

Disclaimer

This research work belongs to

Mr. P. R. Dichwalkar

No content of this work can be
circulated or used for any commercial
purpose without the consent of
respective researcher



HAFKINE INSTITUTE LIBRARY,
MUMBAI.

Call No.

Acc No

432

Price

Source Purchased.

Donated

**BIOCHEMICAL AND IMMUNOLOGICAL
STUDY OF HUMAN FIBRINOGEN AND
CRYOPRECIPITATE (AHF, FACTOR VIII)**

A THESIS

**SUBMITTED TO
THE UNIVERSITY OF BOMBAY**

**FOR
THE DEGREE**

**OF
DOCTOR OF PHILOSOPHY**

**IN
APPLIED BIOLOGY**

**BY
*P. R. DICHWALKAR***

**DEPARTMENT OF IMMUNOHAEMATOLOGY
HAFFKINE INSTITUTE
PAREL, BOMBAY-400 012**

**HAFFKINE INSTITUTE LIBRARY,
MUMBAI.**

1975

**Call No.
Price**

Acc No 432
Source Purchased.
Donated

A C K N O W L E D G E M E N T S

I am extremely grateful to my guide, Dr. H. V. Sant, M.D., F.C.P.S., D.B. (Prague), Ex-Deputy Director and Head, Department of Zoonosis, Haffkine Institute for his valuable advice and able guidance throughout this investigation.

I take this opportunity to express my sincere gratitude and thanks to Dr. B. B. Galtonde, M.D., M.Sc. (Med.), Director, Haffkine Institute, Bombay for providing facilities to carry out this research work.

I am greatly indebted to Dr. K. V. Kulkarni, M.B.B.S., Assistant Director, Shri G. J. Karkhanavala, M.Sc., Senior Scientific Officer, Department of Immunohaematology, Dr. A. J. Baxi, M.Sc., Ph.D., Senior Research Officer, Blood Group Reference Centre (I.C.M.R. Unit), Haffkine Institute for their keen interest, untiring help and encouragement throughout the work.

I have pleasure in acknowledging my thanks to Shri V. M. Patankar, M.Sc., Junior Scientific Officer, Department of Immunology, Shri A. V. Mutho, M.Sc., Junior Scientific Officer, Mrs. J. M. Gokarna, M.Sc., and Shri B. G. Power, Department of Immunohaematology for their suggestions and technical assistance.

Lastly I thank Shri G. G. Karkhar for carrying out typing work, Shri D. V. Vengarkar, for photography, all my colleagues and other staff members of the Department of Immunohaematology (Processing) for continuous and everready help throughout the work.

P. R. Dikhwalkar

C O N T E N T S

<u>CHAPTER</u>	<u>Pages</u>
I. <u>Introduction</u>	1 - 65
Theories of blood coagulation	3 - 12
Fibrinogen	12 - 15
The molecular structure of fibrinogen	15 - 16
Abnormal forms of fibrinogen	16 - 20
Action of whole venom on fibrinogen	20 - 22
Properties of fibrinogen	22 - 25
Preparation of fibrinogen	25 - 33
Thrombin	33 - 35
Thrombin fibrinogen reaction	36 - 38
Measurement of fibrinogen	38 - 39
Congenital deficiency of factor VIII (Haemophilia)	39 - 45
The concept of haemophilia	45 - 49
Treatment in haemophilia	49 - 50
Antihæmophilic globulin (Factor VIII)	50 - 51
The measurement of factor VIII levels	51 - 52
Biochemistry of factor VIII	52 - 54
Physiology of factor VIII	54 - 55
Immunology of factor VIII	55 - 56
Abnormal forms of factor VIII	56 - 57
Materials for the treatment of patients with deficiency of factor VIII	57 - 64
Proposed plan of work	64 - 65
II. <u>Material and Methods</u>	66 - 104
Material	66 - 81
Methods	82
Protein estimation - Biuret method	82 - 84

<u>CHAPTER</u>	<u>Pages</u>
A batch method, for fibrinogen estimation	84 - 87
Thrombin clotting time	87
A simple method for the assay for factor VIII	87 - 91
Single radial immuno-diffusion	91 - 94
Gel diffusion technique	94 - 96
Electrophoresis	96 - 99
Agar gel electrophoresis	99 - 100
Immunoelectrophoresis	100 - 102
Gel filtration using Sephadex G-200	102 - 103
Titration of antibodies in AHF	103 - 104
 III. <u>Experimental</u>	 105 - 124
Preparation of fibrinogen	105 - 113
Purification of fibrinogen	113 - 117
Preparation of anti-fibrinogen	118
Preparation of antihæmophilic globulin (AHF, Factor VIII)	118 - 123
Purification of AHF	123
Preparation of thrombin	124
 IV. <u>Results</u>	 125
Antifibrinogen	132
Titration of anti-fibrinogen (crude)	133
The titre of antiserum	133
Titration of fibrinogen	134
Paper electrophoresis	134



<u>CHAPTER</u>	<u>Pages</u>
Radial immunodiffusion	136
Gel diffusion	136
Immunoelectrophoresis	136
Quantitative estimation of fibrinogen	139
Antihæmophilic globulin	139
Effect of storage on AHG	139
Anti-AHF	140
Gel diffusion of AHF	140
Immunoelectrophoresis of AHF preparations	142
Titration of antibody (anti-A and anti-B)	142
Quantitative estimation of AHF activity	146
Clinical trials	146
Quantitative estimation of AHG in plasma by radial immunodiffusion.	146
 <u>V. Discussion</u>	 147 - 157
 <u>VI. Summary and Conclusions</u>	 158
 Bibliography	 1 - xxix

.....

I N T R O D U C T I O N

Clotting of blood results from the conversion of a soluble plasma protein, fibrinogen (Factor I) into fibrin, an insoluble network of fibres. The jelly like appearance of blood clots is due to the entrapment of cells and serum within the meshes of network in fibrin. The mechanism of coagulation is still not clearly understood in details. Malpighi (1666) was one of the first to consider this problem in the eighteenth century. Alexander Schmidt (1892) proposed the concept that the coagulation of blood is the consequence of a chain of reacting factors culminating in the conversion of fibrinogen into fibrin. The formation of fibrin is catalysed physiologically by a hydrolytic plasma enzyme, thrombin. Thrombin is present in the form of prothrombin. Recognition of part played by calcium led to the development of the classical theory of the coagulation mechanism (Moravits 1905) in which four factors were regarded as necessary, thromboplastin, calcium, prothrombin and fibrinogen.

In modern concept of coagulation blood clotting is conceived as a dynamic process in which positive forces leading to coagulation are antagonised by negative forces, the latter including natural anticoagulants and agents which remove formed clot, (Howell 1910, Helf 1908). There are at least three distinct phases in the clotting of blood, namely the formation of thromboplastin, the formation of thrombin and the production of fibrin. Modern theories of coagulation will be more easily

understood if the various factors are considered separately. The various factors will be described more or less in reverse of the order of their formation.

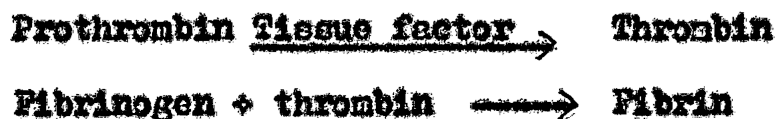
The inherent complexity of coagulation process has been confounded by the great number of terms which have been applied to the various factors involved. Fortunately an international committee has agreed upon uniform terminology which is as follows -

Synonyms for various coagulation factors

International Nomenclature	Synonyms
Factor I	Fibrinogen
Factor II	Prothrombin, tissue extract
Factor III	Thromboplastin
Factor IV	Calcium
Factor V	Proaccelerin, labile factor, accelerator globulin (ACG), thrombogen
Factor VI	Accelerin
Factor VII	Proconvertin, stable factor, serum prothrombin conversion accelerator (SPCA), auto-prothrombin I
Factor VIII	Antihæmophilic factor (AHF), Antihæmophilic globulin (AHG), Platelet cofactor I, antihæmophilic factor A
Factor IX	Plasma thromboplastin component (PTC), Christmas factor, platelet cofactor II, autoprothrombin II, antihæmophilic factor II
Factor X	Stuart - prower factor
Factor XI	Plasma thromboplastin antecedent (PTA)
Factor XII	Hageman factor (HF)
Factor XIII	Fibrin stabilizing factor (FSF), Laki - Lorand Factor (LLF) Fibrinase

Theories of blood coagulation

The discovery of prothrombin formed the basis of classical theory of blood coagulation. This observation was noted by Schmidt (1861, 1862, 1892). He was able to precipitate out the active coagulant, fibrin ferment or thrombin from the serum. This observation showed that an active coagulant was present only during the coagulation process but its precursor (prothrombin) is present in an inactive form in the plasma. This gave rise to the theory of coagulation postulated by Schmidt (1892), Morawitz (1905) and Prid and Spiro (1904).



The chemical reaction involved in the conversion of fibrinogen to fibrin was not known. Heuber (1905) said that an intramolecular rearrangements took place.

Owren postulated the presence of a proconvertin in plasma useful in converting the prothrombin to thrombin.

The classical theory involves five clotting factors out of which fibrinogen, thrombin and calcium were already isolated from either plasma or serum and were

accepted as real clotting factors. Prothrombin was considered to be hypothetical substance. Eagle (1935) and Owren (1947) showed that the amount of thrombin formed was directly proportional to the prothrombin present.

Prothrombin

The existence of prothrombin as a real coagulation factor is found out by two lines of approach (1) Bordet and Delang (1914) observed that after the adsorption of prothrombin by aluminium hydroxide, barium sulphate or calcium phosphate from plasma and then on carrying out coagulation test by recalcification of adsorbed plasma it was delayed markedly and (2) the adsorbent was eluted and the eluting solution was added to "adsorbed plasma" and coagulation test by recalcification was performed. It was observed that adsorbed plasma clotted rapidly on addition of eluting solution. These two observations supported the existence of prothrombin.

Tissue Factor

The role of tissue extracts in the blood coagulation was recognised long ago. It was found that plasma rich in cells or whole blood coagulated more rapidly than those with few cells. The tissue factor differs from thrombin as it is resistant to boiling, secondly it cannot

coagulate fibrinogen solution and thirdly it is not consumed during coagulation of blood i.e. it is enzymatic in nature. Eagle and Harris (1937), Tagnon et. al. (1942), Tagnon (1942) and Purguson (1949) observed that proteolytic enzymes could form thrombin from prothrombin. This supported the enzymatic nature of tissue factor.

Platelets

The thromboplastic activity of platelets was recognised by Bordet and Delang (1912). The recent studies suggest that the platelets are required to react with plasma constituents to release their thromboplastic activity.

There are some elements naturally occurring in the body which tend to keep the blood in liquid phase in vitro as well as in vivo. Such inhibitors are antithrombin, heparin and antithromboplastin.

Schmidt (1892) observed that thrombin disappeared rapidly after the blood coagulation due to conversion of thrombin to "meta thrombin" and that thrombin could be restored on addition of alkalis or acids. Quick (1938) found that antithrombic activity was associated with albumin fraction of plasma. Astrup and Darling (1942) studied the mechanism of inactivation of thrombin

by antithrombin and found the inactivation process to be very slow.

Heparin was discovered by Mclean (1916), Howell and Mollanby (1935). Quick (1938) observed that heparin inhibits the thrombin fibrinogen reaction. The inhibition was very little when pure solutions of thrombin and fibrinogen were used.

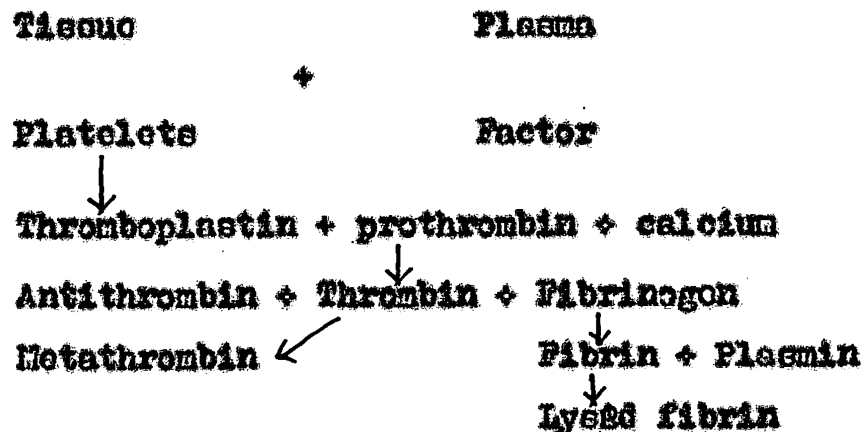
Howell and Holt (1918) found that its action was dependent on albumin fraction of plasma which is precipitated at one third and one half saturation with ammonium sulphate. It is a mucopolysaccharide and is present in most of the tissues of body and also in granules of most cells. Generally heparin is detected in the body in normal conditions, but it appears in large amounts in the cases of anaphylactic shock, exposure to ionising irradiation and some haemorrhagic disorders.

The existence of an antithromboplastin was postulated by Collingwood and MacLohan (1912) and it was supported by Tocantine (1943 a & b, 1944 a & b, 1945) and Tocantine and Carroll (1949). They also presumed that excess of antithromboplastin is present in haemophilic patients. As there is no direct evidence confirming the existence of antithromboplastin, it is considered as a hypothetical substance.

Fibrinolysis

It has been observed that after surgical operation or with the hypersecretion of adrenalin as a result of fear or after adrenalin injection the clot dissolves rapidly on standing at 37°C within a few hours. The factor responsible for the lysis of the fibrin clot is an enzyme known as plasmin or fibrinolysin factor. This property of fibrinolysin factor was observed by Helf (1908 b, 1928). The exact role of fibrinolysin factor is still in dark but it is known that it plays an important part in dissolving the intravascular clots.

All the observations discussed above regarding the mechanism of coagulation of blood, existence of inhibitory substances and the phenomenon of fibrinolysis, support the classical theory according to which prothrombin is converted to thrombin with the help of calcium and thromboplastin in the initial phase and in the next phase. Fibrinogen is converted to fibrin. This can be diagrammatically stated as -



Deviation from classical theory

Inability of classical theory to provide satisfactory answers to certain experimental conditions and to explain the causes of haemorrhage in some disease gave it a serious set back. Modifications of classical theory were suggested in order to provide explanation for these situations.

Woodrigo (1883, 1889) and Mille Quest (1921) presumed that tissue factor itself acted as coagulant. They also believed that two types of fibrinogen A and B are present. The fibrinogen A was derived from tissues and fibrinogen B from plasma and the direct combination of two lead to coagulation of blood. This theory did not receive much attention as it had no experimental support.

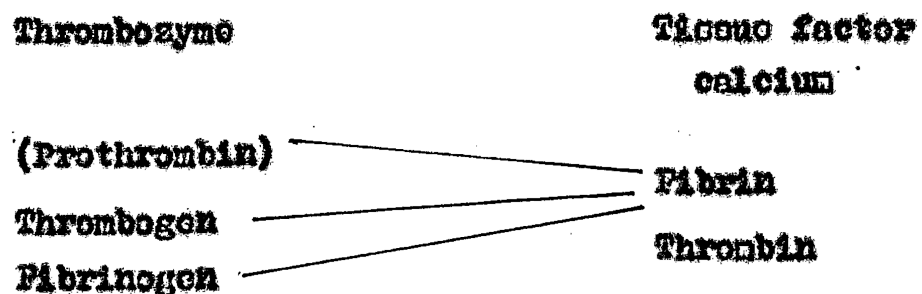
Nolf (1908 a) and Fischer (1934) assumed that prothrombin was derived from tissue. This theory was contradicted as it was already known that prothrombin could be isolated from plasma and secondly the tissue extract has an enzymatic nature.

Stuber and Lang (1930) observed that the process of glycolysis was similar to the phenomenon of blood coagulation. They found that substances which promoted

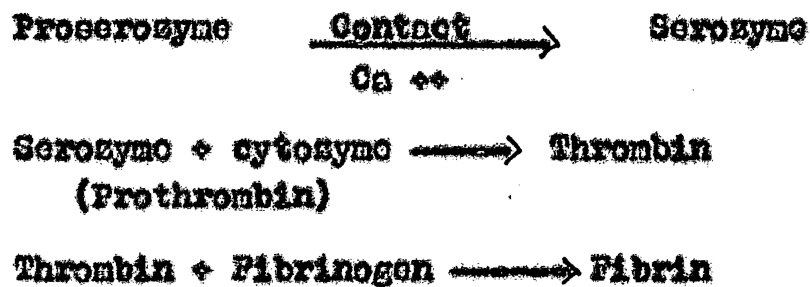
glycolysis shortened the clotting time and the one which delayed glycolysis lengthened the clotting time. These observations were proved to be wrong when the sugar free blood was shown to clot within normal time and the process of glycolysis in case of haemophilia was found to be quite normal.

Howell (1935) put forth a theory that thrombin is formed from prothrombin only by the calcium and in vivo the reaction is inhibited by the combination of heparin and prothrombin. In the presence of thromboplastin, prothrombin was set free to react with calcium. Hottelby (1935) contradicted these views by showing that formation of thrombin was possible even in the presence of heparin.

