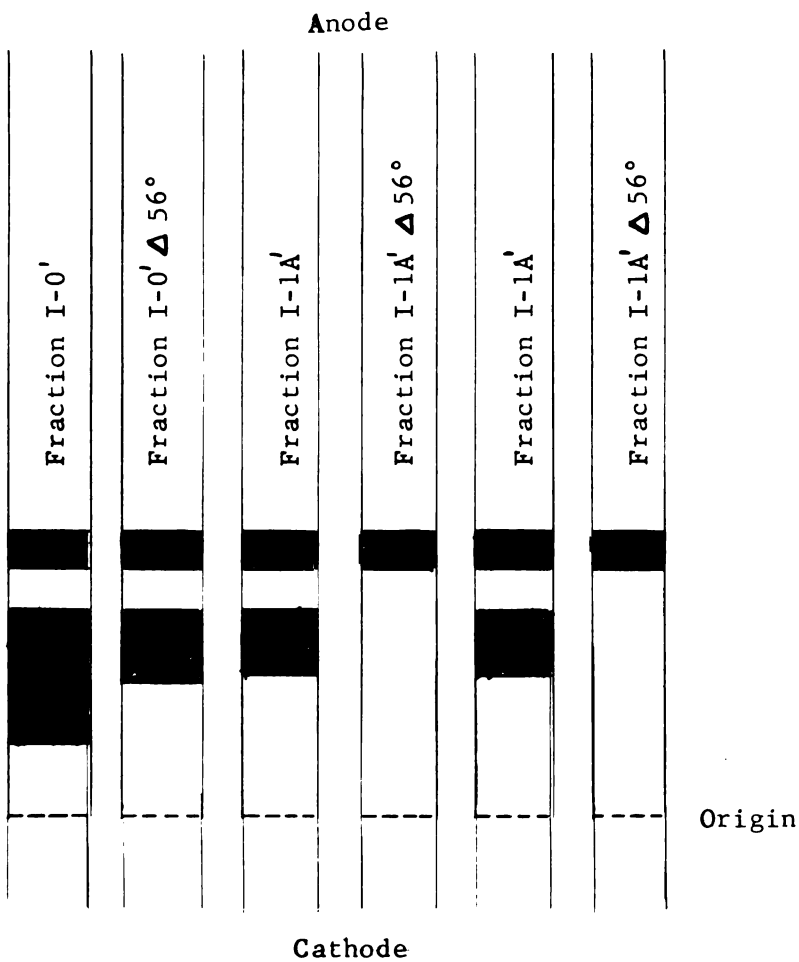

Fig. 11. Starch gel electrophoresis patterns of bovine Factor VIII at various stages of purification.

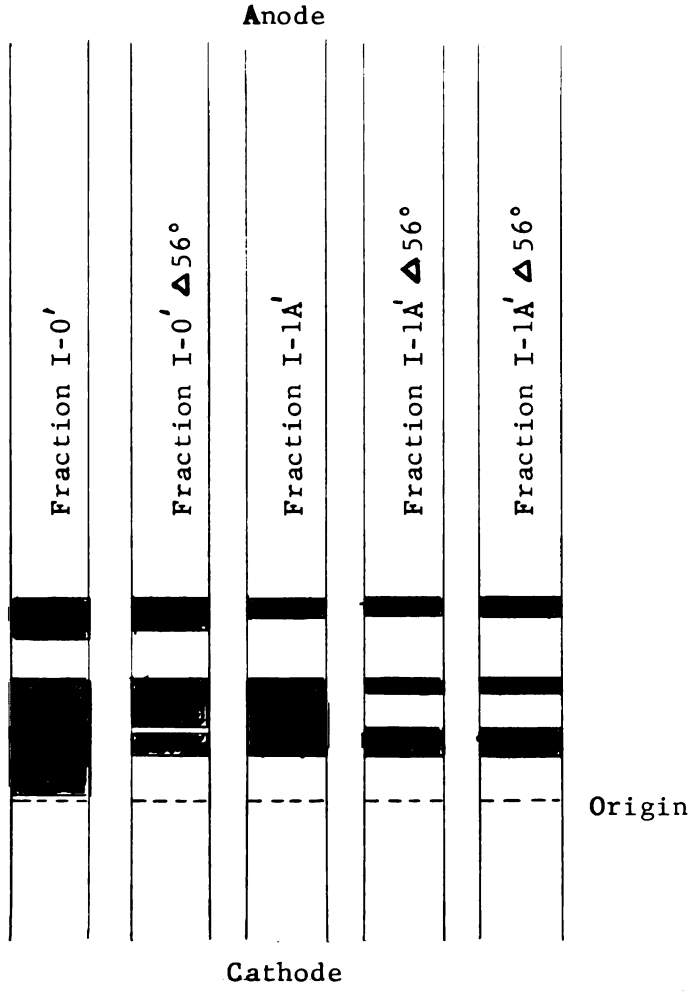

Fig. 12. Urea starch gel electrophoresis patterns of bovine Factor VIII at various stages of purification.

No attempt was made to determine at this point which component or components contained Factor VIII activity. We must tentatively conclude from these results that further purification beyond the Fraction I-1A'Δ 56°C stage is needed to reduce, if possible, bovine Factor VIII to a single electrophoretic component.

E. Gel Filtration of Bovine Factor VIII

Urea gel electrophoretic and immunoelectrophoretic studies showed that highly purified bovine Factor VIII preparations (Fraction I-1A'Δ 56°C) consisted of at least three components. No attempts have been made to show at this point in which of these components the Factor VIII activity is concentrated. For this purpose, the components must be separated in sufficient quantity to study this aspect. Separation was attempted by gel filtration studies using G-200 Sephadex in the hope that the molecular size difference of the components would be sufficient to permit separation in this resin.

Three gm of dry G-200 Sephadex was allowed to hydrate for four days in 150 ml of pH 6.8 Imidazole-saline buffer at room temperature. A column was established and equilibrated in a 1 meter by 1.2 cm glass tube to a total bed height of 0.71 meters or a total bed volume of 80.5 cc. The void volume of the column was estimated at 24 cc based on the manufacturer's data for this gel.

A 10 ml sample of bovine Factor VIII (Fraction I-1A'Δ 56°C for 5 minutes) was applied to the column, and eluted with the

equilibration buffer (Imidazole-NaCl). Fractions were collected in the amount of 1 ml per fraction at an average flow rate of 0.1 ml per minute. The protein peaks were located by determining the absorbancy of selected fractions at 280 mu. The protein peaks were visualized by plotting absorbancy (ordinate) vs fraction (abscissa).

Due to the optical interference of the Imidazole ring structure at 280 mu, subsequent gel filtration studies were performed using the column equilibrated against 0.055M citrate buffer at pH 6.8. All fractions containing protein were assayed for Factor VIII activity, and selected fractions analyzed by immunodiffusion and urea gel electrophoresis.

In the initial gel filtration studies with G-200 Sephadex (in Imidazole buffer), there was a considerable loss of protein. This loss seemed to be due to a fraction insoluble in the cold, precipitating and accumulating at the top surface of the resin bed. The nature of this material was studied to see what effect its removal would have on the activity of the purified preparation. This was accomplished in the following experiment.

Fraction I-1A Δ 56°C was cooled to the range of 0-4°C over a period of 30 minutes to precipitate the cryoglobulins, which were then removed by centrifugation at 20,000 x G for 30 minutes at a temperature of 0-2°C. The supernatant material was decanted and the remaining precipitate redissolved to one-fourth the original sample volume with saline. The starting material, the supernatant fraction, and the redissolved cryoglobulin fraction were assayed for

Factor VIII activity, and analyzed by immunodiffusion techniques. The supernatant material was used as the starting material in gel filtration studies. Assays performed on the materials obtained from the cryoglobulin removal studies are summarized in Table VII.

The data showed that there was an average loss of 17% of the Factor VIII activity in the cryoglobulin removal step that was not recovered in the redissolved precipitate. This is possibly explained by the poor resolution of the precipitate, or by irreversible denaturation.

The interesting aspect of these data was the fact that there was no detectable loss of protein with the removal of the cryoglobulin. This cold insoluble material had the characteristics of a cryoglobulin, but there was insufficient material available for analysis and identification as a cryoglobulin.

Figure 13 shows a typical elution pattern of protein from the G-200 Sephadex column. Superimposed on this is the pattern of assayable Factor VIII activity in the eluate fractions, the dotted portions were plotted to visualize the two closely associated peaks appearing in the eluate fractions.

It can be readily seen from examination of Figure 13 that the starting material was partially separated into at least two components. The slight shouldering tendency of the trailing edge of the major peak might indicate a possible third component. Second, the Factor VIII activity peak is coincident with the major protein peak.