Helf (1908 a) proposed a valuable theory of blood coagulation. Three plasma proteins (i) thrombozyme (prothrombin of other workers), (ii) thrombogen and (iii) fibrinogen were concerned in blood coagulation. He showed that thrombozyme would be adsorbed by aluminium hydroxide, barium sulphate or calcium phosphate and thrombogen was left in supernatant adsorbed plasma. Helf's theory can be diagrammatically expressed as -



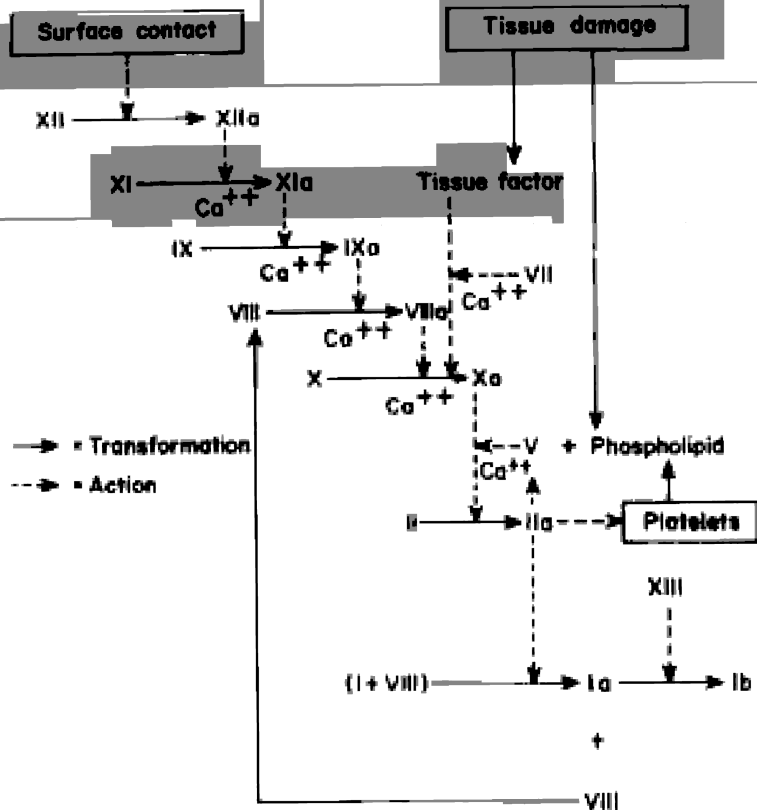
Bordet (1920) put forward a new theory as his experimental observations could not be answered by classical theory. He observed that blood collected in paraffin lined container clotted very slowly than the blood collected in ordinary glass container. This observation suggested that some substance was activated due to the contact with foreign surface. On the basis of this experiment, he supposed the presence of precursor in plasma a hypothetical substance. The theory of Bordet can be expressed as -



Modern theory of blood coagulation

For the last 48 years of this century the classical theory of the blood coagulation received much attention and was confirmed over and over again. But classical theory would not explain the reason why clotting factors get activated on contact with foreign surfaces. Secondly it also could not explain how haemorrhagic condition occurred in cases where the clotting factor recognised by classical theory were within normal limits. This was the first blow brought by Owen (1947) to classical theory.

FIG. 1



A blood coagulation scheme based on existing evidence, and including the autocatalytic effects of thrombin $1a =$ fibrin and $1b =$ stabilized fibrin.

Taken from Human Blood Coagulation,
Haemostasis and Thrombosis
Edited by Rosemary Sims
page, 22.

The development of the experimental work enabled the knowledge as regards the requirement of coagulation factor V, VII & X in addition to tissue factor and calcium for conversion of prothrombin to thrombin in vitro. It is also observed that in vivo the role of tissue factor was played by an activator, thromboplastin, which was derived from blood itself.

The prothrombin activator derived from tissues (brain, spleen, liver etc.) was called extrinsic thromboplastin or prothrombinase or extrinsic prothrombin activator or intrinsic thromboplastin. The extrinsic thromboplastin was found to be formed by the group of whole series of reactions in which at least eight clotting factors take part, namely factors V, VIII, IX, phospholipids, Hageman factor, plasma thromboplastin antecedents (PTA) and calcium. In the modern theory of blood coagulation the blood clotting is supposed to occur in three phases - (Fig.1)

- 1) Surface contact
- 2) Formation of thrombin
and
- 3) Conversion of fibrinogen to fibrin

Congenital factor I (Fibrinogen) deficiency

This disease is extremely rare and is probably inherited as an autosomal recessive condition and affects both sexes. Cases have been described by

Macfarlane (1938), Pinniger and Prunty (1946), Prentice (1951) and Hardisty and Pinniger (1956). A surprisingly large number of all the cases described have been the off spring of consanguineous marriages. The condition may manifest itself at birth with prolonged bleeding from the umbilical stump. There is a tendency to easy and excessive, bruising and cuts, scratches and vein puncture sites tend to bleed more than normal. The haemostatic defect in the afibrinogaemic patient is less marked than in the severely affected haemophilic and haemorrhoses are extremely rare, as is crippling. The plasma level of fibrinogen required for haemostasis is thought to be in the region of 50 - 100 mg./100 ml.

This level can usually be obtained by transfusion of fresh or stored plasma or freeze-dried fibrinogen concentrates, since the half life of transfused fibrinogen is 4 - 6 days (Madden and Gould 1952, Giltin and Borges 1953) transfusions for the control of haemorrhages after injury or surgery need to be given only every 2-3 days. The most striking laboratory findings are the infinitely prolonged whole blood clotting time and one stage prothrombin time and the failure of the blood to clot on the addition of a strong solution of thrombin.

Fibrinogen

Biologically this protein can be defined as the plasma protein which is clotted by thrombin and is

normally present in concentrations of 200-500 mg. per 100 ml. of plasma. Fibrinogen is one of the largest proteins found in normal plasma.

The role of fibrinogen in the living organism is not easily narrowed down to the area of haemostasis, when we consider that low levels of fibrinogen can be tolerated without excessive bleeding i.e. in afibrinogenemia and defibrinated states. Fibrinogen may play a role not only in haemostasis, but also in fibroblast, proliferation and defence mechanism against infection. Human fibrinogen has a molecular weight between 3,30,000 and 3,40,000 (Caspary Kokwick 1957, Schulman 1953).

The molecular structure of fibrinogen

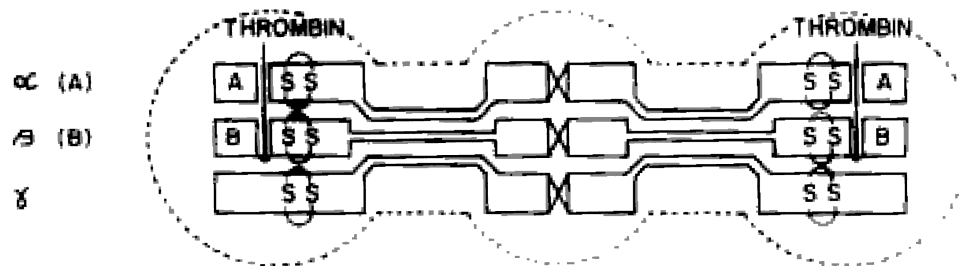
The current concept of fibrinogen is that it is an elongated molecule with high intrinsic viscosity. H_2N - terminal amino acid analysis (Blomback and Yamashina 1959) indicates that it is most likely a dimer built up by two identical halves. Each half consists of three polypeptide chains α (A), β (B) and γ which are linked by disulphide bridges. The two halves are also joined by disulphide bonds. Each chain has a free $COOH$ terminal group.

Fibrinogen has 56-58 half cystine residues all of which are involved in disulphide bridges (Henschen 1964 b). The treatment of fibrinogen with sodium sulphite, to cleave the $-S-S-$ bonds, yields three components with different electrophoretic mobilities (Clegg and Bailey 1962, Henschen 1964 c). The molecular weight of sulphitolyzed fibrinogen in various denaturing solvents lies between 50,000 and 65,000 (Henschen, 1964 a, Haschemeyer and Kadane 1963, Johnson and Mihalyi 1965) which suggest that fibrinogen is composed of three pairs of chains, which is in agreement with the N-terminal amino acid analysis, from this it follows that fibrinogen normally exists in a dimeric form and so far attempts to obtain the monomeric form have failed (Johnson and Mihalyi 1965).

Electron micrographs show that fibrinogen is composed of three modules 50-70 Å in diameter connected by a thin strand about 15 Å thick (Hall and Slayter 1962). The length of the fibrinogen molecule appears to be dependent on the pH of the solution from which it is dried showing a minimum of 230 Å at isoelectric point and increasing to 400 Å at pH 11.9 (Hall 1963).

Electric birefringence studies suggest that the two A peptides, are situated at each end and on the same side, of the fibrinogen molecule. The B peptides on the other hand are symmetrically located about the centre of the molecule (Haschemeyer and Tonoco 1962, Haschemeyer 1963).

FIG. 2



A model of the fibrinogen molecule (Blombäck *et al*, 1968). The dotted line represents the shape of fibrinogen molecule proposed by Hall & Slavter (1962).

Taken from Human Blood Coagulation
Haemostasis and Thrombosis
Edited by Rosemary Digg
page, 33.

Recently Blomback et.al. (1968) have found that the three chains of fibrinogen are cross linked in the N-terminal region by means of disulphide bridges (disulphide knot) and that there are two such regions per molecule of fibrinogen. The proposed model (Fig.2) of the fibrinogen molecule which can be related to the electron micrographs of Hall and Slayter (1962).

The central module may be the region in which the fibrinogen monomer are associated.

There is an evidence that fibrinogen in normal blood consists of a population of slightly different molecules. Thus we can distinguish fibrinogen of low, intermediate, and high solubility in ethanol water mixtures (Blomback and Blomback 1956, Morfess^y et.al. 1948, Morfesson et.al. 1967). The fibrinogen of low solubility that forms complexes with cold insoluble globulin has not until recently (Morfesson et.al. 1970) been obtained in high purity, therefore its molecular structure is not well understood. Human fibrinogen of intermediate solubility has been shown to contain molecules of different chromatographic behaviour (Finlayson 1963) or with differences in H₂N-terminal pattern or with a different degree of phosphorylation (Blomback et.al. 1966). The high solubility fibrinogen described by Morfesson et.al. (1967) has a lower molecular weight and is most likely produced by limited proteolysis of fibrinogen of intermediate solubility by plasmin (Morfesson et.al. 1967, Morfesson et.al. 1971).

When intact fibrinogen is exposed to plasmin there seems to be degradation of the α (A) chains and later of the β (B) chains (Mills and Karparkin 1970, Gaffney 1971). The γ -chains seem to be the most resistant. After long exposure of fibrinogen to thrombin there is also a change in the α (A) chains, as revealed by SDS - polyacrylamide gel electrophoresis (Mills and Karparkin 1970). Several dysfunctional fibrinogens have also been described. Some of these show differences in electrophoretic mobility as compared with normal fibrinogen, and in one fibrinogen Detroit - a point of mutation occurred in the H_2N - terminal portion of the molecules (Blomback et.al. 1968, Lammén et.al. 1969).

Abnormal forms of fibrinogen

Although hereditary deficiencies of fibrinogen have been known for some years (Macfarlane 1938) attention has recently been focussed, on the recognition of molecularly abnormal fibrinogens. Fanconi in 1944 postulated the existence of dysfibrinogenemia states due to the synthesis of molecularly abnormal fibrinogens and Biggs (1951) suggested that there was a difference between the fibrinogen of adults and newborn infants because plasma from the latter reacted more slowly with thrombin. Ingram (1955) studied two patients with prolonged thrombin clotting times and suggested that dysfibrinogenemia might be an hereditary trait. At least eight families with dysfibrinogenemia have now been described in which the affected patients had a normal

concentration of fibrinogen but their plasma reacted more slowly than normal plasma with different concentration of thrombin.

Menache (1964) described the first case of a patient with a clear cut abnormally reacting fibrinogen. The plasma of the patient did not produce a fibrin clot with intrinsic thromboplastin, extrinsic thromboplastin or normal amounts of thrombin. The addition of calcium and normal amount of thrombin resulted in translucent clot and higher concentration of thrombin produced clotting of the plasma.

The venom of *B jararaca* either with or without calcium produced no clot and the patients plasma inhibited the clotting of normal plasma by venom, thrombin or extrinsic thromboplastin. It was shown that the abnormality was confined to the fibrinogen and when this was separated by salt or alcohol precipitation and added to normal plasma, inhibition of thrombin fibrinogen reaction of the normal plasma occurred.

At this time no chemical studies were undertaken to identify the amino acids constitution of the α (A), β (B) or γ -chains of the fibrinogen, although it was clear that this constituted an aberrant molecular form of fibrinogen. It is interesting that this patient had no clinical history of abnormal bleeding. Beck et.al. (1965) described a patient with a mild

haemorrhagic diathesis in which the abnormality could be attributed to the patient's fibrinogen and in which the findings were similar to those described by Menacho. However, it showed some minor differences from those described by Menacho in that the patient's plasma did not interfere with the thrombin fibrinogen reaction of normal plasma and minor immune-electrophoretic differences distinguished the fibrinogen from normal fibrinogen. Beck et.al. (1965) recognized this as an abnormal variant of fibrinogen and adopting the nomenclature previously applied to normal haemoglobins, proposed the descriptive name fibrinogen. Patients with plasma having a prolonged thrombin clotting time attributed to the presence of an abnormal fibrinogen molecule have also been described by Von Felton et.al. (1966), Mannon et.al. (1968), (Fibrinogen Detroit) and Forman et.al. (1968), (Fibrinogen Cleveland). The abnormal fibrinogens are inherited as autosomal dominant traits, some of the patients have a mild bleeding tendency whilst others are symptom free and in two cases episodes of thromboembolism and post operative thrombophlebitis have been recorded. It is not clear yet exactly what precise differences exist in the eight families so far studied. Forman et.al. (1968) have compared their case with those of Beck et.al. (1965), Von Felton et.al. (1966) and Mannon et.al. (1968) and suggest that the abnormality is associated only with the aggregation of dissolved fibrin monomers or polymers.

Monacho suggested that the defect in the case involved the stage of hydrolysis of the fibrinogen by thrombin. Fibrinogen Detroit (Mammon et.al., 1968) has now been more extensively investigated than other fibrinogen variants. Ultra centrifuge studies gave the same molecular weight as normal fibrinogen and after sulphite cleavage the average molecular weights of the three chains were the same as in normal fibrinogen but carbohydrate analysis showed significantly lower values for neutral sugars, sialic acid and hexosamines than the normal fibrinogen. More recently Blomback et.al. (1968) have shown differences in the amino acid sequence of the $\alpha(A)$ chain of fibrinogen Detroit. After reduction and alkylation the N-terminal $\alpha(A)$ chain fragment was separated by factor on Sephadex G-100. This fraction was then digested with trypsin and the tryptic digest subjected to two dimensional electrophoresis - chromatography.

Comparison of the "fingerprint" of this fraction from normal fibrinogen and fibrinogen Detroit showed that one peptide was missing in the map from the abnormal fibrinogen. A second peptide spot was of a different colour with the ninhydrin reagent suggesting that this consisted of elements of the normal peptide in this position and elements of the missing peptide. The complete absence of one of the normal peptide indicated that the patient was homozygous for the fibrinogen Detroit trait. Further analysis of the

differeently coloured peptides indicated that the N-terminal part of the α (A) chain of the abnormal fibrinogen had arginine, residue 19 of a normal α (A) chain replaced by a neutral amino acid, probably serine. Blomback et.al. (1968 b) point out that the replacement of strongly basic amino acid by a neutral hydroxy acid in the N-terminal portion of the α (A) chain may result in considerable conformational changes in the N-terminal disulphide knot of fibrinogen and these changes may directly or indirectly affect the active site of polymerisation. More recently Blomback and Blomback (1969) have investigated the tryptic fingerprints of the α (A) chain fragments of fibrinogen Paris, Zurich, Baltimore and Louvain,. These fibrinogens appear to contain only normal tryptic fragments.

Action of whole venom on fibrinogen

Malayan pit viper crude venom has a thrombin like action on fibrinogen (Reid et.al. 1963 b; Mahas et.al. 1964) in man (Reid et.al. 1963 b) and in experimental animals (Regoeezi et.al. 1966, Reid 1967, Marshall and Bonouf 1968). It depletes plasma fibrinogen by converting the majority of the intravascular pool to fibrin with the formation of microclots probably localised in the capillary net works of the lungs, muclos, liver and spleen. Rapid removal of the microclot takes place re-establishing the potency of the vessels without tissue necrosis. In vitro, crude venom has a

powerful coagulant action (Chan et.al. 1965). The venom converts fibrinogen to fibrin directly both in plasma and in partially purified fibrinogen solutions, in venom concentration between 1 to 10 μ g. per ml. complete conversion of fibrinogen to fibrin appears to take place as the addition of thrombin does not produce further coagulation. Approximately 10 μ g. of whole venom has an equivalent coagulation activity of I.N.I.H. Unit of thrombin. Venom resembles thrombin in the conversion of fibrinogen to fibrin as it produces clot formation in the absence of other coagulation. However, the optical density of clots produced by venom is less than that produced by thrombin suggesting that only a fraction of the total fibrinogen is forming a clot (Regoceni 1966). In contrast to thrombin, heparin in therapeutic concentrations does not inhibit the coagulant activity of the venom in vivo (Chan et.al. 1965, Regoceni 1966) or in vitro (Regoceni et.al. 1966). Serum has a variable inhibitory effect on the coagulant activity of venom (Chan et.al. 1965).

Crude venom contains two enzymes which are known to have a direct action and the other with a powerful fibrinogenolytic effect (Regoceni 1966). Although like thrombin the coagulant fraction of venom acts directly on fibrinogen to produce fibrin, their actions are not identical. Regoceni (1966) suggested that unlike thrombin which releases fibrinopeptides A and B

from fibrinogen in the formation of fibrin, the coagulatory enzyme from the venom has no effect on the B chain of fibrinogen. The lytic component of venom although powerfully active in vitro, is of secondary importance in the production of hypofibrinogonemia in vivo (Rogocsi 1966, Rogocsi et.al. 1966).

Properties of fibrinogen

- 1) This is a protein which distinguishes plasma from serum having α characteristics of a globulin which is easily fractionable.
- 2) It is coagulated by heat at 56°C (serum globulin at about 70°C).
- 3) A plasma protein clottable by thrombin
- 4) Molecular weight 3,30,000 to 3,40,000 (Gospy and Kellwick 1957, Shulman 1953).
- 5) Fibrinogen molecule is 3 to 4 times larger than the other plasma globulins. It is elongated measuring 700-1000 Å in length and 30-50 Å in width.
- 6) Fibrinogen belongs to the fibrillar group of proteins.
- 7) Precipitated by $\frac{1}{5}$ to $\frac{1}{5}$ saturation with ammonium sulphate from human plasma.
- 8) The iso-electric point at pH 5.5
- 9) Destroyed by heat at 47°C

- 10) It is probably synthesised in the liver, as its level is decreased when this organ is removed.
- 11) The clotting of pure fibrinogen is inversely proportional to thrombin concentration.
- 12) The clotting of fibrinogen is due to the polymerisation of altered fibrinogen molecules.

Fibrinogen is used in the preparation of fibrin film and fibrin foam. It is used also in surgery, skin and nerve grafting and in treatment of burns.

Preparation of fibrinogen

Many procedures have been described for the preparation of fibrinogen (Kohwick 1964). The methods used depend upon the precipitation in cold organic solvents such as ethanol or ether, which is then followed by further reprecipitation step or by chromatography with Sephadex or Cellulose. The chromatography removes the last traces of factor XIII and plasminogen.

General methods of fractionation of proteins can be grouped under the following heads.

- 1) Salt precipitation method
- 2) Method using organic solvents such as ethanol, acetone and ether

- 3) Preparative electrophoretic method
- 4) Continuous electrophoresis
- 5) Linear polymers of high molecular weight (polyethylene glycol)
- 6) Chromatography using ion-exchange resins and cellulose ion-exchangers and sephadex etc.

These methods are discussed briefly with their advantages and disadvantages.

1. Salt precipitation method

Increase in the salt concentration in an aqueous solution causes a decrease in the solubility of proteins and they precipitate out. In protein fractionation, salts most commonly used are sodium chloride (Hammersten, 1879 and 1880), sodium sulphate (Howe 1921 and Kohn 1940) and ammonium sulphate (Chick and Martin 1913), Sorenson (1925, 1930), Cohn et.al. (1940), Ritschmann (1960) used polyphosphate of these ammonium sulphate is the most commonly used because of its higher solubility even though sodium sulphate was proved to be more effective protein precipitant than ammonium sulphate.

Usually the solubility of protein increases with increasing temperature within the certain limits.

pH plays an important role in the protein precipitation because each protein has its isoelectric point at which it is least soluble. The protein precipitation also depends on the dielectric constant of the solution. The proteins are large molecules with many positively and negatively charged groups, such as $-NH_3^+$ and $-COO^-$. These groups can interact electrostatically with each other, or with small ions of opposite charge in the solvent, they can also react with water; i.e. they are hydrophilic. Water has a relatively high dielectric constant (78.54 at $25^\circ C$) and in aqueous solution, the interaction between the charged groups with water molecules is enhanced, thus favouring solubility. Some of the proteins like albumin and pseudoglobulins are soluble in salt-free water because the protein-water interaction is stronger than protein-protein interaction. On the other hand, euglobulins are insoluble in salt-free water because the protein-protein interaction is stronger. At low salt concentrations, the charged groups of the protein interact with the inorganic ions, the euglobulins are brought into solution and the protein is said to be "salted in". When more salt is added, the ionic strength is increased and the effective concentration of water (activity) is decreased, until a point is reached where the protein-protein interaction begins to exceed the protein-water interaction. The protein is said to be "salted out".

Disadvantages of salting out method

- 1) The methods of salting out do not sharply separate a series of homogeneous fractions and several reprecipitations have to be carried out to obtain a pure fraction which results in loss of material.
- 2) So the salting out method can be used only as a preliminary method to purify partially the desired fraction which may be further purified by other methods.
- 3) Complete removal of salt which is used for the protein precipitation is difficult for which the dialysis is required.
- 4) The chances of bacterial contamination are more during the dialysis.

2. Low temperature methods using organic solvents

Organic solvents such as acetone, ether and ethanol lower the dielectric constant which causes a decrease in the solubility of the proteins.

The conditions which have to be maintained during such fractionation procedures are :-

- 1) Salt content (ionic strength)
- 2) The temperature to be maintained at 0° to - 5°C.

- 3) pH should be as near the isoelectric point of protein as possible.
- 4) Concentration of protein
and
- 5) Concentration of ethanol, acetone or other

Mollayⁿ (1908) and Hardy and Gardiner (1910) first developed this method using ethanol as a protein precipitant. Cohn (1941) made a systematic study of ethanol fractionation method and developed the procedure to fractionate human plasma. Pilenor (1943) developed the method using methanol while Kekwick (1946) used ether for plasma fractionation. Cohn et.al. (1946) modified the method for plasma fractionation and established the following optimum conditions for the separation of a component from a mixture of plasma proteins. According to Cohn, the following five important factors are essential to get best results :-

- 1) Salt concentration is maintained as low as possible in order,
 - a) To take advantage of the diversity of protein electrolyte interactions
 - b) To avoid the necessity of dialysis
- 2) Concentration of ethanol:- The ethanol concentration should be kept below 40%.
- 3) Concentration of protein maintained

- a) Sometimes as low as possible to minimize protein - protein interaction.
 - b) Other times as high as possible in order to render large scale processing.
- 4) pH near the isoelectric point
 - 5) Temperature is kept as low as possible between zero and the freezing point of the solution.

Advantages of cold ethanol method

- 1) When acetone or ether is used as protein precipitant the processing has to be done under very strict conditions and more precautions have to be taken to avoid chances of explosions. This is not the case when ethanol is used as a protein precipitant.
- 2) The plasma protein fractions can be obtained on a large scale in a purer state if conditions are properly maintained by this method than by any other method.
- 3) All fractions viz. fibrinogen, gamma globulin and albumin prepared by this method can be utilized clinically.
- 4) The cold ethanol method has got another advantage over the salt precipitation method. In the latter, the high salt concentration necessary for protein precipitation can be diminished only by dialysis,

a procedure which is time consuming and involves much work when a large volume of a liquid is to be dialysed and exposes the proteins to higher temperature which may give rise to denaturation of proteins.

Further more, during the process of dialysis, when salt concentration is diminished, conditions become favourable for bacterial growth and protein solution is likely to be contaminated.

- 5) If ethanol is used as a protein precipitant instead of salt, it may be readily removed because of its volatility. The safest method for such removal appears to be the rapid freezing of the solution at very low temperature with subsequent drying from the frozen state in vacuum i.e. lyophilisation.

3. Electrophoretic methods

When proteins are subjected to an electric field they move according to their electrophoretic mobility at that particular pH. The mobility of the protein depends on the net charge on the protein molecule at the pH used. This method was developed by Tiselius (1937) to separate serum proteins. The method is generally used for the qualitative and quantitative analysis of protein mixtures. The original method of

electrophoresis has found number of variations in last few years. In 1956 Gini used preparative agar electrophoresis to isolate proteins in a homogenous form. This method, though it gives very pure fractions, cannot be adopted for large scale manufacture.

4. Continuous electrophoresis

A method was evolved by Haugard and Kerner (1948) to separate proteins using continuous electrophoresis on a paper curtain. The proteins separate according to their electrical charge. The apparatus consists of an electrolyte reservoir. The filter paper sheet hangs from the reservoir. The electrodes are placed along the sides of the paper. The bottom edge of the filter paper is serrated to afford drip points. At a point on the top, the paper is cut and folded back to form a tab to which the sample is fed by means of a Wick. The electrolyte flow is continuous. The electrolyte comes from the gravity and the current is passed at right angles to the flow of electrolyte. This results in a separation of the mixture of the proteins in a continuous manner. It is important to maintain a relatively uniform field of potential distribution on the paper. There should be no evaporation of water or an increase in temperature resulting in the

precipitation of the proteins on the paper during electrophoresis. For this reason the concentration of the electrolyte in the buffer should be low enough to avoid deposition on drying of the sample. The protein mixture to be separated should not be too concentrated.

5. Use of water soluble polymer (polyethylene glycol)

The protein can be fractionated by the use of high molecular weight linear polymer. Polson et.al. (1964) made use of polyethylene glycol molecular weight 6,000 to isolate homogeneous plasma fractions such as fibrinogen and gamma globulin. The products prepared by this method retained satisfactory biological properties. Paul (1967) developed the method to isolate immunoglobulins from commercial gamma globulin preparations and from human serum, using polyethylene glycol molecular weight 5,700. This is another preparative method and cannot be used for large scale processing.

6. Ion exchange chromatography and gel filtration methods

Ion exchangers are insoluble compounds which possess the power of adsorption and have a charge. The structure of an ion exchanger consists essentially

of a polymeric Skeleton to which are attached the charged groups. The charge on the molecule is neutralized by electrostatic charge on the polymer and the whole molecule is thus made electrically neutral. Thus ion-exchange chromatography involves the electrostatic binding of proteins on to a resin suspended in buffer and packed into a column. Elution may be effected either by changing the pH of buffer passing through the column, thus affecting the charge on the protein molecules, or by increasing the molarity of the buffer, thus providing more salt ions to compete with the proteins for charged groups on the resin. Conditions for chromatography are selected to take advantage of net charge differences between serum proteins and to secure a net charge on the resin opposite that of proteins.

Synthetic ion exchange resins have been used extensively for the separation of many substances like serum albumin by Boman (1955), purification of enzymes by Beinert et al. (1952), and for the separation of amino acids by Stein and Moore (1952).

In 1956 Peterson and Sober introduced cellulosic ion exchangers, as ion exchange resins were not found to be very useful in the separation of large molecules like proteins, amino acids, lipids and sugars. The cellulosic ion exchangers have a larger exchange

capacity than that of ion exchange resins. Diethyl aminoethyl (DEAE) cellulose is an anion exchanger and used with buffer system above pH 7.0. Carboxymethyl (c.m.) cellulose is a cation exchanger and used with buffer systems below pH 7.0. By using DEAE cellulose pure IgG was isolated from human and rabbit sera.

Gel filtration chromatography was introduced by Porath and Flodin (1959, 1960). This method has been widely used to isolate antibodies of different molecular weights and to separate the heavy and light polypeptide chains of the immunoglobulin molecule (Fleischman et.al. 1962).

These methods though they give very pure fractions cannot be adopted for large scale manufacture.

Thrombin

Thrombin may be defined as the natural coagulant of fibrinogen. Thrombin appears in blood following the activation of the clotting system, as the result of proteolytic action on its parent prothrombin. Thrombin can be conveniently separated from activation mixtures by making use of the fact that it is less negatively charged than prothrombin or the residue

left after thrombin has been formed. The most highly purified preparations were first obtained by chromatography on columns of Amberlite IRC-50 (Rasmussen 1955) more recently columns of phosphorylated cellulose have been used (Soegars and Landaburu 1960).

The primary sequence of thrombin has almost been elucidated (Magnusson, 1971), and it has been found that, whereas prothrombin consists of a single polypeptide chain, thrombin consists of two chains of unequal length joined by a disulphide bridge. This means that the conversion of prothrombin to thrombin involves the cleavage of the molecule in two places.

The A-chain of thrombin contains forty nine amino acid residues and has no carbohydrate. This chain is derived from the central portion of the original prothrombin molecule. The B-chain, on the other hand, has about 225 residues and shows extensive sequence homology, with the pancreatic protease such as chymotrypsin. The B-chain is derived from the C-terminal end of prothrombin and carries the active centre of the enzyme. Thrombin, like chymotrypsin, has a reactive serine residue at its active centre, which can be characterised by its reaction with diisopropyl-fluorophosphate (Gladner and Lohr 1956). Assuming that there is one active centre per molecule of thrombin, Keady, Lorand and Miller (1969) calculate a molecular weight of 32,600 for thrombin.

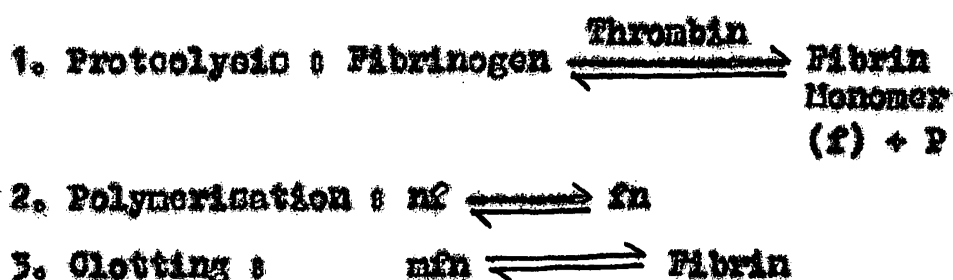
which agrees reasonably well with that obtained by other procedures.

Thrombin hydrolyses arginyl-glycine bonds in fibrinogen but does not hydrolyse these bonds in insulin (Bailey and Bettelheim, 1955) or the isolated B-chain of insulin (Bailey *et.al.* 1954). This apparent specificity for the substrate may be determined by the distance between the binding site, on the substrate, and the arginyl-glycine. It is interesting that the fibrinopeptides released from porcine secretin by the action of thrombin all have a phenyl-alanine residue from the C-terminal arginine (Blomback, 1966, Malt *et.al.* 1965). It may be that this phenylalanine residue is the binding site for the thrombin. The binding site on thrombin for macromolecular substrates may be destroyed by acetylation, since acetylation inhibits the activity of thrombin on fibrinogen while leaving reactivity of the active centre unaffected (Lamshurst and Soogero 1959).

Thrombin besides acting on fibrinogen, has an "activating" effect on factors VIII, V & XIII. The mechanism by which this "activation" is achieved is not yet explained but it does seem to depend on the thrombin being enzymically active.

Thrombin fibrinogen reaction

This reaction gives rise to the only visible phenomenon in coagulation and it is not surprising that it is used as indicator for either of the reactants, and the most commonly used end point for the reaction is the gelation of the plasma. When one adds to purified fibrinogen increasing concentrations of purified thrombin and plots the clotting time of the mixtures against the thrombin concentration one obtains a hyperbolic curve. This curve can be converted into a straight line if the clotting time is plotted against the reciprocal of the thrombin concentration. The visible phenomenon which one observes is in fact the summation of three distinct phases in the interaction of the two proteins (Shoraga and Lackowski 1957). These three phases are visualised as three separate reversible steps viz -



P denotes the release of peptides from fibrinogen due to the enzymic action of thrombin. These peptides were designated A & B (Bettolheim 1956, Bettolheim and Bailey, 1952) Blomback and Vestermark (1958) showed that

the release of peptide A parallels the rate of fibrin formation, while the release of peptide B seems to be minimal when the formation of fibrin has ceased. Two other peptides are also released from fibrinogen. These are designated AP and Y (Klembek 1966). These peptides are analogues of the A peptide. The AP peptide has a phosphorylated serine residue and the Y peptide is the A peptide less the N-terminal alanine. The significance of these peptides is not yet understood. The molecular weights of the A and B peptides from bovine fibrinogen are 1,500 and 2,400 respectively (Gladner et.al. 1959).

In all fibrinogens which have been investigated thrombin cleaves an arginyl-glycine bond when releasing the fibrinopeptides, which are all negatively charged (Klembek, 1966). It is the loss of these peptides, and thus negative charge, which initiates the second phase of the reaction, namely polymerization.

The mechanism by which fibrin monomer polymerises has perplexed chemists and physical chemists for a considerable number of years. In essence the problems surrounding the reversible polymerization of fibrin are that during polymerization, there is a change in the ionisation of the amino acid side chains and that the process is exothermic. One possible explanation for this is that these changes are the result of the action of factor XIII catalysing the reaction between glutamine and lysine side chains. However, Endres and Shorger (1968)

have shown that when this reaction is prevented by the prior reaction of fibrinogen with hydroxylamine there is no significant difference between the behaviour of modified and unmodified fibrinogen. It may be that the observed changes in ionisation and evolution of heat are the result of several different types of interchain interactions. Any type of interaction that is proposed must take into account that the polymerisation of fibrin monomer can be reversed by urea.

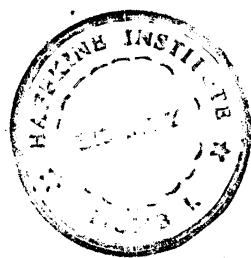
Measurement of fibrinogen

The determination of purity of fibrinogen is linked with the problem of the measurement of fibrinogen. Most methods for the determination of fibrinogen depend on the assumption that thrombin will quantitatively convert fibrinogen to fibrin. During this conversion peptides are released from fibrinogen, which amount to about 3 per cent of the total fibrinogen nitrogen (Lorand 1952). If the amount of fibrin formed is estimated from dry weight or nitrogen determinations, then the estimate will be low by an amount which is equivalent to the amount of peptide released. It however the fibrin is estimated from measurements of the optical density of the redissolved clot at 280 mμ. the loss of the peptides will not be detected since they only have a low optical density at 280 mμ. The fibrinogen is also estimated biochemically by Biuret method and immunologically by Radial Immunodiffusion

Technique having antisera against fibrinogen.