Table VII. Comparison of Factor VIII Activity of Fraction I-1A Before and After Cryoglobulin Removal

Material	Lot 2				Lot 4			
		Factor VIII Activity				Factor VIII Activity		
	Protein mg/ml	units/ml	units/mg Protein	Recovery %	Protein mg/ml	units/ml	units/mg Protein	Recovery %
Fraction I-1A 56°C	.275	71.5	260	100	.281	191	680	100
Fraction I-1A Cryoglobulin Supernatant	.275	60.0	218	84.5	.281	155	553	81.5
Cryoglobulin Precipitate Redissolved	0	1.17	-	-	0	0.88	-	-
Corrected to Original Volume		0.292	-	0.41	-	0.22	-	0.115

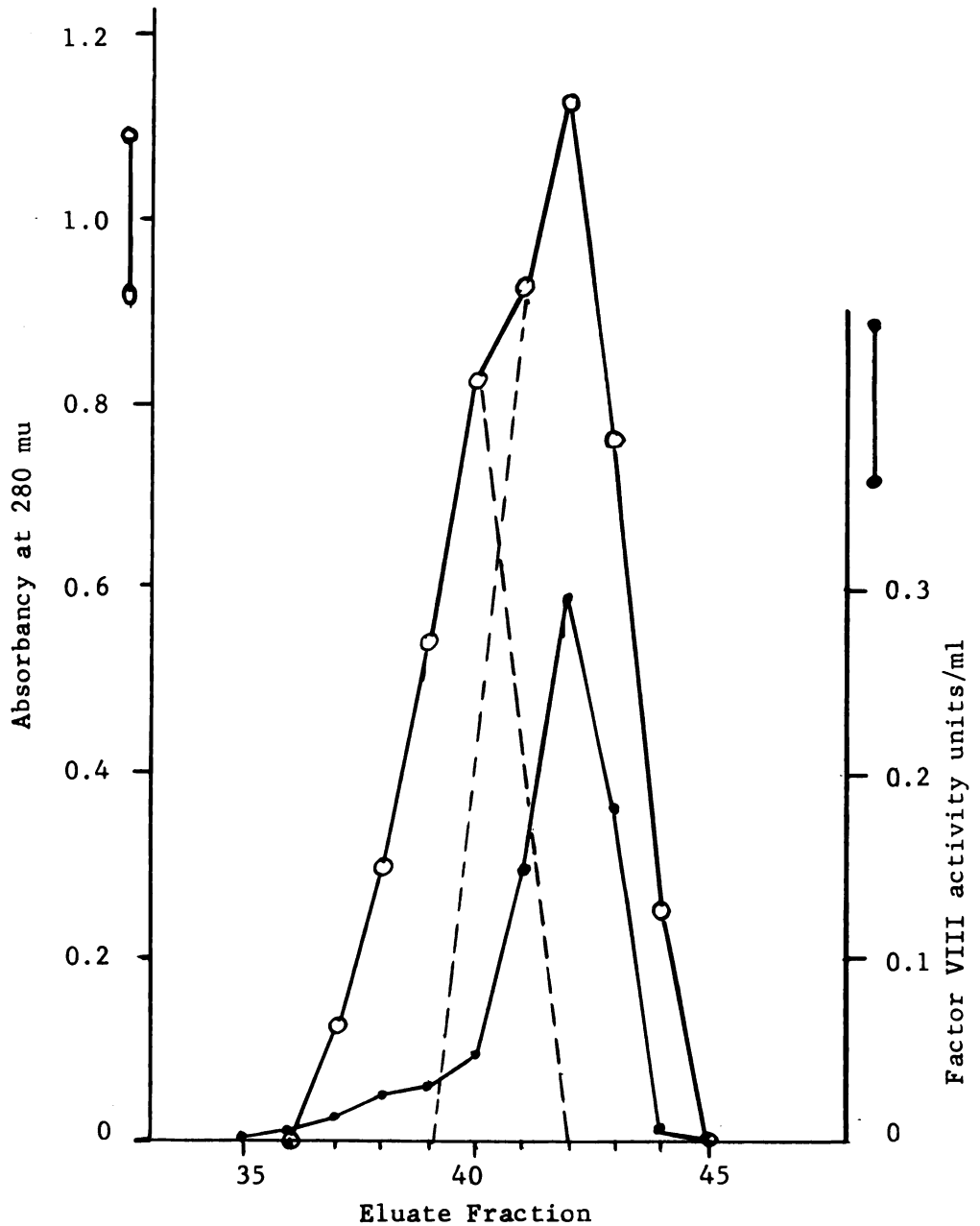

Fig. 13. Elution patterns of protein and Factor VIII activity form gel filtration of Factor VIII (Fraction I-1A 56°) on G-200 Sephadex.

Immunodiffusion studies were performed on the fractions from the cryoglobulin study, and on selected eluate fractions from the gel filtration studies. The support medium for immunodiffusion was cellulose acetate (Millipore Cellotate), and the precipitin lines were developed with antibovine Factor VIII serum. Table VIII summarizes the results of the immunodiffusion studies.

Table VIII. Results of Immunodiffusion Studies on Factor VIII Preparations After Heat Treatment, Cryoglobulin Removal, and Gel Filtration

Material	Number of Precipitin Lines
Fraction I-1A'	3
Fraction I-1A' Δ 56°C	3
Fraction I-1A' Δ 56°C Cryoglobulin Supernatant	3
Cryoglobulin Fraction	1
Eluate Fractions:	
Fraction 38 fast component	1
Fraction 41 mixed peaks	2
Fraction 42 slow component peak	1
Fraction 43 slow component	1

These data confirm that there are two distinct components present in the eluate fractions, and that their separation, however incomplete, has been affected. Complete separation might be possible by applying a smaller sample volume to the column. Thus, assuming the gel filtration studies are valid, bovine Factor VIII has been isolated in small quantities as a single immunologic entity. More

extensive studies of this aspect of the purification of bovine Factor VIII preparation are being planned, using refinements and extension of these techniques.

F. Tyrosine-Tryptophane Ratios

Tyrosine-tryptophane ratios were determined on Fraction I-0' and Fraction I-1A' from all but the first lot by the method of Beavan and Holiday (1952). Table IX gives the values of the calculated constants, molar ratios and molar concentrations of tyrosine and tryptophane for all determinations.

The data show there was greater variation in molar concentrations of tyrosine and tryptophane than in the molar ratios. These variations would indicate that the individual lots are significantly different.

G. Amino Acid Analysis

1. Glycine Removal

Four lots were filtered through G-25 Sephadex to separate the protein and contaminant glycine carried over during the fractionation procedure. This step was necessary to insure that glycine content, determined by amino acid analysis, was not in error due to the presence of free glycine. Figure 14 shows a typical elution pattern obtained from gel filtration of Fraction I-1A' Δ 56°C through G-25 Sephadex. The eluates representing the protein peak (Fractions 65-110) were pooled, and the protein concentrated by precipitation with 5% (v/v) final concentration of trichloroacetic acid, in three

Table IX. Results of Tyrosine-Tryptophane Ratio and Content Determination by the Beavan and Holiday Method

Lot No.	Material	Protein mg/ml	Molar Absorptivity a		Molar Absorption Constant K		Tyrosine Molar Concen.	Tryptophane Molar Concen.	M Tyro. / M Tyrp. From K	M Tyro. / M. Tryp. From OD
			280 mu	294.4 mu	280 mu	294.4 mu				
2	Fr. I-0'	0.360	1.516	1.217	3.49	2.803	0.741	0.442	1.676	1.697
3	"	0.339	1.598	1.269	3.68	2.923	0.763	0.471	1.619	1.62
4	"	0.347	1.551	1.231	3.57	2.835	0.739	0.457	1.617	1.62
5	"	0.345	1.505	1.197	3.47	2.757	0.720	0.443	1.625	1.62
6	Fr. I-0	0.390	1.565	1.246	3.60	2.870	0.749	0.462	1.621	1.63
	Average		1.547	1.232	3.56	2.837	0.742	0.455	1.632	1.637
2'	Fr. I-1A 56°C	0.105	0.94	0.805	2.17	1.86	0.529	0.255	2.07	2.03
3'	"	0.298	1.73	1.58	3.986	3.645	1.11	0.429	2.59	2.63
4'	"	0.293	1.49	1.34	3.44	3.08	0.92	0.38	2.41	2.31
5'	"	0.127	2.345	2.071	5.4	4.77	1.4	0.609	2.3	2.29
6'	"	0.234	1.6	1.41	3.69	3.25	0.945	0.418	2.32	2.32
6	Fr. I-1A 56°C	0.38	1.655	1.475	3.8	3.4	1.01	0.421	2.4	2.34
	Average		1.626	1.447	3.748	3.334	0.819	0.419	2.45	2.32