Genonital deficiency of factor VIII (haemophilia)

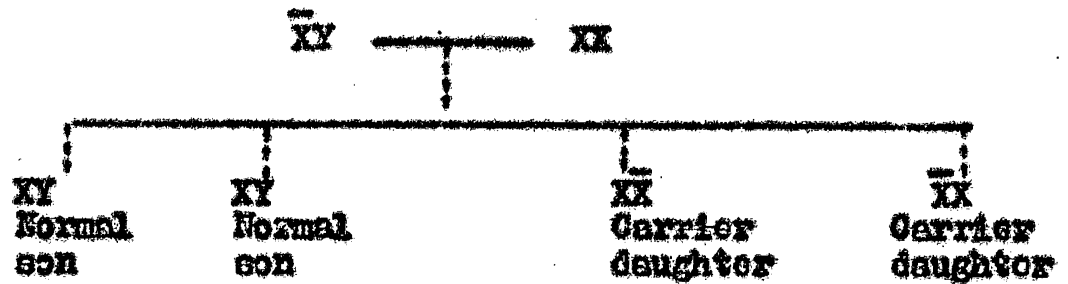
Haemophilia is relatively uncommon disease and is found its severe form in 1 per 25,000 of the population. During last few years, important changes have taken place in the concept of haemophilia. Previously haemophilia was regarded as a well defined clinical entity with a characteristic inheritance and symptomatology caused by a specific defect of blood coagulation. A great variety of names were proposed for such haemorrhagic conditions. Recent advances in the knowledge of intrinsic prothrombin activator system of blood coagulation have altered those views and it seems now that the term haemophilia used in past covered a group of haemorrhagic conditions in which similar clinical manifestations were produced by the basic defect in blood coagulation mechanism. The term haemophilia A is now used to describe the disorder when antihæmophilic globulin (AHG, Factor VIII) is deficient and haemophilia B is used to describe the disorder when PTC is deficient. Haemophilia A is seven times more common than haemophilia B. Haemophilia A and B are not genetically related but both are inherited as sex linked recessive character because the genes for the disorder are carried by the X-chromosome. Thus females carrying a gene for haemophilia on one of



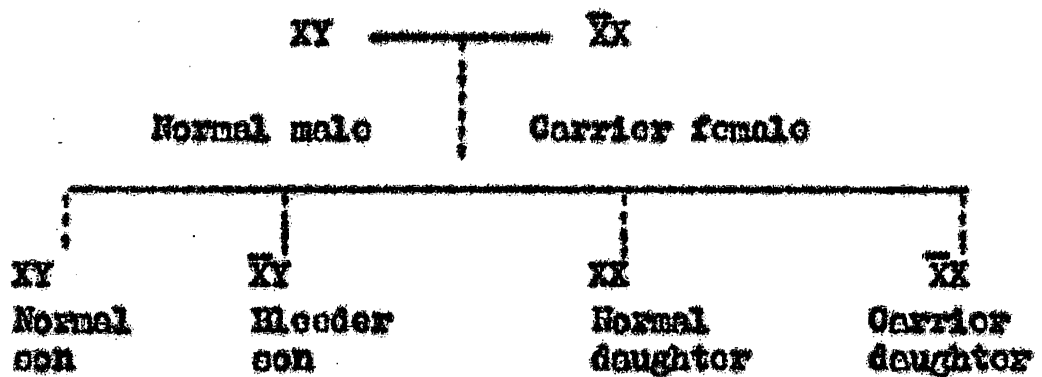
their two X-chromosomes transmit the gene to half of their female offspring and half of their male offspring according to the law of chance. On the other hand haemophilics having only one X-chromosome, transmit the gene to all their female offspring, but to none of their male children. Theoretically it was assumed that a female receives one defective X-gene from the haemophilic father and another defective X-gene from her carrier mother than she may be bleeder. But series of experiments with dogs proved that above conclusion is not true. According to the theory, a single X-chromosome carrying the haemophilia gene in female would not produce a deficiency of factor VIII, because it would be eliminated by the unaffected X-chromosome. In addition to the fact the bleeder conductor marriage could not be expected to have occurred often. It was suspected further that the presence of a double quantity of defect might be lethal and inhibit the development of embryo.



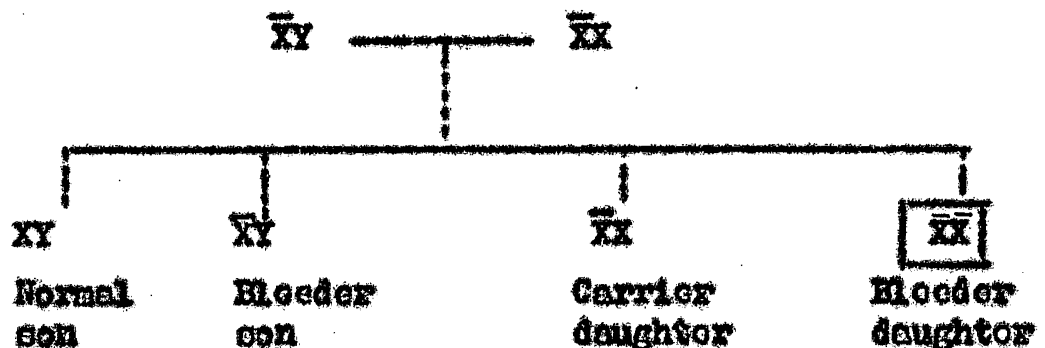
I Haemophilic marrying normal female



II Normal male marrying carrier female



III Haemophilic marrying a carrier female



Severely affected patients suffer prolonged bleeding after injury and characteristically experience

spontaneous haemorrhages into deep muscular tissues and joints. The more mildly affected patients still bleed after trauma but in general are spared the painful and crippling haemorrhages into joints and muscles experienced by the severely affected patients. Superficial cuts and abrasions rarely bleed excessively although deeper wounds may bleed on and off for several weeks unless antihæmophilic replacement therapy is given.

X-linked hæmophilia

One of the most famous inherited abnormalities in man is the bleeder disease, hæmophilia. It is one of several genetic conditions which may result in excessive bleeding at points of injury or stress, usually as a result of delayed clotting which is due to a deficiency of certain components of the blood. Different abnormal genes, some autosomal and others X-linked, are responsible for the deficiency of different components. In classical X-linked hæmophilia, hæmophilia A, "antihæmophilic globulin" is lacking or present in small quantities. In another recently discovered X-linked bleeder disease, called hæmophilia B, or Christmas disease there is a deficiency for another substance the "plasma thromboplastin component". Only recently has it become possible to distinguish the

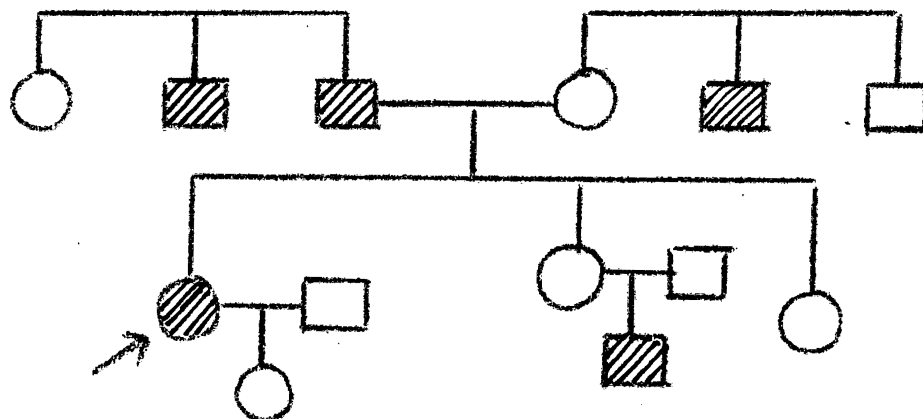
X-linked haemophilia, and it has not yet been decided whether the corresponding genes occupy one or two loci. Haemophilia is well known even among laymen because of its seriousness and of the mystical feelings which are still commonly associated with true or imagined properties of blood, and because, during the past hundred years it has occurred in many members of the royal families of Europe.

Transmission of haemophilia, based on the discovery of sex chromosomes and their recognition as carriers of sex-linked genes, became possible only in the twentieth century.

It has often been wondered whether female bleeders actually exist, even though some women of this type have been listed in the rare autosomal pedigrees of bleeding diseases. Until 1951, no X-linked haemophilic female had been diagnosed, a fact which led to the suggestion that hh females either die before birth or that they live but are phenotypically normal non bleeders. Geneticists were not inclined to accept either hypothesis, but pointed out that hh females would only occur among the offspring of heterozygous (Hh) women and affected h men and that the very small number of such marriages had, by chance not produced hh daughters. This view was strengthened when typical X-linked haemophilic was discovered in a kindred of dogs and when matings between

the heterozygous female and affected male dogs produced haemophilic females as well as males. Studies on the blood of these dogs contributed greatly to the understanding of haemophilia in man.

Soon after the haemophilic dogs had become known, human females were described who were hh homozygous and exhibited, according to careful tests, all symptoms of true X-linked haemophilia. The best established case is that of a woman who had had a history of easy bruising and free bleeding but who had successfully given birth to a child. She probably escaped serious bleeding at the time of delivery because, as is generally thought, the control of haemorrhage from the placental site is by muscular contraction of the uterine wall and not by coagulation of blood. This haemophilic woman's father had died of haemophilia, as had one of her mother's brothers. Her mother may therefore be classified as a carrier, and the genotype of the parents as Hh and h.



Pedigree of haemophilia containing a haemophilic woman

X-linked genes are generally regarded as having no allele in the y-chromosome, and this chromosome can consequently be neglected in the discussions of X-linked transmission. It would however, be possible to assume that the y-chromosome contains recessive alleles for all X-linked genes. If this were true a nonhaemophilic man, for example, would not be homozygous for normality, but heterozygous for the normal allele in the X-chromosome and the recessive haemophilia allele in the y-chromosome, and a haemophilic man would be homozygous for the abnormal allele because of its presence in both sex chromosome. It is unlikely that such a view of the localization and nature of X-linked genes is correct. It does not seem to explain anything that the simpler interpretation of the homozygous nature of X-linked genes in the male cannot and in any case it can at present neither be proven nor disproven.

The concept of haemophilia

The existence of haemophilia, the commonest of the relatively rare disorders of coagulation, has been the main impetus for the studies that have led to our present knowledge of factor VIII. A familial type of bleeding which tends to be severe and some times fatal.

The earliest recorded account of the complications of minor surgery performed on haemophiliacs is found in the 5th century Talmud (1969). In the Rabbinic

instruction, a mother is excused from circumcision of her child if the first two have died as a result of this procedure. John Conrad Otto (1803), an American physician, published a detailed account of haemophilia in 1803 that was widely read. He recognised that the unaffected mother transmits the disease to her affected son, referred to as a "blooder" but a more complete collection of cases was presented by Hasse (1820). He also formulated the law of transmission of haemophilia. The term "haemophilia" was applied to the condition by Hopff, a student of Schönlein's in 1828. The final establishment of haemophilia as a specific clinical entity was made by Bullock and Fildes (1911). Haemophilia has been referred to in the popular press as the disease of royalty because it has affected a number of males descended from Queen Victoria (1966) although one who treats haemophiliacs in America is impressed by its tendency to impoverish the family transmitting it.

Wright invented the whole blood clotting time as a diagnostic test for haemophilia in 1893. In 1911 Addison demonstrated that the prolonged clotting time of haemophilic blood could be shortened by the addition of a fraction of normal plasma. This fraction prepared by dilution and acidification of normal plasma, was named antihaemophilic globulin by Patch and Taylor in 1937. Patch and Stetsen in 1935 showed that the clotting time of a haemophilic reverts to normal when he is

transfused with normal blood, an effect, Brinkhouse later demonstrated to be due to the more rapid conversion of prothrombin to thrombin in 1939. The plasma coagulant that is deficient in haemophilia has also been called antihæmophilic factor (AHF) and most recently factor VIII (Wright 1959). Biggs and associates (1952) described seven cases of supposed hæmophilia, but their AHG concentration was found to be normal though their hæmorrhagic conditions were similar to that of hæmophilia. They named this bleeding tendency as "CHRISTMAS DISEASE" and the factor responsible for the abnormality was named "CHRISTMAS FACTOR". Simultaneously Aggeler et.al. (1952) represents a similar hæmorrhagic condition, but the patient had normal AHG and PTC levels. This bleeding abnormality could not be explained by classical theory and was called "PTA DEFICIENCY" (PTA- plasma thromboplastin antecedents) and the factor deficient was called PTA or factor XI.

Clinical manifestations

Except in mild cases, the hæmorrhagic tendency appears in infancy and remains a life long affliction. Bleeding may occur from or into the external surfaces of the body, from nose, mouth, gastro-intestinal or urinary tracts may continue for days and weeks leading



in some cases to death from exsanguination. There may be haemorrhage into tissue following trauma or infection resulting often into the formation of numerous haematomas. In addition to blood loss tissue haemorrhage may cause pressure effects. The blood supply to the limbs or abdominal organs may be occluded with subsequent necrosis or atrophy. Patients remain anaemic due to repeated blood losses.

In any individual the severity of the basic disorder tends to remain relatively constant. There may be periodic phases during which patients seem to be rather more or less liable than usual to develop haemorrhages, but such phases are very indefinite and cannot be correlated with the demonstrable changes in clotting mechanism.

With growing age there appears to be a loosening of the tendency to bleed. However, this may be due to years of discretion when injuries are avoided rather than any substantial improvement in basic pathology.

There are wide variations in the severity from patient to patient, though in a single family the affected members usually have the similar severity. On clinical grounds a patient will be graded as "severe" if he suffered repeated haemorrhages with serious crippling and deep tissue haemorrhages, with



little provocation "moderate" if he had a few haemorrhosis, occasional haematoma and no serious crippling, the "mild", if he had no haemorrhosis or other spontaneous bleeding and merely gave a history of abnormal bleeding after definite injury.

Treatment in haemophilia

Before recognition of the exact nature of the clotting defect in haemophilia, the treatment consisted of attempts to control excessive bleeding by local measures such as cauterization of wounds by heat diathermy or chemicals, application of tourniquets or tight dressing and sometimes tissues were sutured tightly over the bleeding points in the hope of achieving haemostasis. The result was almost always extensive tissue destruction and bleeding from a larger area than before, the formation of dangerous haematomas or actual gangrene.

Recently the treatment of haemophilia has aimed at a correction of clotting defect in vivo, i.e. raising the AHC level. Many coagulant substances were tried without success. It was also found that spontaneous bleeding in severely affected cases was easy to control rather than traumatic bleeding. This is because factor VIII level between 10-15 per cent suffices to control

spontaneous bleeding but for traumatic bleeding much higher levels (about more than 50 percent are needed).

In 1840, Lane suggested whole blood transfusion therapy but this could correct the AHF levels to very little extent. Later on fresh plasma was used to raise the AHG levels. The main difficulties encountered in treating the patient in this manner was the volume of plasma that could be transfused i.e. maximum 1000 ml. of plasma can be given to the patient which raises the AHG level by 20-35 per cent temporarily. Now-a-days AHG concentrates are being manufactured from human, bovine and pig plasma. Biggs and Macfarlane (1962) observed that AHG concentrates which is also known as "cryoprecipitate" are three to five times more active than fresh plasma. This cryoprecipitate can be stored frozen at -20°C . for several months without much loss of activity.

Antihæmophilic globulin (Factor VIII)

Factor VIII is a protein of large molecular weight greater than 1,00,000 not absorbed by $\text{Al}(\text{OH})_3$, BaSO_4 , absent in serum. It separates with fibrinogen in fractionation procedures, may be separated from each other by continuous electrophoresis. The substance

is highly unstable. Some preparations may be stored without loss of activity in the deep freeze for years at -20°C . The activity disappears during clotting and is rapidly destroyed by thrombin and proteolytic enzymes, congenital deficiency of factor VIII causes haemophilia. Horshgold et.al. (1971) analyzed a human fraction of high purity and found it to contain 75% amino acids, 10% hexose, and 11% lipid. Marchosi et.al. (1972) also found carbohydrate (5%) and lipid (5%) in human preparation. Mackee's (1970) human fraction was stated to contain only 5% neutral sugars. Schmer et.al. (1972) found that bovine factor VIII preparations purified 10,000 times contained 20% carbohydrate, but no lipid.

The measurement of factor VIII levels

Estimates of amounts of factor VIII in plasma have been based largely on three assay procedures. The earliest involves measuring the improvement of the impaired utilization of prothrombin in haemophilic whole blood after the addition of varying amounts of the plasma being tested (Graham, et.al. 1951). The estimate is made by comparing the effectiveness of the test plasma with a standard plasma in bringing the prothrombin concentration of a haemophilic plasma to some preselected value under standard conditions say to 50% in 30 minutes at 28°C and pH 7.0. Another

assay of factor VIII is based on the observation that the prolonged "partial thromboplastin" (or cephalin) clotting time (PTT) of haemophilic plasma can be reduced by addition of small amounts of normal plasma in reducing the prolonged clotting time and the effectiveness expressed as per cent of normal (Langdell et.al. 1953). The third method (Biggs and Douglas, 1959), the thromboplastin generation test (TGT) measures the amount of "thromboplastin" generated in a clotting system in which the amount of AHF is the limiting factor (Biggs, et.al. 1955). Each method has disadvantages that need to be recognized and the decision as to which one to use ultimately rests with investigator.

The two stage method is based on the thromboplastin generation test. In this test system dilutions of the test plasma are compared with normal in their activity to form intrinsic prothrombin activator. Two stage method gives less random errors than the other methods.

BIOCHEMISTRY OF FACTOR VIII

1. Stability with changes of temperature and pH

At pH 7.2 more than 90% of the activity persisted for 7 days at 4°C., while at both 28 and

37°C. there was a 50% loss in 24 hrs. Loss was more rapid at higher temperatures, in 30 minutes at 45°C. - 50% loss, at 55 - complete loss. Complete destruction at pH 4 and 10 at 37°C for 30 minutes. 50% destruction at pH 5 and 9, and no destruction at pH 7.

2. Stability during storage: Quick freezing at very low temperatures, and storage at temperature - 30°C or below promote retention of activity.
3. Effects of chelators on activity: The stability of factor VIII varied with the type of chelating agent; most rapid loss in plasma collected in EDTA, considerable loss when plasma collected in oxalate, Factor VIII activity persisted longer in citrated plasma and longest in citrated plasma with added $MgCl_2$.
4. Electrophoretic mobility: Factor VIII was reported to migrate in the β_1 or β_2 globulin region. (Hardisty and Ponniger 1956). Lewis et.al. (1959) found that factor VIII in prealbumin and $\alpha_1 - \alpha_2$ regions in continuous electrophoresis. Barkhan et.al. (1963) located it in the α_2 - globulin zone in starch block electrophoresis. Factor VIII has mobility ranging from prealbumin to slow gamma globulin.