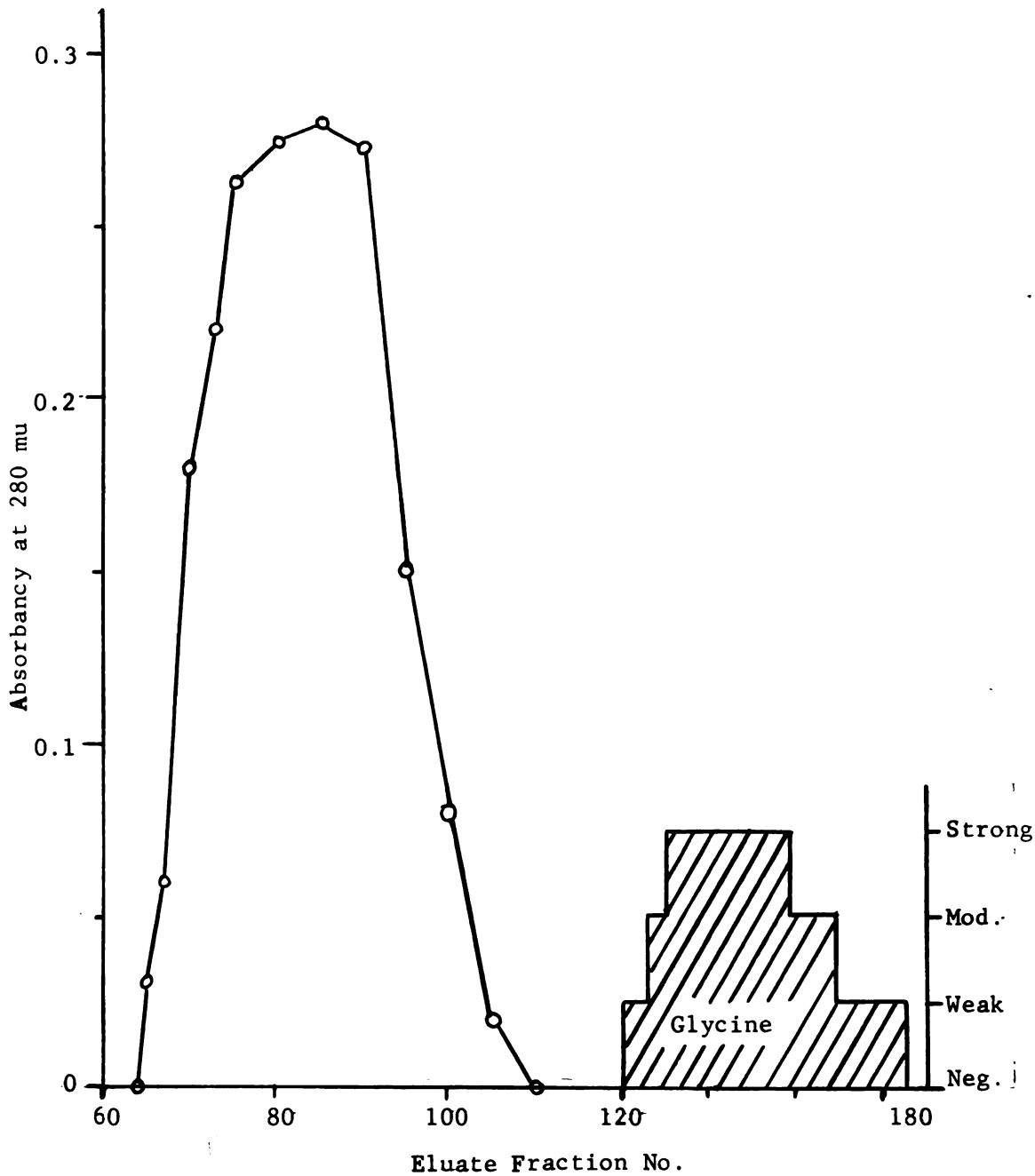

Fig. 14. A typical elution pattern obtained from the separation of Factor VIII and free glycine by gel filtration through G-25 Sephadex.

aliquots. Two aliquots were used for protein determinations by micro-Kjeldahl, and the other was submitted for amino acid analysis.

2. Amino Acid Analysis

Three of the four samples submitted for amino acid analysis were analyzed for amino acid content. The other sample was ruined during drying of the hydrolyzed sample. The resulting chromatograms of the three lots were analyzed by the half height method to determine the content of the individual amino acids. The amino acid contents (mg amino acid/gram of protein) were derived from the protein content of the original sample before hydrolysis. Corrections for buffer dilution of the hydrolyzed samples were made to determine protein content of the samples applied to the resin columns of the amino acid analyzer. In the case of one sample (Lot 2), the total weight of recovered residue was 30% lower than expected recovery based on protein content of the original sample.

It was thus assumed that the original protein value was in error, and the calculation of amino acid content (mg/gram protein) was based on actual recovery. Table X summarizes the data of amino acid analysis.

Nitrogen content of the three lots was calculated from the amino acid analysis data. The calculations were made after making the necessary corrections for the nitrogen content of lysine, histidine, arginine and tryptophane, and were based on the total recovered amino acids. The results of these calculations are summarized in Table XI.

Table X. Results of Amino Acid Analysis of Three Lots of Bovine Factor VIII

Amino Acid	Lot 2		Lot 6'		Lot 6	
	mg/gm* Prot.	m-mole per gm	mg/gm Prot.	m-mole per gm	mg/gm Prot.	m-mole per gm
Lysine	40.79	.279	41.1	.281	46.6	.319
Histidine	20.17	.13	20.35	.131	19.5	.126
Ammonia	16.14	.948	14.9	.875	18.3	1.08
Arginine	60.62	.348	62.6	.359	64.1	.378
Aspartic Acid	88.91	.668	94.5	.711	97.4	.73
Threonine	83.67	.702	85.7	.72	89.1	.747
Serine	66.84	.636	63.25	.602	69.6	.663
Glutamic Acid	125.8	.885	125.3	.852	131.1	.89
Proline	74.03	.643	63.2	.55	67.1	.583
Glycine	44.87	.597	47.6	.635	48.8	.651
Alanine	31.36	.352	30.75	.345	31.4	.353
Half Cystine	32.56	.271	21.45	.179	24.1	.201
Valine	72.51	.691	66.9	.562	71.6	.61
Methionine	11.79	.079	13.5	.091	11.95	.08
Isoleucine	38.83	.295	38.8	.296	40.5	.309
Leucine	58.24	.444	51.3	.10	59.2	.453
Tyrosine	57.07	.315	54.75	.302	57.1	.316
Phenylalanine	33.0	.201	31.9	.195	31.4	.191
Tryptophane**	31.65	.155	26.6	.13	27.5	.135
Totals	988.8		954.45		1006.35	

* Lot 2 data based on calculated recovery.

**Calculated from molar ratio data.

Table XI. Nitrogen Content of Three Lots of Factor VIII Calculated from Amino Acid Analysis Data

Lot No.	Total Amino Acids Micrograms	Total Nitrogen Micrograms	% Nitrogen
2'	988.8	139.5	14.1
6'	1,222.4	174.5	14.25
6	1,762.3	251.3	14.25

Comparison of the data of amino acid analysis (Table X) with the results of tyrosine-tryptophane ratio and content obtained by the method of Beavan and Holiday (1952), in Table IX, show significant differences in regard to tyrosine and tryptophane content. Table XII summarizes these variations in results obtained by the two methods.

Table XII. Comparison of Tyrosine and Tryptophane Content from Amino Acid Analysis and the Beavan and Holiday Technique

	Amino Acid Anal.		Beavan and Holiday		Difference	
	1	2	3	4		
Lot No.	Tyro. m-moles per gm	Tryp. m-moles per gm	Tyro. m-moles per gm	Tryp. m-moles per gm	Tyro. $\frac{3}{1}$	Tryp. $\frac{4}{2}$
2	.315	.155	.529	.255	1.67	1.64
6'	.302	.13	.945	.418	3.13	3.21
6	.316	.135	1.01	.421	3.2	3.12

Because of these differences, another spectrophotometric method for the determination of tyrosine and tryptophane molar ratios and content was investigated. In the past, this procedure had shown good correlation with tyrosine content determined on the amino acid analyzer. The method was first described by Bencze and Schmid (1957) as an improvement on the earlier methods of Holiday (1936 and 1938), and Goodwin and Morton (1946), by eliminating errors caused by the bathochromic shift of absorption spectra of tyrosine and tryptophane. The method consists of determining the absorption spectrum between 270 mu and 350 mu. The slope of a line tangent to the two characteristic maxima of the absorption curve was calculated as follows:

$$\text{Slope (S)} = \frac{(\Delta A/\Delta \text{mu}) \times 10^3}{A \text{ max}}$$

where: A = absorbance

A max = maximum absorbance

mu = wave length

Figure 15 is a typical example of the spectrum and tangent line used in this method.

The extinction coefficient ($E^{1\%}_{1\ cm}$) and molar ratio of tyrosine-tryptophane (R) were derived from a table of values prepared from determinations on known mixtures of the two amino acids. Where necessary, the values were obtained by interpolation for values of S not in the table.