5. Molecular size of intact factor VIII in plasma

Shulman et.al. (1960) arrived at a figure of 196,000 daltons for the molecular weight of "platelet cofactor I" (Factor VIII). Koco et.al. (1969) precipitated factor VIII from plasma and filtered through agarose gel column and estimated the molecular weight more than 2 million.

6. Minimum molecular size: Molecular size have ranged from 25,000 to 27,000 daltons. Barrow and Graham (1968) estimated molecular size of the purified factor VIII lie between 24,000, 28,000 daltons. McKee (1970) found minimum molecular weight was 927,000. Logan et.al. (1972) found between 90,000-1,00,000.

PHYSIOLOGY OF FACTOR VIII

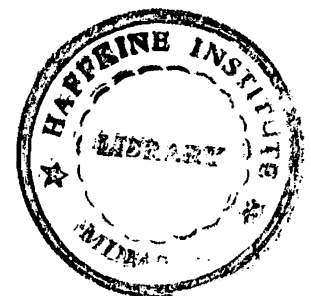
1. Half time survival in transfused subjects: The survival times reported for factor VIII in all types of subjects varied from 10 to 26 days (Schulze 1966).
2. Sites of synthesis: The spleen, liver and kidney may be involved in the synthesis of factor VIII (Norman et.al. 1967, Webster et.al. 1967, Dodds U.J. 1969).



3. Role of clotting mechanism: Factor VIII is converted to factor VIIIa by IXa. It is also activated by thrombin. The experiments do not prove that factor VIII is not an enzyme. The preparations of highly purified factor VIII contains lipid which is necessary for its activity. Incubation of factor VIII with phospholipase C resulted in rapid destruction of factor VIII (Hershenfeld et.al. 1971).
4. Increased levels in plasma: Factor VIII levels are increased during pregnancy with use of oral contraceptives, patients with leukemia (lymphoblastic), patients suffering from renal disease and also by exercise. (Brandt et.al. 1973, Crowell et.al. 1971, Rosenthal and Sloan 1967).
5. Activation in vitro : The enzyme thrombin has two effects on factor VIII, when it is added to whole blood factor VIII activity disappears when it is added to plasma, fibrinogen is converted to fibrin and factor VIII is destroyed (Alexander et.al. 1954, Penick 1957, Rissa 1957).

Immunology of factor VIII

The patients with haemophilia develop antibody against factor VIII. These human anti-factor VIII





antibodies are designated as "inhibitors". These antibodies neutralize factor VIII. These inhibitors belong to γ -G immunoglobulins having sedimentation constant 7S. The inhibitor is quantitatively removed by an antihuman γ -globulin. The inhibitor activity is exhausted by incubation with factor for 4 to 5 hrs.

Abnormal forms of factor VIII

The haemophiliacs possess a functionally inactive form of factor VIII. The transfusion in haemophiliacs develops factor VIII antibodies, which suggest that there are two groups of patients, one group in which the plasma is completely deficient in protein bearing any immunological relationship to factor VIII and a second group in which the plasma contains a functionally inactive but immunologically similar form of factor VIII to that found in normal persons. As there is no antigen in the first group, infused factor VIII may develop antibodies (Daussot, 1959, Shanberg and Goro 1957, Usaynoki 1966). To test factor VIII antigen in haemophilic plasma samples is to see whether the samples are able to neutralize factor VIII antibodies using standard coagulation assays to measure residual inhibitor after mixing antigen and inhibitor. Adelson *et al.* (1963) showed that haemophilia A plasma was unable to neutralize antifactor VIII antibodies whilst Shanberg and Goro (1957)

and Piper Schreier (1964) concluded that haemophilic plasma had the same capacity as normal plasma to neutralise factor VIII antibodies. Berglund (1962) also obtained equivocal results using thromboplastin generation test as a method of measuring factor VIII inhibitor neutralization.

With the discovery of two forms of haemophilia B it became apparent that perhaps there are two variations of haemophilia, in one of them immunological equivalent is present. Certainly this hypothesis would explain the conflicting and confusing reports of earlier workers. Recently Hoyer and Brockenridge (1968) and Duncan *et al.* (1969) have provided evidence to show that this is indeed the case. Duncan *et al.* applied a similar terminology to that which had been applied to haemophilia B namely haemophilia A⁺ for patients whose plasma contained a functionally inactive but antigenic equivalent substance to factor VIII and haemophilia A⁻ for patients whose plasma was totally lacking in immunologically recognizable factor VIII.

Materials for the treatment of patients with deficiency of factor VIII

- i) Plasma
- ii) Cryoprecipitate
- iii) Factor VIII rich fractions
- iv) Animal AHG



- i. Plasma: Plasma of the blood collected in A.C.D. from ten donors of groups O, A., B and AB pooled in the ratio 4:4:1:1 or concentrated human AHG. Plasma is kept frozen at -40°C . The loss on freezing is about 20% (Preston 1967). Factor VIII in fresh frozen plasma at -30°C . for six months, 73% activity survives for at least 20 months at -30°C (Britton and Grove Rasmussen 1966).

Potency of frozen plasma: Thaw fresh frozen plasma - 80% activity Pool and Shanon (1965) - 65% activity.

- ii. Cryoprecipitate (Pool and Shanon 1965): Plasma is frozen below -30°C with ethanol and dry carbondioxide snow mixture. The precipitate so formed is dissolved in citrate saline 10-20 ml. warming to a temperature not exceeding 30°C .

Composition of cryoprecipitate

Factor	Name	% recovery (approx.)
VIII	AHF	56.0
I	Fibrinogen	23.0
II	Prothrombin	1.5
V	Proaccelerin	1.0
VII	Proconvertin	1.8
IX	PTC	5.5
X	Stuart's prower	1.4
XI	PTA	1.1
XII	Hageman	2.0

111. Human antihemophilic factor prepared by fractionation of Plasma

The principal agents of fractionation are as follows -

- (1) Alcohol (2) Ether (3) Cryoprecipitation
(4) Polyethylene glycol (5) Amino acids

1. Alcohol precipitation

(a) Cohn fraction (Cohn et.al., 1946): Fibrinogen and factor VIII are precipitated in the first fraction (ethanol 8% v/v, - 5°C, pH 7.0 to 7.2). Blood was collected in A.C.D. and rapid separation was done to yield high concentration of factor VIII. To avoid transmission of serum hepatitis, two donors' plasma was used. Recovery of factor VIII was 31 to 55%.

(b) Blombach procedure (Blombach 1958): The crude fraction I was purified by extraction with an ethanol glycine - citrate mixture. Impurities were dissolved leaving the factor VIII and most of the fibrinogen in the precipitate. The final solutions were sterilized through a millipore filter. The yield is about 16%. On a large scale 80-90% yield is readily achieved.



2. Ether precipitation (Kohnick and Mackay 1946)



Ether was used instead of alcohol which resulted in the preservation of much of the factor VIII in the fibrinogen fraction, sooptie precautions are taken. The ether fractionated antihæmophilic factor has now been replaced by a product based on the Elombach procedure (Ellis 1969).

3. Cryoprecipitation

(a) Crude : The plasma is frozen at -30°C and the precipitate is lyophilized, 20 to 30% activity is lost on freeze-drying. According to Pool and Shanen (1965) the yield of the cryoprecipitation procedure on a large scale is as low as 25 per cent. Forty six per cent of the activity of fresh plasma can be achieved. Weaver et al. (1967) have suggested that the factor VIII activity of "out dated" blood is sufficiently high (more than 50 per cent).

(b) Partially purified Johnson and his co-workers used as their first step cryoprecipitation accompanied by the addition of ethanol to 3 per cent v/v, they called this step cryoethanol

precipitation (Johnson et.al. 1966), Johnson Karpatskin and Herman, 1969, 1970). The factor VIII is extracted from this precipitate with very low ionic strength tris buffer (0.02 M tris pH 7.0). Other clotting factors are adsorbed on aluminium hydroxide gel, sterilised by millipore filtration.

4. Polyethylene glycol : Polson, et.al. (1964), Johnson, et.al. (1966) introduced linear polymers of high molecular weight. The starting material is a solution of the "Intermediate potency AHF". Polyethylene glycol of molecular weight 4,000 is used as fractionating agent. The pH is adjusted to 6.2 with 0.02 M citric acid. Much of the fibrinogen is precipitated by the addition of solid PEG to 4% w/v at 20°C. The supernatant contains most of the original factor VIII activity, which is then precipitated out by increasing the P.E.G. concentration to 12 per cent w/v. The precipitate is rinsed in 0.02 M tris pH 7.0 containing 8 per cent v/v ethanol at -2°C and dissolved in about 1/200 of the original plasma volume of a solution of 0.02 M tris and 0.02 M sodium citrate pH 7.0 centrifuged and sterilized by filtration and lyophilized. According to Johnson et.al. (1969), the final product is 200-400 fold purified, containing 50% of the activity of the starting plasma.



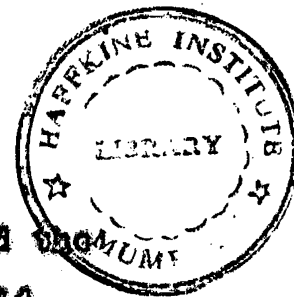


Glycine acid

Wagner, Mcnester, Smith and Brinkhouse (1964) prothrombin group of coagulation factor was removed by adsorption on aluminium hydroxide gel and fibrinogen by adsorption on fibrinogen. The precipitation of factor VIII was effected by glycine, β -alanine and α -amino-butyric acid. β -alanine was not used as residual β -alanine gave toxic effects. The glycine was used as a precipitant (Wobster *et.al.* 1965), yield was 70-100 per cent, comparison of glycine and ethanol procedures showed the yields 80 and 65% respectively. Glycine removes albumin. High potency factor VIII is prepared by glycine using polyethylene glycol as in the method of Johnson *et.al.* (1966, 1968, 1969). The yield is 50 per cent. The material was said to be free from fibrinogen.

Tannic acid

The use of tannic acid as a precipitating agent for the purification of factor VIII was introduced by Simonetti, Casillas and Pavlovsky (1961). The starting material for fractionation is P 1-0 of Blomback (1958). Tannic acid was added as a 1 per cent solution



(0.5 - 1.0 mg. - tannic acid per ml.). The final purification of factor VIII was about 200 fold and the stage yield from the Blomback fraction I-0 was said to be 80-100 per cent.

Agarose

Hershey and Sprawls (1966) found that factor VIII could be separated from fibrinogen by filtration through agarose gel. This method has not yet been developed on a regular production scale.

Most of the material in regular use is of low or intermediate potency. It must be administered by intravenous drip. Factor VIII activity may be lost more rapidly from the circulation after a dose of highly purified material compared with the rate of loss after a similar dosage of a cruder product (Johnson *et al.* 1969, Shanderson, 1969) while clinically effective material of lower potency remains in short supply.

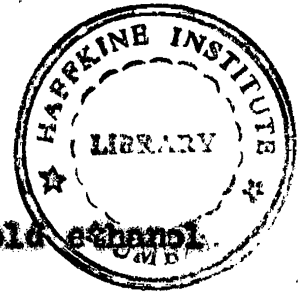
PROPOSED PLAN OF WORK

It is clear from the foregoing that fibrinogen (factor I) cannot be easily separated from other cocontaminants (which are otherwise useful) particularly the antihæmophilic globulin (AHG). In spite of the fact that these two coagulation factors act complementary to each other in clinical treatment of certain hæmorrhagic disorders, there exists the need of separating these fractions in purified form to be of sufficient effectiveness. Further, it is imperative to develop the procedures whereby accurate levels of fibrinogen and antihæmophilic globulin could be measured which may help in the laboratory evaluation of these hæmatologic disorders.

It is therefore proposed that the following plan of work be initiated :-

1. Preparation of fibrinogen and antihæmophilic globulin by wide varieties of methods involving salts and organic solvents at ordinary and near zero temperatures. Agents used for the preparation of these two factors are (a) ammonium sulphate (b) phosphate buffer





(e) ethyl ether (d) sodium chloride (c) cold ethanol.
(f) polyethylene glycol.

2. Further purification was carried out by column chromatography using Sephadex G-200.
3. Immunologic and immunochemical tests of purity of fibrinogen and antihæmophilic globulin.
4. Production of heteroimmune serum against fibrinogen and antihæmophilic globulin in animals and preparation of monospecific antibodies there of.
5. Quantitation of fibrinogen and antihæmophilic globulin using monospecific sera by radial immuno-diffusion method of Mancini and colleagues (1965).
6. Finally clinical studies are undertaken on patients in an attempt to delineate differences in fibrinogen and antihæmophilic globulin in these disorders.



CHAPTER - II

M A T E R I A L



1. Preparation of siliconized glassware

3 ml. of silicon fluid is diluted to 100 ml. with Hexane to give a 3% solution (V/V). The cleaned glassware is rinsed with this solution. The excess of solution is allowed to drain off. The glassware is then heated in a dry sterilizer for half an hour at 160-200°C. If there is no coating of the solution, the process is repeated again.

Bottles, pipettes, syringes and needles are siliconised by the above process and then they are rinsed with distilled water and then sterilized in the usual manner.

2. A.C.D. solution

22.0 gms. sodium citrate (Analar)

5.0 gms. citric acid (R. Merck)

22.4 gms. dextrose (I.P.B.P.)

were dissolved in glass distilled water and volume

is made to one litre and distributed in small bottles in 50 ml. quantity. Bottles are then sterilized at 115°C. for 15 minutes at 10 lbs pressure. The sterility and pyrogen testing is done before use.



3. Normal human plasma

Human blood was collected at Haffkine Institute and at different centres.

300 ml. of blood is taken from each donor by a clean venopuncture. Continuous gentle agitation ensured thorough mixing of the blood and the acid citrate dextrose anticoagulant throughout the bleeding. The blood bottles are cooled at 4°C in the refrigerator. The bottles are then centrifuged for 30 minutes at 1,600 G. to obtain the maximum yield of plasma in a refrigerated centrifuge. The plasma is separated and pooled in 400 ml. quantity in each bottle.

4. Ammonium sulphate

A saturated solution of pure ammonium sulphate is prepared (sp.gr. 1.246 and pH 6.6) by dissolving,

72 gms. of ammonium sulphate in 100 ml. of distilled water.



5. Organic solvents

(a) Alcohol: Commercial absolute alcohol is freshly distilled at boiling point 78.5°C which is 100% (Sp. gravity 0.7939).

(b) Ethyl ether (I.P.): Commercially prepared ether is distilled and used.

6. Phosphate buffer (2 M. pH 6.6)

2 M. phosphate buffer at pH 6.6 is made by dissolving 817 gm. of anhydrous KH_2PO_4 in 1,000 ml. of distilled water to which has been added 750 ml. of 4 M KOH (169 gm.). After warming to dissolve, the volume is made up to 3,000 ml. and the solution is filtered. One molar and M/4 solutions are made from this stock solution.

7. 0.055 M sodium citrate, pH 7.4 Morch Reagent





8. BaSO_4 (U.S.P.), Baker Analytical Reagent Powder
9. Glycine (NH_3 -free) (E. Merck)
10. Magnesium sulphate $7\text{H}_2\text{O}$ cryst, reagent (E. Merck)
11. Polyethylene glycol (M. wt. 6,000, M. wt. 4,000)
12. Phosphate citrate buffer pH 7.1

Stock solution

A : 0.1 M solution of citric acid

(19.21 gms. in 1,000 ml.)

B : 0.2 M solution of dibasic sodium phosphate

(28.39 gms. of Na_2HPO_4

or

71.7 gms. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1,000 ml.)

6.5 ml. of A + 43.6 ml. of B diluted to 100 ml.

= Phosphate citrate buffer pH 7.1

13. Sodium chloride (I.P.)

14. Sodium chloride (0.5 M)

0.5 M sodium chloride solution is used to increase the ionic strength in gradient solution. The solution is prepared by dissolving 58.45 gms. of sodium chloride in one litre of distilled water.



15. Sulphate sulphite reagent

Anhydrous sodium sulphate (BDH)

(Na_2SO_4) ... 203 gms.

Sodium sulphite (Na_2SO_3) (BDH) 70 gms. is dissolved in distilled water and diluted to one litre.

16. Biuret reagent

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (BDH) ... 2.5 gms.

Rochell salt

(Sodium potassium tartrate) ... 10 gms.

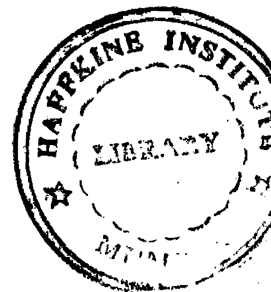
Potassium iodide ... 1 gm.

10% NaOH ... 350 ml.

is dissolved in CO_2 free distilled water and made to one litre.

17. Phosphate buffer M/5, pH 6.4

1.82 gm. KH_2PO_4 + 0.94 gm. Na_2HPO_4 is dissolved in distilled water and diluted to 100 ml.



18. Thrombin solution

Thrombin prepared is diluted with 0.85% NaCl by adding equal volume of glycerol to give 500 units per ml. Kept in the refrigerator.

19. Calcium chloride

A molar solution is prepared and standardised by titration. 0.05 N, 0.025 N or any other weaker molarity may be obtained by suitable dilution.

1% calcium chloride is prepared by dissolving 1 gm. of calcium chloride in 100 ml. of distilled water.

20. Sodium hydroxide (3%) is made by dissolving 3 gms. of sodium hydroxide (E. Merck) in 100 ml. of distilled water.

21. Sodium chloride solution

- i. 0.85 gm./100 ml.
- ii. 10 gms./100 ml.
- iii. 20 gms./100 ml.