The total concentration of tyrosine and tryptophane was calculated as follows:

$$\text{C tyrosine + tryptophane\%} = \frac{A \text{ max}}{E^{1\%}_{1\ cm}}$$

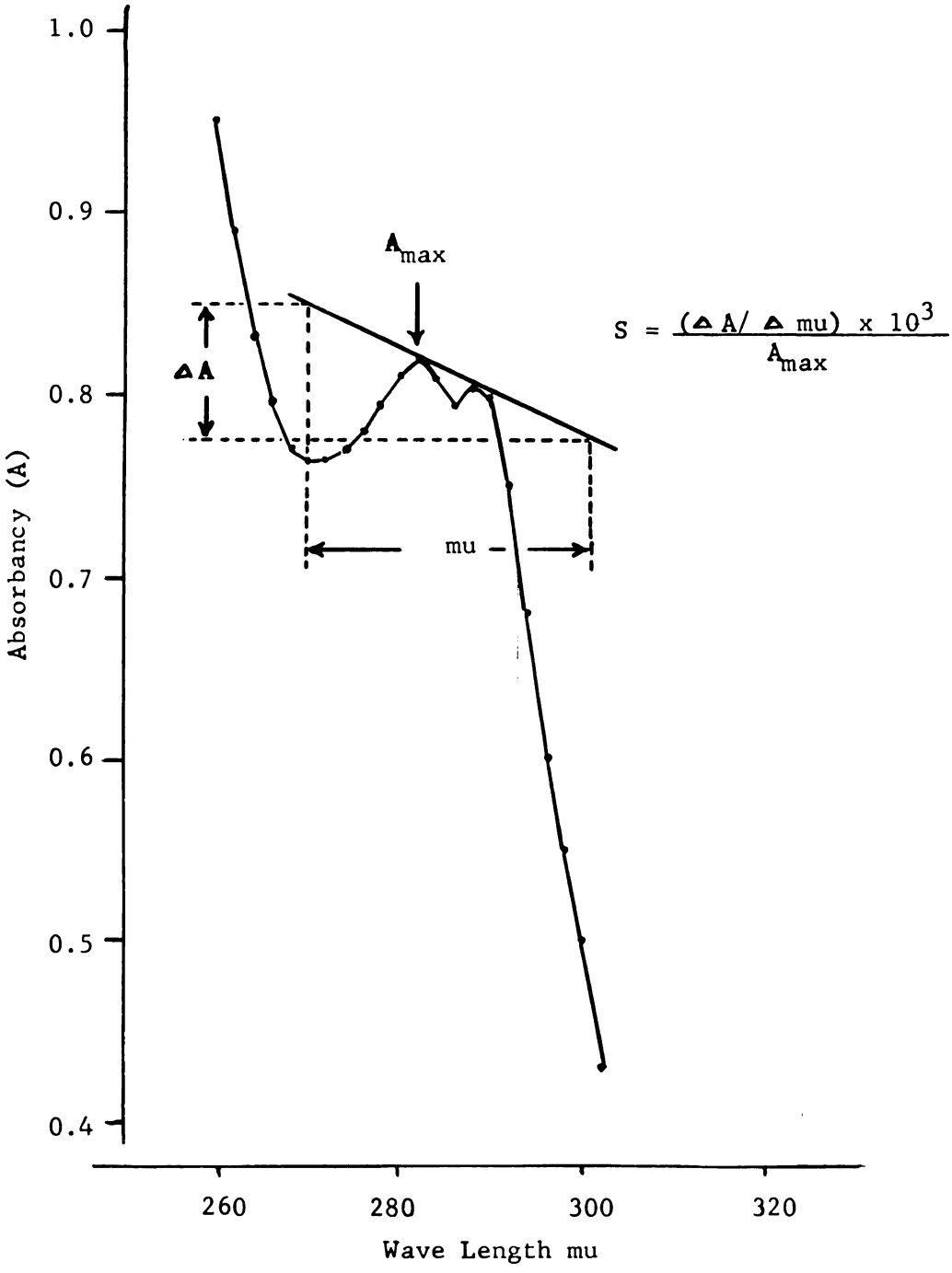

Fig. 15. A portion of a typical absorption spectrum as used to calculate tyrosine-tryptophane ratios by the method of Bencze and Schmid (1957).

The molar concentrations of tyrosine and tryptophane were calculated using molar ratios and total concentration data. Table XIII summarizes the calculation of tyrosine-tryptophane ratios and content by the above method. Inspection of the data shows even greater disagreement than that obtained by the method of Beavan and Holiday (1952).

The lack of agreement of the three methods indicates that one of two situations exists: 1) one method is correct and the other two are in error, or 2) all three methods are in error. It must be pointed out, however, that when the original calculations of amino acid analysis data were made, the molar ratios determined by the Beavan and Holiday (1952) technique were used to determine tryptophane content. Re-examination of these data shows that the values obtained for tryptophane, assuming the protein determinations were accurate, gave good correlation with the total protein recovered. For example, in the case of Lot 6, 1.75 mg of protein was placed on the column and 1.762 mg was recovered. According to the calculations of the chromatograms, this total includes the calculated value of tryptophane based on the molar ratio. This represents an error of 0.6%. For Lot 6', the recovery error was 4.5%, and Lot 2, 1.1%. Thus, it seems that the ratios obtained by the Beavan and Holiday (1952) technique are reasonable, while the molar concentrations calculated are not in agreement with the amino acid analysis data. Due to the limited amounts of material, this disagreement could not be resolved by determination of tyrosine content by other available techniques (i.e., the Folin-Ciocalteau phenol method).

Table XIII. Calculation of Tyrosine-Tryptophane Molar Ratios and Concentrations

$$\text{Slope} = \frac{(\ A/\ mu) \times 10^3}{A_{max}} \qquad C_{\text{Tyrosine-Tryptophane}\%} = \frac{A_{max}}{E^{1\%}_{1\ cm}}$$

Lot No.	Protein mg/ml	Slope S	$E^{1\%}_{1\ cm}$	R Tyrosine / Tryptophane Molar	A_{max}	C Tyro.+Tryp. mg/gm	Tyrosine m-moles per gm x 10^3	Tryptophane m-moles per gm x 10^3
2'	0.495	-14.45	248.8	0.212	0.814	3.28	2.48	13.5
4'	0.368	- 5.88	201	0.72	0.425	2.11	4.56	6.29
5'	0.316	- 3.67	191.4	0.921	0.725	3.79	9.42	10.2
6'	0.302	- 2.87	189.19	0.994	0.635	3.36	8.7	8.73
6	0.479	- 2.72	188.7	1.008	0.875	4.64	12	12

V. DISCUSSION

A. Modification of the Blomback Procedure

The purification of Factor VIII from human or bovine plasma requires that it initially be isolated in crude form. The isolation of the crude factor is probably the simplest of all the steps required in the purification of this protein. The initial preparation is contaminated with most of the other plasma proteins either as trace components occluded in the precipitate, (i.e., albumins, α, β and γ globulins) or as coprecipitates in large quantities, (i.e., fibrinogen). Blomback (1958) devised methods for removal of the trace contaminants by extraction of the crude precipitates with glycine-containing buffers, and developed methods for the partial separation of Factor VIII and fibrinogen (Factor I) which is truly the major component of the crude preparation.

The product (Fraction I-1A) resulting from this partial separation step is still grossly contaminated with fibrinogen, containing about 15% of the fibrinogen initially precipitated in the crude fraction (Blomback, 1958).

The removal of fibrinogen and the other contaminants from Fraction I-1A while maintaining Factor VIII activity has been the prime goal of the present research. The removal of fibrinogen from protein solutions is readily accomplished by heat treatment at 56°C for a period of 2 to 5 minutes, depending on the fibrinogen content.

When this procedure was used on fractions prepared according to the Blomback procedure, significant loss of Factor VIII potency was encountered. At the Fraction I-0 stage, the loss of activity due to heating varied from 72 to 78%, and resulted in a 1.25 to 5 fold increase in specific activity. At the Fraction I-1A stage, there was loss of activity due to heating, varying from 65 to 82%, and an 0.8 to 1.7 fold increase in specific activity. The observations of Simonetti et al. (1961) that citrate ions have an adverse effect on the heat stability of Factor VIII preparations, seemed to be a logical starting point for modification of the Blomback procedure.

The first comparative study of the Blomback procedure and the initial modification, was made to test the validity of Simonetti's observations. The results of this study showed that there was a reduction in activity loss due to heat treatment at 56°C for two minutes. This aspect of the modification was overshadowed by an unexpected, yet more significant, gain in apparent purification of the heat-treated material as seen in the increased specific activity. Table XIV summarizes the differences of the Blomback and initial modified procedure in regard to the effect of heat treatment.

Table XIV. Comparison of the Effects of Heat Treatment for Two Minutes at 56°C of Fraction I-0 and Fraction I-1A, Prepared by the Blomback Procedure and the Initial Modified Procedure (Mercer)

	Procedure			
	Blomback		Init. Mod. (Mercer)	
Fraction Heat Treated at 56°C	Factor VIII Activity Loss %	Specific Activity Increase	Factor VIII Activity Loss %	Specific Activity Increase
Fraction I-0	78	1.25	54	2.6
Fraction I-1A	65	1.7	71	4.8

Thus, it is apparent that although the data on Fraction I-0 substantiates the observation of Simonetti, the more important aspect is the actual increased purification obtained by reduced citrate levels in the initial modified procedure.

The complete modification, which entailed the elimination of citrate from the purification procedure, resulted in even smaller heating losses of Factor VIII activity.

Table XV summarizes the differences in the Blomback procedure and the modified procedure (second comparative study) in regard to the effects of heat treatment of fractions at 56°C for five minutes.

Table XV. Comparison of the Effects of Heat Treatment for Five Minutes at 56°C of Fraction I-0 and Fraction I-1A Prepared by the Blomback Procedure and the Modified Procedure (Mercer)

Fraction Heat Treated 56°C 5 Minutes	Procedure			
	Blomback		Modified (Mercer)	
	Activity Loss %	Specific Activity Increase	Activity Loss %	Specific Activity Increase
Fraction I-0	72	5	29	11.25
Fraction I-1A	82	0.8	33	1.7

As in the first study, the data showed that the significant advantages of citrate reduction or elimination were decreased heating losses and increased purification of heat-treated materials. The significance of the modification is further substantiated by examination of the data in Tables III and IV which compare the two studies. Both studies show

the same trends of increased activity and purification of Factor VIII by the modified procedure. The variations between results of the two experiments appear to be, in part, a function of the variation in assay systems.

Shinowara (1966) studied thermal denaturation of Factor VIII activity in crude (Fraction I) and highly purified Factor VIII preparations. His experiments were carried out in the presence of citrate-phosphate buffer at pH 7.3, ionic strength .092. Although the citrate concentration used by Shinowara was lower than that used in the Blomback procedure, the results of thermal denaturation compared with those obtained with the Blomback Factor VIII preparations (See Tables XIV and XV) are strikingly similar.

Shinowara reported that after five minutes at 56°C, only 15% of the original Factor VIII activity remained. It would be interesting to know what effect the absence of citrate would have had on his findings.

From his studies, he has postulated the presence of two forms of Factor VIII having different rates of thermal denaturation; the less labile D form had a thermal half life of six minutes and the labile M form a thermal half life of 0.6 minutes. The data obtained in this present study on bovine Factor VIII shows that thermal stability of Factor VIII is greatly dependent upon the ionic species present in the solution. Although no attempts were made to determine the "thermal" half life of Factor VIII, it has been established that the presence or absence of citrate certainly influences thermal stability of Factor VIII, and that in the absence of citrate, the half life is certainly longer than five minutes.

The results of the four fractionation runs with the modified procedure (See Table II) show some variations in protein content of the various fractions. These variations are based on variation in the starting plasma (individual variations of animals). The small variations of protein distribution in the fractions, as indicated best by protein recovery values, show that there was only minor variability between runs. The greatest variation seemed to be found in the fluctuation of Factor VIII activity from run to run, which again can be partly attributed to individual variations among animals. This is indicated by variations in the Factor VIII activity of the starting plasmas.

There is, however, one disturbing fact shown by the data. That is the poor recovery of activity obtained in the final product. These great losses in activity during the purification procedure can be attributed to any number of causes, i.e., mechanical loss in precipitation of the crude fraction in that 9% is lost in Super I and only 45% is recovered at the Fraction I-0 stage. The goal of this present work was to obtain significant purification regardless of such losses.

B. Preparation of Antisera

Antisera prepared against bovine plasma and human plasma were of good quality and titer. These antisera proved valuable in purity determinations of Factor VIII preparations. The antiserum against bovine plasma was the most valuable in determining the number of components present in purified Factor VIII preparations. In many cases,

these same determinations were not possible with the specific antisera prepared against Factor VIII.

The preparation of antisera against bovine Factor VIII was hampered by the low protein content of the injected antigen, resulting in low circulating levels of antigen. Distribution studies were not made on the injected antigen, but had such studies been made, the results would probably have shown that the disappearance rate of antigen was such that inadequate levels of antigen were maintained for full antibody response.

The antisera obtained following the injection of adjuvant-Factor VIII emulsions were considerably better. These were valuable for two reasons. Although as prepared they were not specific for a single antigen, the titer was sufficient to develop precipitin patterns against antigens of low concentration. The second advantage of preparing antisera against partially purified proteins was that the number of components present in the original antigen could be qualitatively determined indirectly by reacting the antiserum against bovine plasma by immunoelectrophoretic or immunodiffusion techniques.

C. Immunoelectrophoretic Analysis

The initial immunoelectrophoretic studies performed were made using commercially prepared antisera. These antisera were unsuitable due to the high degree of cross reaction with heterologous proteins. Immunoelectrophoresis performed with antisera prepared in our laboratory was helpful in determining the degree of purification obtained

at various stages of the procedure. There was one limitation to its use that was overcome in immunodiffusion studies, that is, the protein dilution suffered during electrophoresis prevented the development of strong precipitin patterns.

The results of several studies, (summarized in Table VI), show that there were still two components present at the Fraction I-1A' Δ56°C stage of purification. These two components were present in patterns developed either against bovine plasma antiserum or Factor VIII antiserum. An interesting aspect of these data was the difference in the patterns obtained against Fraction I-0 with the two antisera. Three components were present when the pattern was developed against antibovine plasma. The pattern developed with Fraction I-0 against Factor VIII antiserum showed only two components. From this it was concluded that the γ precipitin line represented fibrinogen, since this antigen was present in plasma but not in the Factor VIII antigen used in the initial preparation of the antiserum.

D. Gel Electrophoresis

Starch gel electrophoresis of plasma and serum gives much greater resolution than that obtained with the more conventional methods (i.e., moving boundary electrophoresis and electrophoresis on paper strips). Serum is resolved into seven components by moving boundary electrophoresis, whereas in starch gel, at least twice this number can be visualized.

Bovine Factor VIII preparations before fibrinogen denaturation were resolved into two components in starch gel. The major component was assumed to be fibrinogen, and the minor component assumed to be Factor VIII. With the removal of fibrinogen by heat denaturation, the major component was absent on subsequent electrophoretic analysis. Thus, it was assumed that Factor VIII was resolved to one component.

The inclusion of urea in gel electrophoresis was reported to increase resolution. The suggested mechanisms for this increased resolution are partial denaturation with the subsequent splitting of the subunits. The presence of urea further prevents aggregation of the denatured protein subunits (Wake and Baldwin, 1961).

Bovine Factor VIII preparations found to contain one component by conventional starch gel electrophoresis were further resolved into three components of approximately equal concentrations when 7M urea was included in the gel. It was thus concluded from these results that further purification was required to obtain Factor VIII as a single component.

E. Gel Filtration Studies

The results obtained from immunoelectrophoresis and urea-starch gel electrophoresis gave evidence that further purification of Factor VIII was required. Earlier observations on attempted small scale purification of human Factor VIII on G-100 Sephadex showed that the exclusion limit (MW=100,000) of this gel was too low to effect detectable separation of Factor VIII and its contaminants

(Mercer et al., 1963). The estimated molecular weight of human Factor VIII has been reported as 196,000 (Shulman et al., 1960) and later by Aronson et al. (1962) as 180,000. If these estimates are good, then gel filtration through a resin with an exclusion limit of 200,000 M.W. should prove fruitful for separation provided that there are significant differences in the molecular weights of Factor VIII and its contaminants. Such was the rationale for selecting G-200 Sephadex. The results of the gel filtration studies show that separation of the components of Factor VIII was accomplished. The separation was only partial in that there was mixing of the peaks (Figure 13).

Immunodiffusion studies on various fractions representing the peaks confirm the presence of single immunologic components represented by the peaks and the presence of two components in the mixed peaks fractions. These preliminary data lend great promise for future research. It was concluded that, based on these data, the two components separated have molecular weights under 200,000 with the major peak (Factor VIII) having the lower molecular weight of the two components. The data, in part, agree with earlier estimates of Factor VIII molecular weight (Shulman et al., 1960; Aronson et al., 1962).

F. Cryoglobulin Studies

The removal of cryoglobulins (cold insoluble proteins) from purified Factor VIII preparations was done for two reasons. First, it was apparent from earlier observation with gel filtration that material was precipitating at the surface of the resin bed. This

insoluble material at the surface was undesirable in that it could, if allowed to accumulate in the column, tend to slow and eventually stop the gel filtration procedure. Second, it was essential to know what this protein loss represented in terms of Factor VIII activity. Was it contributing to significant loss of Factor VIII in the eluates? The results showed that cryoglobulin removal represented a loss in Factor VIII activity of 15.5% to 18.5%, with no detectable loss of protein in either case. The total material lost by visual observation of the precipitate was slight, and it is thus possible that it was below the sensitivity of the protein determination. The loss of activity could have been due to the loss of a small quantity of high potency Factor VIII in the precipitate, that was not detected due to the poor resolution of the precipitate.

A purely speculative, but not too probable answer to this problem is found in some work on the subfractionation of Fraction I-1A reported by Blomback et al. (1961). They reported that when Fraction I-1A was adsorbed on tricalcium citrate, and the bulk of the protein eluted with 0.1M EDTA, there was a phospholipid remaining in the tricalcium citrate precipitate. The precipitate, when extracted with organic solvents, exhibited a low level of Factor VIII activity in the extract. This lipid could not, in itself, account for the total activity loss from the eluate. When the lipid and eluate fractions were recombined, the resulting activity was greater than the sum of the two separate activities. In the present case, this explanation would not be valid in that if the cryoglobulin

were purely phospholipid, it would have risen to the surface instead of sedimenting. Secondly, lipids are not readily separated by cooling alone as are certain lipoproteins.