22. Solid CO_2 snow

23. Citrate HCl buffer

Trisodium citrate $5 \text{ H}_2\text{O}$	... 8.00 gms.
Sodium chloride	... 3.40 gms.
Glucose	... 13.00 gms.
Concn. HCl. (Sp. gravity 1.19)	... 0.35 ml.

are dissolved in distilled water and diluted to 1,000 ml. pH adjusted to 6.3.

24. Tris buffer: 0.02 M tris buffer (extracting buffer)

2.42 gms./litres of trishydroxy methyl amino ethane is dissolved in distilled water and pH is adjusted to 7.0 with concn. HCl.

25. Reconstituting buffer

0.02 M tris
 0.02 M sodium citrate
 0.075 M NaCl
 40 ml. of 0.5 M sodium citrate
 + 4.4 gms. of sodium chloride
 + 960 gms. of extracting buffer and pH

adjusted to 7.0 with 0.1 N HCl.



26. 0.02 M citric acid solution

4.203 gms. of citric acid dissolved in one litre of distilled water.

27. 0.02 M citrate solution

5.882 gms. of sodium citrate dissolved in one litre of distilled water.

28. Al (OH)₃ for adsorption

1 gm. of alumina gel (BDH) is suspended in 4 ml. of distilled water.

29. Imidazole saline buffer

(0.02 M imidazole, 0.14 M sodium chloride, pH 6.5).

0.136 gm. of imidazole and 0.819 gm. of sodium chloride dissolved in 100 ml. of distilled water.



30. Sodium citrate: 3.8% is made by dissolving the appropriate weight of tri-sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) in distilled water.
31. Bovine serum: Bovine blood is collected at the slaughter house from animals bled from a stab wound in the neck. The blood is allowed to flow directly into a measured amount of anticoagulant (1 part of 1.85 per cent potassium oxalate to 10 parts of blood); these are mixed thoroughly, avoiding excessive foaming as much as possible. The blood is filtered through clean gauze and centrifuged at 2,000 r.p.m. for 30 minutes to remove the red cells, the plasma is aspirated. About 100 ml. of bovine plasma is recalcified with one-fourth of its volume of 0.1 M calcium chloride; it is allowed to clot at 37°C and maintained at that temperature for 4 hours after coagulation, then placed in the ice-box overnight. The next morning the serum is separated from the clot and stored at -20°C in 1 ml. amount until required. Absence of factor VIII activity was confirmed by inability of the serum to correct the abnormal thromboplastin generation, and the prothrombin consumption test, a haemophilic blood clotted with two-tenth volume of bovine serum. The different batches of bovine serum employed, generally contain about 25 per cent of factors VII, IX and X, 20 per cent of factor V, and less than 1 per cent of prothrombin. The same supply of serum can be used

for tests carried out over a period of several months. The day before a factor VIII assay is to be performed, 1 ml. of serum is diluted 1 in 5 with veronal buffer at pH 7.35 and left at 4°C. Overnight to permit complete activation which takes place after dilution.

32. Test plasma: Blood is obtained from the patients by two syringe technique with the use of clean, siliconised and precooled tubes and syringes. Nine volumes of blood are mixed immediately with one volume of 3.8 per cent trisodium citrate and spun in a refrigerator centrifuge at 3,000 r.p.m. for 30 minutes without any delay. The clear supernatant plasma is separated and adsorbed with one-tenth volume of aluminium hydroxide gel, the mixture is shaken and incubated at 37°C. for 5 minutes. The tubes are then spun at 3,000 r.p.m. for ten minutes, and the supernatant is promptly aspirated into cold, siliconized test tubes. Dilutions are made in citrated saline. The choice of the dilution depending upon the probable content of factor VIII. The plasma dilutions and the other reagents are kept in a bath of melting ice until the moment of being used.

33. Standard plasma: The standard plasma is collected and prepared as was outlined for the test plasma. Six dilutions, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 are

commonly used for making the standard curve. The whole procedure should be done at approximately the same time as the test sample. Standard plasma may also be stored at -20°C . With the following precautions: after being separated from the blood in a refrigerated centrifuge, it is transferred without delay into small siliconized tubes, which are immersed in a CO_2 ice-alcohol mixture (with a temperature of approximately -70°C) to freeze the specimens as quickly as possible. Similarly, the thawing of the frozen plasma should be performed rapidly, the tube being shaken with the frozen plasma, in a waterbath at 37°C , until complete thawing has taken place. With these precautions it has been possible to preserve the AHP activity of the standard plasma for at least a month.

34. Cephalins (Chloroform extract of brain - Bell and Alton 1954). Instead of human brain extract, monkey brain was utilized in this experiment.

A fresh normal monkey brain was collected from Polio Department of the Institute. It was washed with water. The blood vessels and the meninges were removed and the tissue was cut into small pieces. The small pieces of the tissue were macerated into twice or thrice its volume of AR grade acetone. The process was repeated twice or thrice by changing

acetone and the cloudy acetone was decanted off every time. A crude white powder of the tissue was obtained (fine powder was not prepared as it used to rise up while preparing suspension with normal saline). The powdered tissue was then dried and preserved in the small lots in cool, dark and dry place.

0.5 mg. of acetone dried brain was first washed with 20 ml. of AR grade acetone and the mixture was left for two hours. The brain tissue was recovered by centrifugation and the supernatant acetone was discarded. The recovered brain powder was extracted with 20 ml. of chloroform AR grade and kept for two hours. After two hours the suspension (deep yellow in colour) was filtered and the powdered brain was discarded. The filtrate was dried in vacuum and the resulting solid was diluted with 5 - 10 ml. of normal saline. The stock solution was preserved in deep freeze without any loss of activity for two to three months and diluted 1:50 in veronal buffer before use. The suitable dilution must be determined by trial and error in the test.

For thromboplastin generation test 1:100 dilution of chloroform extract of brain was found to give satisfactory results.



35. Substrate: Nine parts of human blood are mixed with one part of 3.8 per cent sodium citrate and centrifuged at 3,000 r.p.m. to 30 minutes to obtain a platelet poor plasma. This is fractionated in convenient volumes and stored frozen until used.

36. Collection of blood for coagulation studies

A readily accessible vein of good caliber on the forearm was chosen and the skin overlying the vein was cleaned. The tourniquet was tied on the upper arm in such a way that the arterial blood flow was maintained undisturbed and the venous return was cut off. The blood was allowed to flow out into the syringe. The entrance of the air bubbles through the hub of the needle and the tip of the syringe and between the piston and the barrel of the syringe were avoided by adjusting the speed of flow. Definite capacity and the type of the syringe and definite gauge of the needles were chosen for blood collection and they were treated with silicon solution before use (Syringe: 20 ml. capacity, luer mount type, all glass fitting tightly with the sharp pointed needle of no. 20 gauge and 3 cms. in length).

Two syringe method was adopted for the collection of blood so as to ensure that the ruptured



tissue or tissue fluid was not mixed with the blood. (Firstly one or two ml. of the blood was collected with one syringe which was discarded and the second syringe was attached and the required amounts of the blood was aspirated). The blood thus obtained was immediately mixed with the anticoagulant by tilting the tube gently.

37. Freund's Adjuvant

The dispersion of water microdroplets in paraffin oil was introduced by Freund, who used several types of water-in-oil stabilizers such as lanolin, lanolin derivatives, Arlacol A etc.

Freund's "complete" adjuvant contains mycobacteria (0.5mg./ml.) adjuvant without mycobacteria is called "incomplete". The choice complete "versus" incomplete emulsions rest on special considerations. A prolonged immunological stimulus is experienced when incomplete adjuvant is used, but it may be somewhat greater with complete adjuvant. Mycobacteria can add a new quality, by synthesizing. Larger amounts of precipitating antibody now termed $7S_2$ where as without mycobacteria the synthesis is largely $7S_1$. Freund's devised his "complete" emulsion, for intramuscular

injection. The modified and effective Freund's Adjuvant is prepared with following constituents.

86 parts liquid paraffin
12 parts lanolin
2 parts aluminium monosterate

The above substances are mixed together and boiled to give a uniform solution. This adjuvant is incomplete. To this mycobacteria were added (H 607) and mixed to give complete adjuvant.

38. Sophadon G-200

39. DEAE CELLULOSE

40. Anti-A, Anti-B human serum

41. A and B group cells of human blood

42. Veronal buffer

pH 8.6, ionic strength 0.0625

1) Barbituric acid 2.3 gmo.
11) Sodium barbitone 12.875 gmo.



The acid was dissolved separately in 50 ml. of glass distilled water at 60°C. The salt was also dissolved separately in the glass distilled water. Both the above solutions were mixed and the final volume was made to one litre.

43. Barbitone acetate buffer

pH 8.6, ionic strength 0.05

- 1) Sodium barbitone 14.76 gms.
- ii) Sodium acetate 9.71 gms.

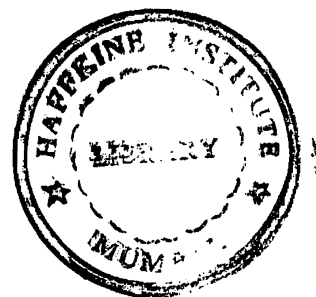
The above salts were dissolved into distilled water and to the solution 90 ml. N/10 hydrochloric acid was added and the final volume was made to 3.0 litres.

44. Bromophenol blue 0.1%

- 1) Bromophenol blue dye 0.1 gm.
- ii) Mercuric chloride 10.0 gms.
- iii) Absolute alcohol 100 ml.

45. Amido black 10B 0.5%

0.5 gm. of amido black dye 10B was dissolved in the methanol, acetic acid and distilled water in the ratio of 50:10:40 respectively.



M E T H O D S



Protein estimation - Biuret Method

Principle: The peptide group in the protein reacts with Biuret reagent to form a violet coloured complex compound the amount of which is measured colorimetrically.

Reagents

- 1) Sulphate sulphite reagent
- 2) Biuret reagent

Procedure: 0.1 ml. of plasma was taken in a test tube and made the volume 5 ml. by adding 4.9 ml. of sulphate sulphite solution and 5 ml. of biuret reagent added making the total volume to 10 ml. In case of "Blank" 5 ml. of each, sulphate sulphite solution and biuret reagent was taken. Allowed to stand for 10 minutes and read on colorimeter at 530 wave length. Standard graph was plotted with 1% human albumin solution.

Determination of fibrinogen in plasma by applying the Biuret reaction to the fibrin clot (Ware et.al. 1947)

Principle: Plasma is diluted with buffered saline containing Ca^{++} . Thrombin is added, causing rapid and quantitative deposition of fibrin. The fibrin clot is removed, washed and the protein determined by the biuret reaction.

Reagents

- 1) Phosphate buffer M/5, pH 6.4
- 2) Thrombin solution 500 units/ml.
- 3) CaCl_2 solution 1%
- 4) NaOH solution 3%



Procedure

1 ml. of citrated plasma + 3 ml. of N.saline + 1 ml. M/5 phosphate buffer and 0.5 ml. of 1% CaCl_2 were mixed in a test tube. 0.2 ml. (100 units) thrombin solution was added to it.

Allowed to stand still at R.T. for 15 minutes.



Removed the clot, washed with distilled water.

5.0 ml. of 3% NaOH added and placed in a waterbath at 37°C.

0.5 ml. of solution was taken in a tube. 4.5 ml. of sulphate sulphite was added and 5 ml. of biuret reagent was added and read on colorimeter at 530 wave length after 15 minutes.

A batch method for estimating thrombin clottable fibrinogen (Farrell and Wolf 1975).

Principle of estimation

Light scattering as described by Thorp (1967) or light absorption measurements of a diluted plasma and its corresponding serum at the same dilutions were compared after heating for 20 minutes at 59°C. The difference between the plasma and serum readings was directly proportional to the concentration of thrombin clottable fibrinogen.



Reagents

- 1) 0.85 gm./100 ml. sodium chloride
- 2) 10.0 gms./100 ml. sodium chloride
- 3) 20 gms./100 ml. sodium chloride
- 4) Human thrombin prepared at this Institute.
Diluted in such a way that one volume of this diluted thrombin clotted an equal volume of normal citrated plasma in 11-13 seconds.
- 5) Citrated plasma obtained by centrifuging the blood at 3,000 r.p.m. for five minutes at 4°C.

Procedure

In two tubes labelled "serum" and "plasma" were pipetted 1.8 ml. of 0.85% sodium chloride and 0.2 ml. of fresh citrated plasma was added.

The contents of the "serum" tube were clotted with 0.2 ml. of thrombin solution and an equivalent volume of 0.85% saline was added to the contents of the plasma tube.

The contents of both tubes were mixed and incubated at 37°C for 20 minutes and the fibrin clot which formed in the serum was removed by winding on to



a wooden applicator stick. All the liquid included in the fibrin was drained back into the tube by pressing the clot on the side of the tube.

Next 2.0 ml. of 20% sodium chloride was added to the "plasma" and "serum" tubes to bring the concentration of NaCl to approximately 10% in each tube.

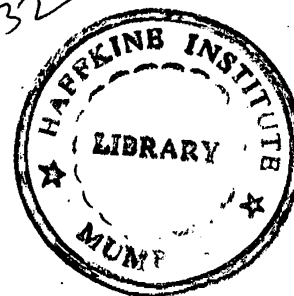
Both tubes were incubated at 59°C for 15 minutes.

Before measurement by light absorption, the tubes were cooled to room temperature. The difference between "serum" and "plasma" tubes was measured without additional dilution at 365 mμ in a Unicam Sp. 600 spectrophotometer.

Identical procedures were followed for the standard plasma in each case. The fibrinogen concentration (mg./100 ml.) of the test sample was given by O.D. difference between test "plasma" and "serum"/O.D. difference between standard "plasma" and "serum" x fibrinogen content of standard plasma in mg. per 100 ml. i.e.

mg.% of fibrinogen

$$\frac{\text{O.D. plasma (T)} - \text{O.D. serum}}{\text{O.D. plasma (S)} - \text{O.D. serum}} \times \text{mg.\% fibrinogen}$$



Thrombin clotting time

Thrombin clotting time was performed in quadruplicate by adding 0.1 ml. thrombin solution (2 NIH units/ml.) to 0.2 ml. sample prewarmed at 37°C for 3 minutes.

A simple method for the assay of factor VIII

Luio Jono. Bergma (1960)

The method for the assay of factor VIII activity, based on the thromboplastin generation test of Biggs and Douglas but does not require hemophilic blood nor special materials and is easily adapted to any laboratory. It consists of a mixture of calcium, cephalin and bovine serum (to provide all the clotting factors required for the generation of thromboplastin except factor VIII) added successively with diluted adsorbed unknown plasma and standard plasma. The correcting effect on the thromboplastin generation (measured in the usual way on a recalcified platelet poor substrate) is compared; the amount of factor VIII can be expressed in per cent of the standard.

The results are expressed according to Biggs, Evelyn and Richards 1955 given in Biggs Macfarlane 1962 Edition.

Materials :-

Bovine serum

Test plasma

Standard plasma

Cephalin

Substrate

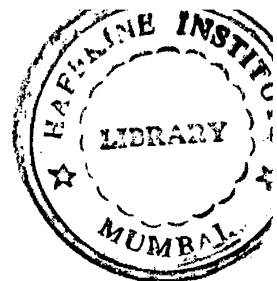
Method :-



Plasma dilution for the factor VIII assay method

Plasma sample	0.5 ml. of absorbed plasma	1 ml. of 1 in 8 plasma	2 ml. of 1 in 64 plasma	1 ml. of 1 in 64 plasma
Citrate saline in ml.	3.5	7	2	3
Final dilution	1 in 8	1 in 64	1 in 128	1 in 256

The reagents are kept in melting ice, are pipetted into a warm 10 x 75 mm. tube; maintained in a waterbath at 37°C, in the following order -



1) 0.1 ml. of cephalin 2) 0.1 ml. of diluted bovine serum 3) 0.1 ml. of diluted test or standard plasma 4) 0.1 ml. of 0.025 N CaCl_2 . At the moment of adding the calcium chloride a stopwatch is started. Exactly 8 minutes after the incubation of the reagents, 0.1 ml. of reacting mixture is transferred to a 10 x 75 mm. tube containing 0.1 ml. substrate plasma, previously warmed in the waterbath and to which 0.1 ml. of CaCl_2 was added, 10 seconds before transferring the generating mixture. The clotting time is determined with a second stopwatch. It will be observed that 8 minutes elapse before the generating mixture is added to the substrate. By starting successive generating tubes at intervals of one minute using the same stopwatch, it is possible to test the six dilutions of the standard plasma and the blank in 15 minutes. The duplication of the standard curve will take another 15 minutes. Then, in the next fifteen minutes, the three dilutions of the plasma under study are tested in duplicate. Any subsequent determination will take another 15 minutes.

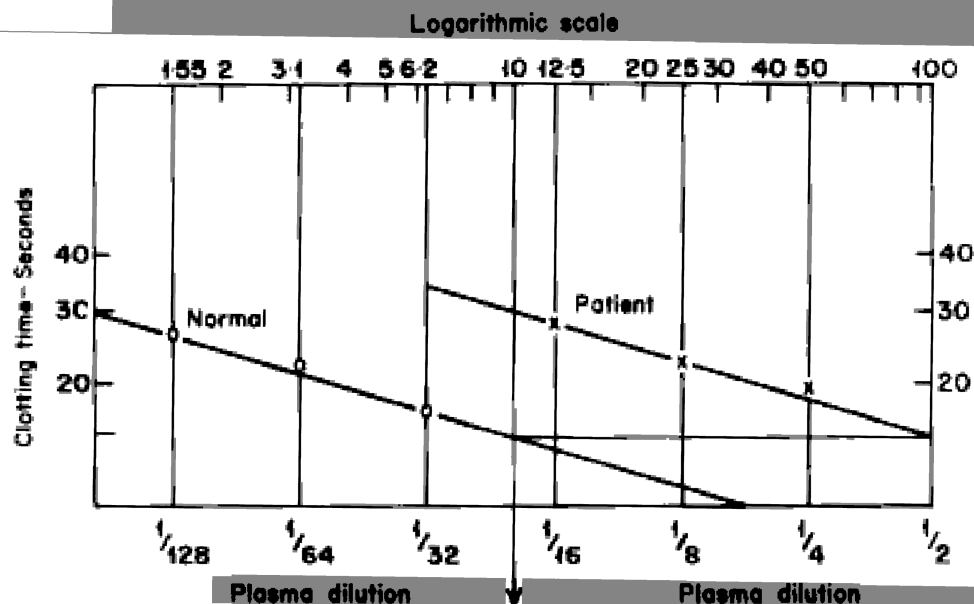
The average values of the two clotting times recorded with each dilution of the standard plasma and test plasma are plotted on double logarithmic paper and the points are found to fall on a straight line.