G. Amino Acid Analysis

Analyses of three lots of Factor VIII (Fraction I-1A Δ 56°C Sephadex eluate) for amino acid composition and content showed that, with the exception of some amino acids, there was good correlation between the individual lots (Table X).

Mihalyi et al. (1964) reported on the amino acid composition of bovine fibrinogen. The preparation analyzed was 94% clottable protein. The differences between their data and the data presented here for bovine Factor VIII are obvious. From their data, tyrosine-tryptophane molar ratios were calculated with the resulting ratio of 1.23. Mihalyi reported the data of Tristram (1949) on human fibrinogen. The molar ratio calculated from these data was 1.85. These data are in fair agreement with the molar ratios reported here (Table IX) for bovine Fraction I-0 which is mainly fibrinogen.

The data for total nitrogen content of the individual lots showed that, in this respect, the agreement was even better. If the results for nitrogen content are valid, then it must be pointed out that in the case of Factor VIII at this degree of purification, the factor, 6, normally used to convert nitrogen to protein from Kjeldahl analysis data is in error. Based on the average nitrogen content of 14.2%, this conversion factor should then be the reciprocal of 14.2, or 7.04.

H. Tyrosine-Tryptophane Ratio and Content

The determination of tyrosine-tryptophane ratios and the molar concentration of these two amino acids was done by two different methods (Beavan and Holiday, 1952; Bencze and Schmid, 1957). The results of these two methods (Table IX and Table XIII) are in complete disagreement with each other, and neither method agrees with the molar concentrations as determined by amino acid analysis (Table X). As mentioned earlier, the tryptophane content for the amino acid analysis data was derived using the molar ratio determined by the Beavan and Holiday procedure. The resulting values gave close correlation between the recovery of total amino acids and the total protein analyzed.

The Beavan and Holiday values for tyrosine and tryptophane concentrations, although not in agreement with amino acid analysis values, are still much closer than the Bencze-Schmid values. Since the calculation of these values was dependent on determination of protein concentration, any error in this later determination would have a great effect on the calculated concentrations of the two amino acids in question. Since it was not in the scope of this thesis to resolve disagreements of these two methods, the data obtained from the Beavan and Holiday procedure was used because of closer agreement to amino acid analysis.

I. Significance

The modification of the Blomback procedure described has resulted in great increases in the purification of Factor VIII, by changing protein distribution during the purification procedure and reducing the heat losses of Factor VIII during the heat denaturation of fibrinogen. The best purification obtained with this modified procedure was 230 fold on a protein basis compared to plasma. This purification was 2.6 fold greater than that obtained by the Blomback procedure from the same starting material.

Gel filtration of partially purified Factor VIII has yielded a fraction containing the Factor VIII activity in a single immunologic component.

Further confirmation of these results and refinements of the techniques used will permit the production of purified Factor VIII on a larger scale. There are many areas yet to be investigated that will be greatly aided by this present study. With a highly purified Factor VIII preparation, it will be possible to prepare a specific antiserum for Factor VIII. This specific antiserum will be used basically in four ways: 1) to develop immunologic assays of Factor VIII in an attempt to resolve the discrepancies of present in vitro clotting assays, 2) to study in vitro distribution of Factor VIII during the purification procedures in an attempt to improve yield of Factor VIII obtained from these procedures, 3) to utilize the antiserum coupled to cellulose derivatives for purification by immunoabsorbant techniques, 4) to use the specific antiserum to study in vivo

distribution and degradation of Factor VIII and determine the sites of Factor VIII synthesis and degradation. The eventual aim is to achieve a method of producing Factor VIII of animal origin that will be suitable for less limited clinical use in man.

VI. SUMMARY

The purpose of this study was to isolate highly purified bovine blood clotting Factor VIII (antihemophilic globulin).

A. This required modifying presently available purification techniques to assure separation of Factor VIII from fibrinogen with minimal losses of Factor VIII activity. The Blomback procedure was modified by replacement of sodium citrate with sodium chloride of equivalent ionic strength. In connection with the modification of the purification procedure, it was found that:

1. The elimination of citrate from the reagents used in the purification procedure developed by Blomback resulted in increased purification of Factor VIII.

2. The observed citrate effect was two fold. First, the reduction or elimination of citrate caused changes in protein distribution in the fractionation and purification procedure, resulting in increased purification. Second, the reduction or elimination of citrate reduced the losses of Factor VIII incurred during heat denaturation of contaminant fibrinogen at 56°C.

3. The mechanism of the citrate effect was not elucidated by this study, but the possibility of heavy metal contamination of the citrate would be an unlikely cause due to the reported low concentrations of heavy metals (0.0001% as Pb) in the citrate used.

B. Studies on purified Factor VIII obtained from the modified procedure were performed to determine the actual purification obtained. From these studies it was found that:

1. Starch gel electrophoretic studies on Factor VIII showed that denaturation of fibrinogen by heating resulted in a preparation purified to a single electrophoretic component.

2. Antisera prepared against the purified Factor VIII preparation showed that the preparation was not homogeneous.

   a. Immunoelectrophoretic studies showed the presence of two immunologic components in the purified preparations.

   b. Immunodiffusion studies on cellulose acetate membranes visualized three immunologic components present in the purified preparations.

3. Urea-starch gel electrophoretic analysis of the purified preparations confirmed the presence of three protein components.

4. Amino acid analysis of several preparations showed that the preparations were quite uniform on the basis of amino acid composition.

C. Further purification of Factor VIII preparations was accomplished on a small scale by cryoglobulin precipitation followed by gel filtration with the following results:

1. A cryoglobulin precipitate was removed from the fibrinogen-free Factor VIII preparations that resulted in minor losses of Factor VIII activity.

2. Gel filtration of the cryoglobulin-free Factor VIII preparation on G-200 Sephadex resulted in a partial separation of two components.

3. Assay of the eluate fractions from gel filtration showed that the peak of Factor VIII activity was coincident with the major protein component.

4. Immunodiffusion studies on the redissolved cryoglobulin precipitate and eluate fractions representing the components from gel filtration showed that each material was a single immunologic component.

REFERENCES

Addis, T. 1910. The Pathogenesis of Hereditary Hemophilia. J. Path. and Bact., 15, 427.

Adelson, E., J. J. Rheingold, O. Parker, M. Steiner, and J. C. Kirby. 1963. The Survival of Factor VIII (Antihemophilic Globulin) and Factor IX (Plasma Thromboplastin Component) in Normal Humans. J. Clin. Invest., 42, 1040.

Aggeler, P. M., S. G. White, M. B. Glendening, E. W. Page, T. B. Leake, and G. Bates. 1952. Plasma Thromboplastin Component (PTC) Deficiency, A New Disease Resembling Hemophilia. Proc. Soc. Exper. Bio. Med., 79, 691.

Alexander, B., and G. Landwehr. 1948. Studies of Hemophilia. II. The Assay of Antihemophilic Clot-Promoting Principle in Normal Human Plasma With Some Observations on the Relative Potency of Certain Plasma Fractions. J. Clin. Invest., 27, 98.

Anderson, H.D. 1964. A Program Which Coordinates the Activities of Blood Banks, Maternity Hospitals, Physicians and the Department of Health Laboratories to Provide All Useful Derivatives in Adequate Quantity with a Minimum of Cost to the Recipient. Proc. 9th Congr. int. Soc. Blood Trans., Mexico 1962. p. 664.

Anderson, H.D. 1964. The Use of Human Blood Derivatives in a Population Where Such Products are Readily Available in Adequate Quantity Without Cost. Proc. 9th Congr. int. Soc. Blood Trans., Mexico 1962. p. 669.

Aronson, D. L., J. W. Priess, and M. W. Mosesson. 1962. Molecular Weights of Factor VIII (AHF) and Factor IX (PTC) by Electron Irradiation. Thromb. Diath. Haemorrhag., 8, 270.

Astrup, T., P. Brakman, P. Ollendorff, and J. Rasmussen. 1960. Haemostasis in Haemophilia in Relation to the Haemostatic Balance in the Normal Organism and the Effect of Pea Nuts. Thromb. Diath. Haemorrhag., 5, 329.

Astrup, T., P. Brakman, and K.-E. Sjølin. 1962. Haemophilia and the Protease Inhibitor in Peanuts. Nature, 194, 980.

Beale, A.J., and P. J. Mason. 1962. Measurement of D-Antigen in Poliovirus Preparations. J. Hyg., 60, 113.

Beavan, G. E., and E. R. Holiday. 1952. Ultraviolet Absorption Spectra of Proteins and Amino Acids. In: Advances in Protein Chemistry, 7, 319. Academic Press, New York.

Bendien, W. M., and S. van Creveld. 1939. On Some Factors of Blood Coagulation Especially with Regard to the Problem of Hemophilia. Acta. Med. Scand., 99, 12.

Bencze, W. L., and K. Schmid. 1957. Determination of Tyrosine and Tryptophane in Proteins. Analyt. Chem., 29, 1193.

Bidwell, E. 1955a. The Purification of Bovine AHG. Brit. J. Haematol., 1, 35.

Bidwell, E. 1955b. The Purification of Antihemophilic Globulin from Animal Plasma. Brit. J. Haematol., 1, 386.

Biggs, R., A. S. Douglas, R. G. MacFarlane, J. V. Dacie, W. R. Pitney, C. Mersky, and J. R. O'Brien. 1952. Christmas Disease; A Condition Previously Mistaken for Hemophilia. Brit. Med. J., 2, 1378.

Biggs, R. 1960. The Treatment of Hemophilic Patients With Animal Antihaemophilic Globulin. Acta Haemat., 24, 124.

Bizzozero, G. 1882. Uber ein neuen Formbestandtheil des Blutes und desen Rolle bei der Thrombose und der Blutgerinnung. Virchows Arch. f. path. Anat., 90, 261.

Blomback, M. 1958. Purification of Antihemophilic Globulin. I. Some Studies on the Stability of Antihemophilic Globulin Activity in Fraction I-0 and a Method for its Separation From Fibrinogen. Arkiv. f. kemi, 12, 387.

Blomback, B., M. Blomback, and I Struwe. 1961. Studies on Blood Clotting Factor VIII. Arkiv. f. kemi, 18, 223.

Boudreaux, H. D., and V. L. Frampton. 1960. A Peanut Factor for Haemostasis in Haemophilia. Nature, (Lond.) 185, 469.

Boyer, S. H., D. C. Fainer, and M. A. Naughton. 1963. Myoglobin Inherited Structural Variation In Man. Science, 140, 1228.

Brakman, P., K.-E. Sjølin, and T. Astrup. 1962. Is There A Hemostatic Effect of Peanuts in Hemophiloid Disorders? Thromb. Diath. Haemorrhag., 8, 442.

Breckenridge, R. T., and O. D. Ratnoff. 1962. Studies On The Nature of a Circulating Anticoagulant Directed Against Antihemophilic Factor: With Notes On An Assay of Antihemophilic Factor. Blood, 20, 137.

Brinkhous, K. M. 1947. Clotting Defect in Hemophilia: Deficiency in a Plasma Factor Required for Platelet Utilization. Proc. Soc. Exper. Biol. and Med., 66, 117.

Brinkhous, K. M. 1964. Hemophilia - Pathophysiologic Studies and the Evolution of Transfusion Therapy. Am. J. Clin. Path., 41, 342.

Campbell, D.H., et al. 1963. Methods in Immunology, W. A. Benjamin Co., New York.

Cohn, E. J., L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor. 1946. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation Into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. J. Am. Chem. Soc., 68, 459.

Collingwood, B. J., and M. T. MacMahon. 1912. The Anticoagulants in Blood and Serum. J. Physiol., 45, 119.

Davie, E. W., and O. D. Ratnoff. 1964. Waterfall Sequence for Intrinsic Blood Clotting. Science, 145, 1310.

Douglas, A. S. 1958. Antihemophilic Globulin Assay Following Plasma Infusions in Hemophilia. J. Lab. Clin. Med., 51, 850.

Duckert, F.,P. Fluckiger, M. Matter, and F. Koller. 1955. Clotting Factor X Physiologic and Physico-Chemical Properties. Proc. Soc. Exper. Biol. and Med., 90, 17.

Ferris, T. G., R. E. Easterling, and R. E. Budd. 1963. Electrophoresis of Serum Proteins in Acrylamide Gel. I. Removal of Background Stain by Means of Electric Current. Am. J. Clin. Path., 38, 383.

Field, R. C., W. F. Enneking, and G. Rothstein. 1963. Elective Surgery in Hemophilia. I. Correction of Talipes Equinus Deformity. J. Amer. Med. Assoc., 185, 628.

Frank, E., and E. Hartmann. 1927. Uber das Wesen und die therapeutische Korrektur der hamophilen Gerinnungsstorung. Klin. Wchnschr., 6, 435.

Frankel, G. J., and G. E. Honey. 1955. Gunshot Wounds in a Hemophilic Patient: Successful Treatment by Animal Antihaemophilic Globulin and Surgery. Lancet, 2, 1117.

Fuld, E., and K. Spiro. 1904. Der Einfluss einiger gerinnungshemmender Agentein auf das Vogelplasma. Beitr. Chem. Physiol. u. Path., 5, 171.

Gallick, H. 1965. The Development of an Assay and Method of Purification for Human Blood Clotting Factor XI. Ph.D. Thesis. Michigan State University.

Gaston, L. W. 1964. The Blood Clotting Factors (In Two Parts). New Eng. J. Med., 270, 236 and 290.

Goodwin, T. W., and R. A. Morton. 1946. The Spectrophotometric Determination of Tyrosine and Tryptophane In Proteins. Biochem. J., 40, 628.

Grabar, P., and C. A. Williams, Jr. 1953. Méthode Permettant l'etude conjugee des proprietés electrophoretiques et immunochimiques d'un anelange de proteines. Application au serum sanguin. Biochem. Biophys. Acta., 10, 193.

Gugler, E. 1961. Zur therapeutischen Anwendung der Fraktion I nach Cohn. Bibl. haemat., 12, 270.

Handley, D. A., N. S. Painter, and M. R. P. Hall. 1961. Surgical Treatment of Duodenal Ulceration in Two Haemophiliacs. Lancet, 1, 482.

Hardisty, R. M., and J. C. MacPherson. 1962. A One-Stage Factor VIII (Antihemophilic Globulin) Assay and Its Use on Venous and Capillary Plasma. Thromb. Diath. Haemorrhag., 7, 215.

Hayem, G. 1896. Du caillot non rétractile: suppression de la formation du serum sanguin dans quelques états pathologique. Comp. rend. Acad. d. Sc., 123, 894.

Holiday, E. R. 1936. Spectrophotometry of Proteins. I. Absorption Spectra of Tyrosine, Tryptophane and Their Mixtures. II. Estimation of Tyrosine and Tryptophane in Proteins. Bioch. J., 30, 1795.

Holiday, E. R., and A. G. Ogston. 1938. The Ultraviolet Absorption and Potentiometric Titration Curves of Serum Proteins and Some Others. Biochem. J., 32, 1166.

Hougie, C., E. M. Barrow, and J. B. Graham. 1957. Stuart Clotting Defect. I. Segregation of an Hereditary Hemorrhagic State from the Heterozygous Group Heretofore Called"Stable Factor". (SPCA, Proconvertin, Factor VII). Deficiency. J. Clin. Invest., 36, 485.

Ingram, G. I. C. 1962. Major Surgery in Haemophilia Use of Animal Antihaemophilic Factor in Britain. Transfusion, 2, 88.

Janiak, A., and J. P. Soulier. 1962. Preparation d'une fraction anti-hemophilique A a partir de plasma ACD standard. Thromb. Diath. Haemorrhag., 8, 406.

Johnson, C.M., J. C. N. Westwood, and M. Beaulieu. 1964. A Continuous-flow Micro Double Diffusion technique with Cellulose Acetate. Nature, 204, 1321.

Jones, A. F. D. 1805. A treatise on the Process Employed by Nature in Suppressing its Haemorrhage of Divided and Punctured Arteries and the Use of the Ligature. Longman, Hurse, Rees, Orme and Brown, London. 1810.

Joules, H., and R. G. MacFarlane. 1938. Pseudo-haemophilia in a Woman. Lancet, 1, 715.

Leitner, A., E. Bidwell, and G. W. R. Dike. 1963. An Antihaemophilic Globulin (Factor VIII) Inhibitor. Purification Characterization and Reaction Kinetics. Brit. J. Haemat., 9, 245.

Lozner, E. L., L. S. Jolliffe, and F. H. L. Taylor. 1940. Hemorrhagic Diathesis with Prolonged Coagulation Time Associated with Circulatory Anticoagulant. Am. J. M. Sc., 199, 318.

Lane, S. 1840. Haemorrhagic Diathesis: Successful Transfusion of Blood. Lancet, 1, 185. Cited by Brinkhous, K. M. Am. J. Clin. Path., 41, 342. 1964.

Langdell, R. D., R. H. Wagner, and K. M. Brinkhous. 1953. Effect of Antihemophilic Factor on One Stage Clotting Tests. J. Lab. Clin. Med., 41, 637.

Lorand, L., and K. Laki. 1954. A Simple Method for Purifying an Activator of Prothrombin (Antihemophilic Factor?). Biochem. Biophys. Acta, 13, 448.

Ma, T. S., and G. Zuazaga. 1942. Micro Kjeldahl Determination of Nitrogen. Ind. Eng. Chem., Anal. Ed., 14, 280.

MacFarlane, R. G., R. Biggs, and E. Bidwell. 1954. Bovine Antihaemophilic Globulin in the Treatment of Hemophilia. Lancet, 1, 1316.

MacFarlane, R. G., P. Mallam, L. Witts, E. Bidwell, R. Biggs, G. Frankel, G. Honey, and K. Taylor. 1957. Surgery in Hemophilia: The Use of Animal Antihaemophilic Globulin and Human Plasma in Thirteen Cases. Lancet, 2, 251.