Expression of results

The clotting times, for the normal standard and for the abnormal samples tested are plotted as parallel lines on double logarithmic paper (Fig. 3). In Fig. 3 is a diagrammatic representation of results in which lines corresponding to 50, 25, 12.5 and 6.2 per cent of factor VIII have been drawn parallel to the normal standard line. It is assumed that the concentration which give the same amount of factor VIII. Thus for the normal standard a clotting time of 20 seconds occurs in the sample diluted 1 in 64. For the 50 per cent sample the clotting time of 20 seconds occurs in the sample diluted 1 in 32 and for 25 per cent sample at 1 in 16 and so on.

In this example the calculation of factor VIII concentration is obvious. In most experiments the concentration tested will not be so conveniently arranged and it will be necessary to make use of the calibrations on the graph paper. The horizontal scale of the paper is used entering the plasma concentrations. It will be observed that, on double cycle paper (which is required), this scale extends 1 to 100, if the least dilution used (1 in 2 in Fig. 3) is entered at 100

Fig. 3



Scale reading 10

$$\text{factor VIII} \frac{10}{100} \times 100 = 10\%$$

An example of the calculation of factor VIII level for a sample containing 10 per cent of factor VIII.

Based on Human Blood Coagulation

Hæmostasís and Thrombosis

Edited by Rosemary Dicks

page, 619.



then the next dilution, the 1 in 4 (which is half the concentration of the 1 in 2 dilution) must be entered at the 50 calibration, the 1 in 8 will be placed at 25 and so on. The logarithmic scale is shown as the upper scale of Fig. 3 and the actual dilution used are on the lower scale. To calculate relative amount of factor VIII activity in the unknown, a horizontal line is drawn to connect the standard line with that for the patient. The graph paper scale reading of each end of this line are recorded. The relative amount of factor VIII will be given by formula.

$$\frac{\text{Scale reading of intersection with normal line}}{\text{Scale reading of intersection with test sample line}} \times 100 \text{ per cent}$$

For example

$$\frac{10}{100} \times 100 = 10 \text{ per cent}$$

Single Radial Immunodiffusion for Quantitation of Plasma Fibrinogen and AHF

Immunological reactions are the only specific methods for demonstration of the majority of these



proteins. Quantitative serum electrophoresis on paper, cellulose acetate or other media is usually not sufficient because of the heterogeneity of the various electrophoretic fractions. Immuno-electrophoresis permits only semiquantitation of the individual proteins.

Recently, relatively simple immunochemical methods have been developed which allow the precise quantitation of individual proteins in serum and other biological fluids. The method of single radial immunodiffusion as modified from Mancini, Carbonara and Heremans (1965), is reliable and practicable.

Principle: The principle of this method depends upon the diffusion of the protein from a small cylindrical well into a thin antibody - containing agar gel layer. As the antigen diffuses into the agar gel a precipitin ring is formed around the antigen well. The diameter of this ring is related to the concentration of the applied antigen.

Materials

1. Agarose gel 1%
2. Human fibrinogen 95% clottable was reconstituted to give 1% solution.

3. Human AHF free from fibrinogen was prepared and 1% solution was made.

4. Antisera

a) Rabbit antihuman fibrinogen antiserum was used by absorbing with human serum. Immuno-electrophoresis showed that the antiserum reacted as a single line against human plasma and did not react with human serum.

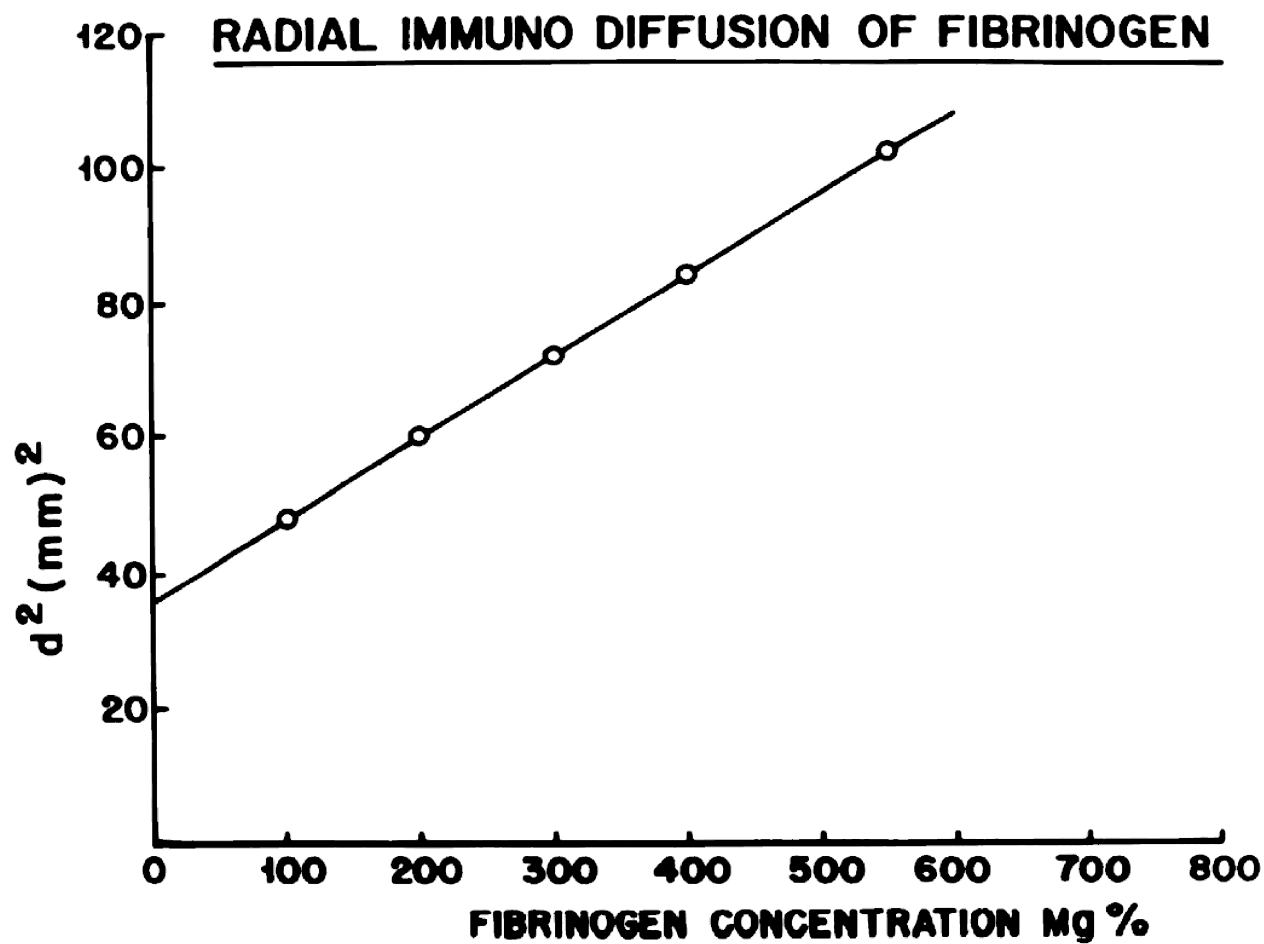
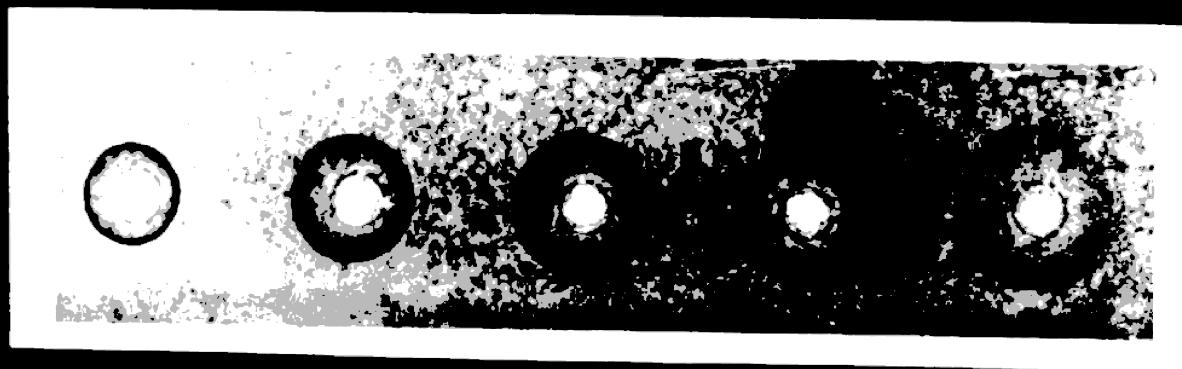
b) Anti-AHF prepared by Hoyer's method was used which gives a single precipitin line against plasma and AHF and not against fibrinogen.

Methods

The optimal antibody concentration in the agar gel varied between antibody lots and was adjusted by trial and error. For fibrinogen 0.8 ml. of antiserum was incorporated in 10 ml. agar solution and for AHF 0.6 ml. of antiserum was mixed in 10 ml. agar solution.

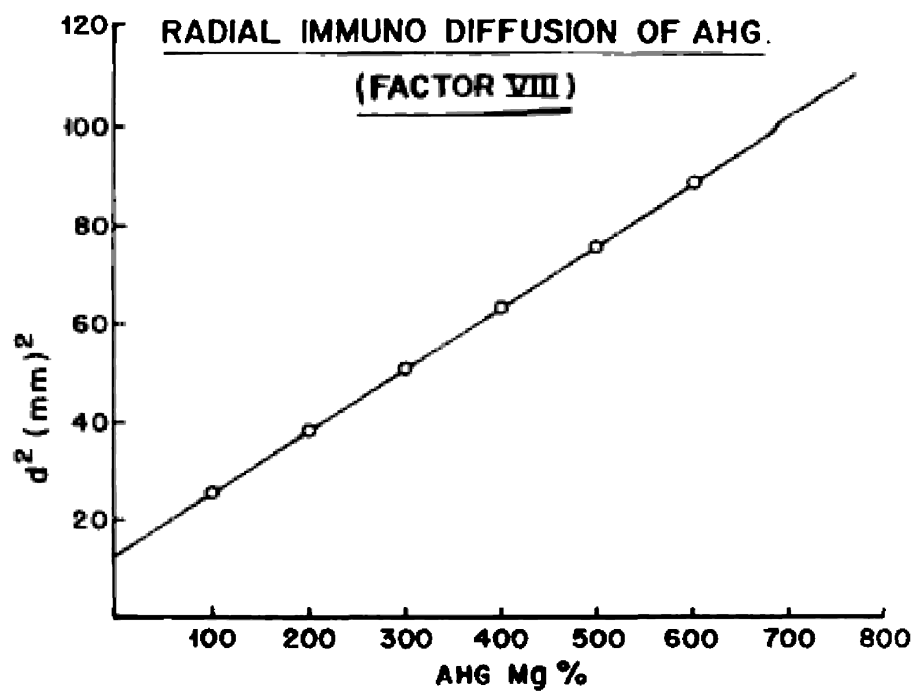
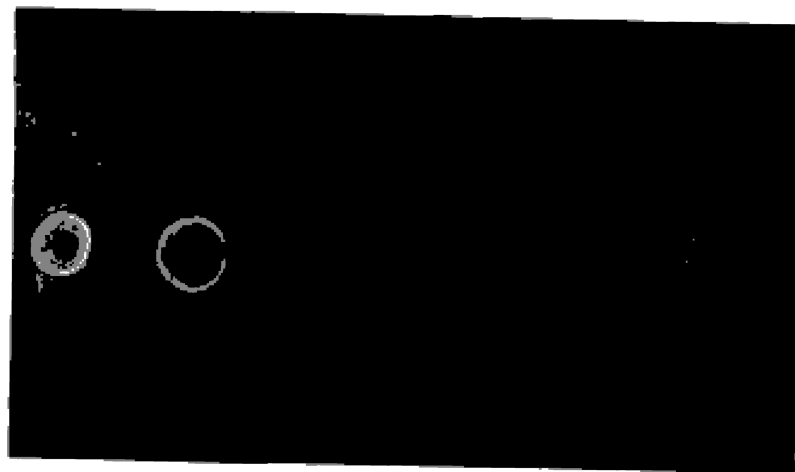
The wells were punched. A micropipette was used carefully to fill each well in 5 μ ml. of the sample to be tested or the standard. The slides are covered

FIG. 4



Typical calibration graph obtained after 24 hours of diffusion of human fibrinogen solution.

FIG. 5



Typical calibration graph obtained after 24 hours of diffusion of human AHG solution.

and allowed to stand at room temperature for 48 hours. The diameters of the circular precipitates were measured with a special scale supplied by a Hoechst Pharmaceuticals alongwith partigon plates.

The unprecipitated protein was eluted for 12 hours with saline solution. The gel was then dried in an oven at 85°C for half an hour; stained with amido black, washed with 2% acetic acid and dried at room temperature.

A standard curve was constructed by plotting the diameter² of the precipitin rings of each dilutions of the standard solutions (abscissa - antigen concentration in mg./100 ml.; ordinate:- diameters in millimeters) on semi logarithmic graph paper. The obtained points usually connect to a straight line. The values of the test samples are read from the graph. Protein concentration on test samples are expressed in mg./100 ml. (Fig. 4 and 5)

Gel diffusion technique

Gel diffusion is a simple and highly useful technique for the antigenic analysis of complex material. The principle underlying this technique is

that when complex antigen and its specific antibody are allowed to diffuse towards each other in a gellified medium, precipitin lines are formed which are insoluble in saline. Each precipitin line represents and indicates the presence of one antigen.

We have followed the method described by Grassett et.al. (1958), which is a modification of Ouchterlony's horizontal gel diffusion technique. In this method, clear microscopic slides 2.5 cm. X 7.5 cm. are used. 3 ml. of 1% agar prepared in normal saline is poured over the slide and is allowed to solidify the gel. After solidification of the gel a seven well pattern was cut in the gel. The wells were 4 mm. apart and 2 mm. in diameter. The wells were punched by suction using a pipette with a rubber test to create vacuum. The wells were then filled with appropriate samples of antigen and antibody. The slides were kept in the petridish along with moist cotton to prevent the gel from drying. The result was scored after 24-48 hours.

Washing and staining

After scoring the result the slides were washed with normal saline for 5-6 days changing the saline

every 24 hours. The final washing was done with distilled water. The slides were dried at 80°C. keeping filter paper over them. The slides were then stained with 0.5% amido black dye 10 B. The excess stain was washed off with 1% glacial acetic acid solution and subsequently with distilled water. The washings were continued till agar layer surrounding the precipitin lines was free from the stain. Final washing was done with glycine solution. Such slides can be preserved indefinitely.

Electrophoresis

Electrophoresis is the movement of charged particles in solution under the influence of an electric field. The movement of the particles is based on the differences in the migration velocities of different proteins in an electric field due to the differences in the net charge on the protein molecules.

There are various methods of electrophoresis, the most widely used method is moving boundaries. It was first applied for the study of proteins by Tiselius (1937) and is therefore known as Tiselius method. The various

supporting media have been used such as glass beads, glass powder (Coolidge 1959), Starch (Tiselius & Flodin 1953) agar gel (Grabbar & Williams 1953, 1955, paper (Kunkel 1954) and starch gel (Smithon 1955). Most commonly used media are filter paper, agar gel and starch gel.

Limitations of the paper electrophoresis -

- a) It is a micromethod
- b) Most proteins are absorbed to the slight extent on the paper.
- c) Variations in the filter paper, buffer concentration, temperature, electrical system, staining methods etc. caused variations.

We have used the electrophoresis chamber supplied by Bijou Adco Company and Shandon Power Unit.

Procedure

Veronal buffer, pH 8.6; ionic strength 0.0525 was filled in the buffer compartments. 4X34 cm. Whatman No.3 filter paper strips were used. The strips were

moistened with buffer and blotted to remove excess of moisture. The strips were then placed in the chamber in such a way that the two ends were dipped in the buffer compartments. 0.05 ml. of the sample was applied in the middle of strips. The chamber was covered with cover. A current of 0.5 mA. per strip was applied and electrophoresis was run for 18 hours.

Drying and staining

The strips were dried in an oven at 100°C. for 15 minutes and then stained with bromophenol blue dye. The strips were kept for 10 minutes in dye and excess dye was removed by washing the strips with 1% acetic acid solution. Final washing was done with distilled water and strips were dried at 80°C. The dried strips were preserved for further testing.

Quantitative estimation of electrophoretically separated protein fractions

The separated protein fractions were cut out as indicated by zones and put into separate test tubes. To each tube was added 5 ml. of 4% sodium carbonate

solution taking care to see that the strips were completely dipped in the solution. After one hour, optical densities of the coloured solutions were read on a Unicam Sp. 200 colorimeter using No. 624 filter having maximum absorption at 520 m μ . The concentration of the protein was calculated with the help of a standard graph of dye concentration against optical density.