MacFarlane, R. G., and R. Biggs. 1959. The Treatment of Haemophilic Patients with Animal Antihemophilic Globulin. In: Brinkhous, K. M.: Hemophilia and Other Hemorrhagic States. Chapel Hill. New York, Van Rees Press, 1959. p. 19.

MacFarlane, R. G. 1964. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. Nature, 202, 498.

Mammen, E. F. 1963. Investigations on the Nature of Hemophilia A. Thromb. Diath. Haemorrhag., 9, 30.

Mammen, E. F. 1964. Concentration of Platelet Cofactor I (AHG) from Bovine Serum. Thromb. Diath. Haemorrhag., 11, 127.

Maycock, W. D'A., S. Evans, L. Vallet, B. Combridge, P. Wolf, B. H. MacGibbon, E. E. French, L. H. Wallet, J. V. Dacie, R. Biggs, D. Handley, and R. G. MacFarlane. 1963. Further Experience With a Concentrate Containing Human Antihemophilic Factor. Brit. J. Haemat., 9, 215.

McLester, W., and R. Wagner. 1965. Antibody to Antihemophilic Factor and its Lack of Reaction with Hemophilic Plasma. Amer. J. Physiol., 208, 499.

McMillan, C. W., L. K. Diamond, and D. M. Surgenor. 1961. Treatment of Classic Hemophilia: The Use of Fibrinogen Rich in Factor VIII for Hemorrhage and Surgery. New Eng. J. Med., 265, 224.

Mercer, J. E., H. Gallick, and L. A. Hyndman. Unpublished observations.

Michael, S. W., and G. W. Tunnah. 1963. The Purification of Factor VIII (Antihaemophilic Globulin). Brit. J. Haemat., 9, 236.

Mihalyi, E., P. A. Small, Jr., and J. P. Cooke. 1964. The Amino Acid Composition of Bovine Fibrinogen. Arch. Biochem. and Biophys., 106, 229.

Morand, S. F. 1736. Mem. d. Acad. Roy de Chirurgie. V Ed., p. 8.

Morawitz, P. 1904. Bietrage zur Kenntnis der Blutgerinnung. Beitr. Chem. Physiol. u Path., 5, 131.

Munro, F. L., and H. W. Jones. 1943. The Detrimental Effect of Frequent Transfusions in the Treatment of a Patient with Hemophilia. Am. J. M. Sc., 206, 710.

Niemetz, J.,C. Weilland, and J. P. Soulier. 1961. Preparation d'une Fraction Plasmatique Humaine Riche en Facteur VIII (Antihemophilique A) et pauvre en Fibrinogene. Nouvelle Rev. Francaise d'Hematologie, 7, 880.

Nolf, P. 1938. Coagulation of the Blood. Medicine, 17, 381.

Otto, J. C. 1803. An Account of an Hemorrhagic Disposition Existing in Certain Families. Med. Reposit., 6, 1.

Owen, C. A., and J. L. Bollman. 1948. Prothrombin Conversion Factors of Dicoumarol Plasma. Proc. Soc. Exper. Biol. and Med., 41, 231.

Owren, P.A. 1947. The Coagulation of Blood: Investigation on a New Clotting Factor. Acta. Med. Scand., 194, 223.

Patek, A. J., and R. P. Stetson. 1936. Hemophilia I. The Abnormal Coagulation of the Blood and its Relation to the Blood Platelets. J. Clin. Invest., 15, 531.

Patek, A. J., and F. H. L. Taylor. 1937. Hemophilia II: Some Properties of a Substance Obtained from Normal Human Plasma Effective in Accelerating the Coagulation of Hemophilic Blood. J. Clin. Invest., 16, 113.

Pavlovsky, A., C. Simonetti, G. Casillas, L. J. Bergna, A. Andino, and A. E. Bachman. 1961. The Use of Factor VIII Freed from Fibrinogen in the Treatment of Hemophilic Patients. Brit. J. Haemat., 7, 365.

Petit, J. E. 1731. Mem. de l'Acad. Roy. des Sci. p. 90.

Pool, J. G., E. J. Hershgold, and A. R. Pappenhagen. 1964. High Potency Antihemophilic Factor Concentrate Prepared from a Cryoglobulin Precipitate. Nature, 203, 312.

Quick, A. J. 1943. On the Constitution of Prothrombin. Am J. Physiol., 140, 212.

Quick, A. J. 1947. Studies on the Enigma of the Hemostatic Dysfunction of Hemophilia. Am. J. Med. Sci., 214, 272.

Quick, A. J. 1957. Hemorrhagic Diseases. Lea and Febiger, Philadelphia.

Ratnoff, O. D., and J. E. Colopy. 1955. A Familial Hemorrhagic Trait Associated with a Deficiency of a Clot-Promoting Fraction of Plasma. J. Clin. Invest., 34, 602.

Rosenthal, R. L., O. H. Dreskin, and N. Rosenthal. 1953. New Hemophilia-Like Disease Caused by Deficiency of a Third Plasma Thromboplastin Factor. Proc. Exper. Biol and Med., 82, 171.

Rosenthal, R. L., L. H. Dreskin, and N. Rosenthal. 1955. Plasma Thromboplastin Antecedent (PTA) Deficiency: Clinical Coagulation, Therapeutic Aspects of a New Hemophilia-Like Disease. Blood, 10, 120.

Sahli, H. 1905. Uber das Wesen der Hamophilie. Ztschr. klin. Med., 56, 264.

Seegers, W. H., R. Landaburu, and R. L. Fenichel. 1957. Isolation of Platelet Cofactor I from Plasma and Some Properties of the Preparation. Am. J. Physiol., 190, 1.

Seegers, W. H., E. Mammen, J. M. Lee, R. H. Landaburu, M. H. Cho, W. J. Baker, and R. S. Shepard. Further Purification of Platelet Cofactor I. In: Brinkhous, K. M. Hemophilia and Other Hemorrhagic States. Van Rees Press, 1959. New York.

Shinowara, G. Y. 1966. Kinetic Studies on the Thermal Denaturation of Human Antihemophilic Globulin (AHG). Abstracts of 14th Annual Symposium on Blood. Wayne State Univ. School of Med., Detroit, Mich., Jan. 22, 1966.

Shulman, S., R. H. Landaburu, and W. H. Seegers. 1960. Biophysical Studies on Platelet Cofactor I Preparations. Thromb. Diath. Haemorrhag., 4, 336.

Simonetti, C., G. Casillas, and A. Pavlovsky. 1961. Purification du Facteur VIII Antihemophilique (FAH).Hemostase, 1, 57.

Smith, P. H. 1965. Management of Splenectomy in Hemophilia: Case of Spontaneous Rupture of Spleen Due to Glandular Fever in a Hemophiliac. Brit. Med. Jour., 1, 296.

Smithies, O. 1959. An Improved Procedure for Starch-gel Electrophoresis: Further Variations in the Serum Proteins of Normal Individuals. Biochem. J., 71, 585.

Spaet, T. H., and B. G. Kinsell. 1953. Properties of Bovine Antihemophilic Factor. Proc. Soc. Exper. Biol. and Med., 84, 314.

Strauss, H. K., S. V. Kerry, L. K. Diamond. 1965. Ineffectiveness of Prophylactic EACA in Severe Hemophilia. New Eng. J. Med., 273(6), 301.

Taylor, F. H. L., C. S. Davidson, H. J. Tagnon, M. A. Adamis, A. H. MacDonald, and G. R. Minot. 1945. Studies in Blood Coagulation. The Coagulation Properties of Certain Globulin Fractions of Normal Human Plasma in Vitro. J. Clin. Invest., 24, 698.

Thorne, C. B., and F. C. Belton. 1957. An Agar Diffusion Method for Titrating B. Anthracis Immunizing Antigen and its Application to a Study of Antigen Production. J. Gen. Microbiol., 17, 505.

Tristram, G. R. 1949. Amino Acid Composition of Purified Proteins. Advan. in Protein Chem., 5, 83.

van Creveld, S., H. A. Veder, C. N. Pascha, and W. F. Kroeze. 1959. The Separation of AHF from Fibrinogen I. Thromb. Diath. Haemorrhag., 3, 572.

van Creveld, S., H. A. Veder, and C. N. Pascha. 1960. The Separation of AHF from Fibrinogen II. Thromb. Diath. Haemorrhag., 4, 211.

van Creveld, S., C. N. Pascha, and H. A. Veder. 1961. The Separation of AHF from Fibrinogen III. Thromb. Diath. Haemorrhag., 6, 282.

Verstraete, M. 1957. Excess of Anticoagulant as a Cause of the Clotting Defect in Hemophilia. In" Brinkhous, K. M. Hemophilia and Hemophiloid Diseases. Chapel Hill. Van Rees Press, New York. 1957.

Wagner, R., W. McLester, M. Smith, and K. Brinkhous. 1964. Purification of Antihemophilic Factor (Factor VIII) by Amino Acid Precipitation. Thromb. Diath. Haemorrhag. 11, 64.

Wake, R. G., and R.L.Baldwin. 1961. Analysis of Casein Fractions by Zone Electrophoresis in Concentrated Urea. Biochem. et Biophys. Acta., 47, 225.


MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 03146 3577