Agar gel electrophoresis

Various methods have been described among which the technique of Gordon et.al. (1950) and Giri (1956) is based on the use of an agar gel. The principle is same as in paper electrophoresis except the supporting medium is agar. This has an advantage over the paper electrophoresis that the structure of an agar gel is more uniform than that of filter paper and moreover the fractions can be eluted for testing its various properties (Grabar 1959).

The chamber was filled with barbitone acetate buffer pH 8.6, ionic strength 0.05. 1% agar gel was prepared by dissolving 1 gm. difco-agar in 50 ml. of buffer and 50 ml. of water. This was heated in boiling

waterbath, for 15-20 minutes. 2.5 ml. of gel was poured on each slide. After solidification the slides were placed on the electrode vessels. The connection between the gel and buffer was made by placing Whatman No.3 strips.

Application of the sample

Whatman No.3 filter paper strips of size 2 cm.x 0.1 cm. were soaked in the sample and placed on the gel layer. A constant current of 2.5 mA per slide was applied. The whole set was covered with glass plate to minimize evaporation and to keep up the humidity. The electrophoresis was carried out for 3 hours. Then the slides were dried in oven at 70°C. for 20-30 minutes.

Staining

The protein components were located by staining with 0.5% amido black, 10 B dyo.

Immunoelectrophoresis

This is a sensitive method for characterization of various proteins after their electrophoretic

separation by their specific precipitation with antibodies. The method was first introduced by Greber and Williams (1953, 1955). The mixture to be studied is subjected to electrophoresis in gel. When the dispersion of the constituents by the electric field is judged to be sufficient, electrophoresis is stopped and immune serum is allowed to diffuse perpendicularly to the axis of migration. The antibodies present in this serum and the antigenic constituents of the analysed liquid diffuse into the gel and when they meet in proper relative proportions insoluble antigen antibody complexes appear and areas of specific precipitation become visible.

Procedure

The agar gel and buffer is same as in agar gel electrophoresis. In the gel two wells are made. Test samples are filled into the wells and the slides then placed in the electrophoresis apparatus forming a bridge with the paper strips dipping in the buffer. The current used was 2.5 mA per slide for 3 hours. The voltage was adjusted to get the required current and was usually 100 volts.

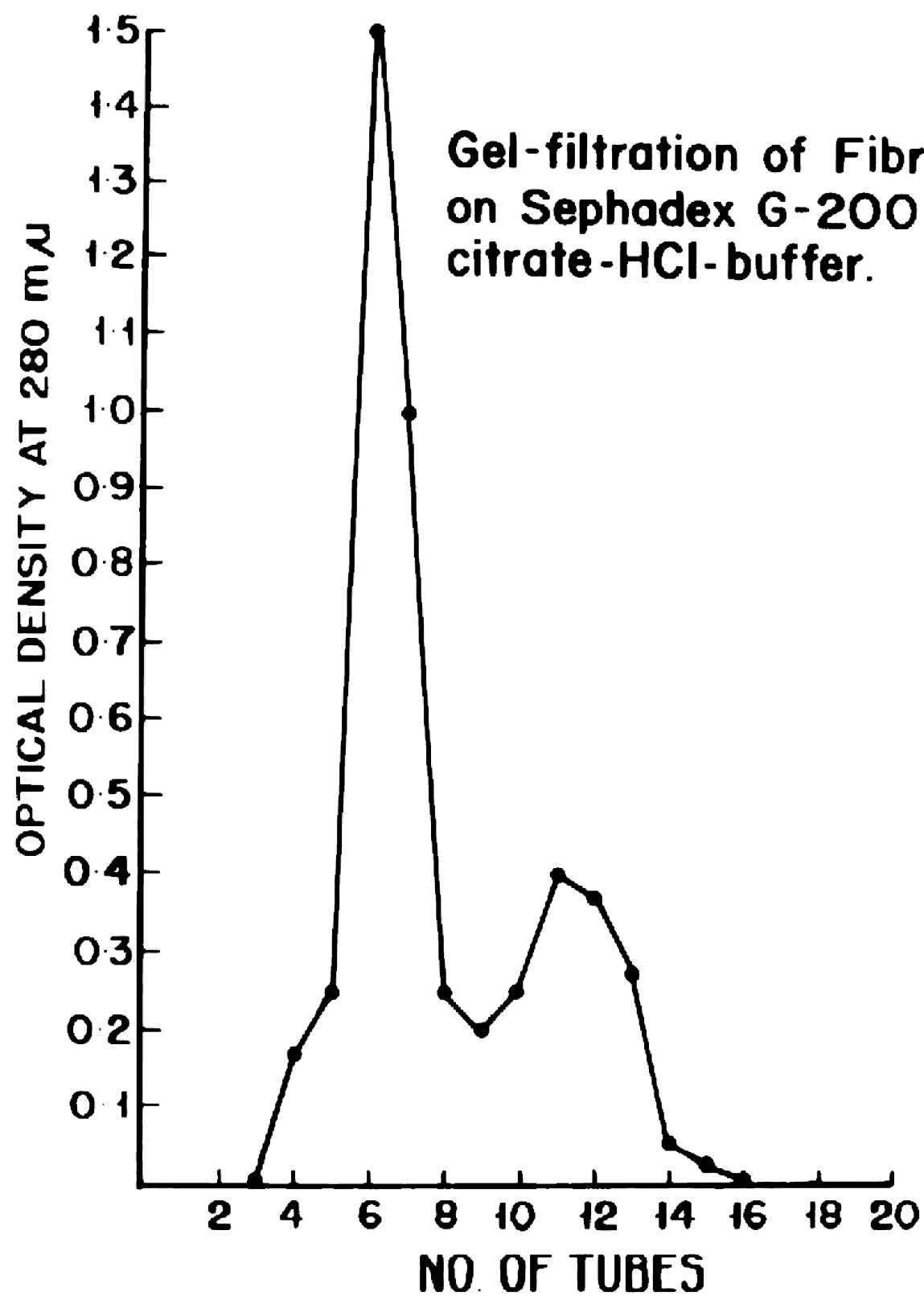
After 3 hours the current is stopped and a trench 2 mm wide is cut in the agar gel parallel to the direction of migration of components equidistant from the wells. The trench is filled with antisera. Sodium azide was sprinkled on the gel to prevent bacterial contamination. The slides were kept in the petridish along with the moist cotton and kept at room temperature. The specific precipitation lines appear within 24-48 hours.

Washing and staining

The slides are washed with normal saline, dried and stained with amido black dye.

Gel filtration using Sephadex G-200

2 gm. of Sephadex G-200 powder was allowed to soak in 500 ml. of normal saline for 48 hours in cold (0° - 4°C). The saline was changed for 2/3 times and the light particles of Sephadex were removed by decanting very carefully. The sephadex was then poured as a slurry into a siliconised glass column measuring 50" x 1" in diameter and allowed to settle under gravity till the column attained a constant height. A volume of 2 ml. buffer was kept above the sephadex column to prevent from drying. The column was kept in a cold room (0-4°C) and washed with citrate HCl buffer. The height of the sephadex column was 30 cm.



The fibrinogen was dialysed in saline and 10 ml. was applied onto the column. The fractions collected at rate of 12-15 ml./hr. and 5 ml. elutes were collected into test tubes. The optical density was measured of each tube on spectrophotometer at 280 m μ and after plotting up a graph the tubes were pooled. (Fig.6)

Two peaks were obtained. The first peak starts from 4th tube extending to the 8th tube. Second peak starting from 10th tube and extending to the 15th tube. The overlapping fractions i.e. 8 tube, 9 was discarded as it was observed to contain mixture of both the fractions i.e. AHF and fibrinogen. Tubes 4 to 8 were pooled and called as fraction I and 10 to 15 fraction II. Both the fractions were concentrated by lyophilization for further studies.

Titration of antibodies in AHF

AHF was prepared from individual groups of plasma and also from pooled plasma by different methods and antibody titre was determined as given below -

To these dilutions, one volume of 2% red cells suspension in saline is added and tubes are shaken (A for Anti-A and B for Anti-B). The tubes are incubated at room temperature for 1 hour and results are read under the microscope. The reciprocal of the highest dilution which will show detectable agglutination is termed as the titre of antibody.

Salino and AHS are mixed as follows

[illegible]

CHAPTER - III

EXPERIMENTALPreparation of fibrinogen

Fibrinogen is precipitated from plasma with alcohol or ether. The methods used for precipitation vary with the consideration of blood clotting and the type of experiment for which the fibrinogen is used.

Hammerston (1879) used the fraction precipitated with saturated sodium chloride solution and removed other plasma proteins by repeated precipitation. Hellanby (1909, 1930) precipitated fibrinogen by dilution and acidification to pH 5.3 and studied the action of thrombin on fibrinogen.

The purity of the products prepared is judged by determining the amount of protein which remain after the fibrinogen is removed by coagulation with thrombin. Fibrinogen prepared by phosphate buffer method was found to be purer than that prepared by precipitation with ammonium sulphate. The fibrinogen prepared by ether is also relatively pure (Kekwick 1946).

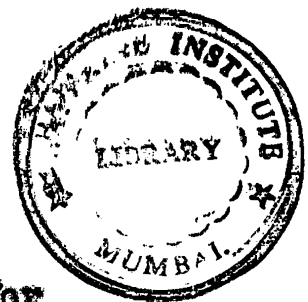
Some methods for the preparation of fibrinogen are discussed here.

METHODS USED FOR THE PREPARATION OF FIBRINOGEN

- 1) Ammonium sulphate method (Mourath et.al. 1943)
- 2) Phosphate buffer method (Jaques 1943)
- 3) Precipitation with ethyl ether (Kekwick & Machay 1954)
- 4) Precipitation with sodium chloride (Polson 1972)
- 5) Precipitation with ethyl alcohol (Cohn et.al. 1946)
- 6) Preparation of fibrinogen from cryoprecipitate supernatant (Nitschmann et.al. 1957)
(Grobelaar 1973)

1. Preparation of fibrinogen by saturated ammonium sulphate

300 ml. of human blood is collected in the bottles containing 50 ml. of Acid Citrate Dextrose. Blood is kept in the refrigerator at 4°C. The plasma is then clarified by centrifuging at 2,000 r.p.m. for 20 minutes. The 400 ml. of pooled plasma was separated. The pH is adjusted to 7.6 by the addition of NaOH. The solution is chilled in an ice box to 0°C. A saturated ammonium sulphate (528 gms. per 1000 ml.) is added to plasma drop by drop from a separating funnel while chilled plasma is being constantly stirred mechanically. For each 100 ml. plasma 33 ml. of ammonium sulphate is added, resulting in 25% saturation. The solution is placed in the ice-box and allowed to stand for at least 12 hours. The precipitated fibrinogen is then separated from



solution by centrifugation at 2,000 r.p.m. for 15 minutes. The supernatant is poured off and the precipitate is washed with a chilled 25% solution of ammonium sulphate. The precipitate is again separated by centrifugation and then suspended in a maximum amount of distilled water. This suspension is placed in a cellophane bag and dialysed in the cold against four successive portions of physiological saline, changed thrice in order to remove ammonium sulphate. The suspended fibrinogen goes into solution during dialysis. The pH is checked and is usually found to be 7.0. Any insoluble material is removed by centrifugation and the clear supernatant is sterilised by passing it through a seitz filter. The solution thus collected is stored in sterile bottle for storage either in the frozen state or in a freeze dried state. The fibrinogen obtained is tested for protein estimation. The clotting time is also studied. The purity of the product is studied by electrophoresis.

2. Preparation of fibrinogen by phosphate buffer method

Contaminated plasma was used for this method. To 1,000 ml. of discarded, contaminated plasma are added 50 ml. of $Al(OH)_3$ suspension. The mixture

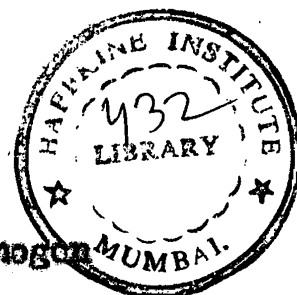
is stirred at room temperature for 15 minutes. The mixture is centrifuged and the precipitate is discarded. The plasma is cooled to 12° - 14°C , and to this is added an equal volume of 2 M phosphate buffer which has been previously cooled to $2-4^{\circ}\text{C}$, in the refrigerator. The mixture is allowed to stand for 15 minutes and to allow the fraction to precipitate. The mixture is centrifuged for 10 minutes at 2,000 r.p.m. and the supernatant is discarded. The precipitate is washed with 1,000 ml. of 1 M phosphate buffer and the precipitate recovered by centrifugation. The precipitate is then dissolved in 500 ml. of M/4 phosphate buffer. The volume is measured and the fibrinogen is reprecipitated by adding an equal volume of 2 M phosphate buffer. The precipitate is sedimented by centrifuging, washed with 500 ml. of 1 M phosphate buffer and redissolved in 500 ml. of M/4 phosphate buffer. The precipitation and washing are repeated once more and final precipitate is dissolved in 150 ml. of citrate saline. (9 parts of 0.85 per cent saline and one part of 3.8% trisodium citrate). The solution is dialysed in the cold overnight against 5000 ml. of citrate saline, the dialysing fluid must be stirred during this time. The product is centrifuged to remove any precipitate that may have formed and freeze dried. The drying is completed in a vacuum desiccator over phosphorus pentoxide.

The dried product is weighed and dissolved in distilled water and reconstituted to original volume. This solution is ready for use.

3. Precipitation with ethyl ether

Fresh plasma is used in this method. The addition of ether was carried out on the same day. The fractionation method is described below.

The serial fractionation commences with precipitation of fibrinogen. The addition of 11% volume of ether to plasma was carried out, maintaining the temperature at 0°C. This brings about the precipitation of 88% of fibrinogen which collects as a sludge at the bottom of the precipitation vessel. The sludge is centrifuged to obtain crude fibrinogen as a firmly packed precipitate. The precipitate may be suspended at 0°C, in 20% of the plasma volume citrate saline to which has been added 8% volume of ether and again centrifuged down to increase substantially the purity of the product. The crude fibrinogen (Fraction I) analysed electrophoretically or by clotting test, contains 50% fibrinogen, the main contaminant being albumin. This material is used



for making fibrin foam. The purified fibrinogen can be used for skin and nerve grafting and contains the ANG also.

4. Precipitation with sodium chloride

Fibrinogen is precipitated from plasma over the range 15-22 gm. NaCl/100 ml. plasma. By initially treating plasma with 25 gms. NaCl/100 ml. plasma a precipitate produced which contains all of the fibrinogen. This may be used as starting material for further purification.

Fibrinogen may be purified from plasma by precipitating with 5% polyethylene glycol (PEG) in phosphate citrate buffer at pH 7.1. The precipitate is removed by centrifugation, redissolved, in the phosphate citrate buffer and reprecipitated with 20% NaCl. When using NaCl as the precipitating agent traces of contaminating PEG remain in the supernatant fraction and the precipitate behaves in a peculiar manner in that it partly sediments out upon centrifugation and partly forms a "Cake" on the surface, which is caused by air trapped in the precipitate.



5. Precipitation with ethyl alcohol

300 ml. each of blood was collected in 50 ml. of A.C.D. solution. The blood bottles are centrifuged in a refrigerated centrifuge and 400 ml. of pooled plasma is separated out in sterile bottles.

Cool the plasma bottles to 0°C.

By means of a syringe and needle, and by puncturing the rubber disc, enough ethanol is added to each bottle to give a final concentration of 8% (9 ml. per 100 ml. plasma). Ensure the mixture by rotating thoroughly. The alcohol should be precooled to -15°C to -20°C. No pH adjustment is necessary.

Let the bottles stand overnight at -2°C.

Centrifuge the bottles in a refrigerated centrifuge at 2,000 r.p.m. for 5 minutes.



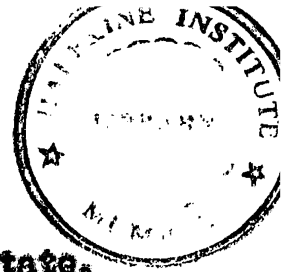
Syphon off the supernatant from the sedimented fraction I using the same technique as before. The supernatant may be used for further fractionation.

The precipitate in each bottle is dissolved in enough sterile citrate buffer to give a total volume of 150 ml. The buffer is transferred by aspiration into the closed bottle through the rubber disc. Solution is achieved by swinging or rolling the closed bottle and is accelerated by cautious warming to 30°C in a waterbath. The buffer contains per 1,000 ml. 8 gms. sodium citrate (5.5 hydrate), enough HCl to give a pH 6.5 (about 0.35 ml. of concn. HCl., d. 1.19), 3.40 gms. NaCl and 13.0 gms. glucose.

When dissolution is complete, the opalescent fluid is drawn into 250 ml. blood bottles and lyophilised immediately by freeze drying.

6. Preparation of fibrinogen from cryoprecipitate supernatant

A better system is to produce fibrinogen from the supernatant plasma after the cryoprecipitate has been extracted. Only about



25% of the fibrinogen is found in the cryoprecipitate, the balance remaining in the supernatant.

Two bottles of freshly collected plasma are pooled into one.

After the cryoprecipitate has been produced, the supernatant is treated with 10% alcohol at -4°C . overnight. This precipitates out the fraction I, i.e. the fibrinogen. The precipitates from 2 of these 400 ml. volume, i.e. from 4 units of plasma, are pooled, dissolved in a buffer and freeze dried. Such a unit will be equivalent to approximately 1 gm. of fibrinogen. There is further advantage with fibrinogen produced from pooled plasma in a large scale fractionation programme that the role of transmitting viral hepatitis is considerably reduced.

Purification of fibrinogen

There are several methods of purifying fibrinogen. The methods depending on salting out with neutral salts, do not provide very pure fibrinogen. This shows that fibrinogen is least soluble protein of the plasma.

and this is true in the case of solvent containing systems which have been devised for fractionating plasma proteins. Cohn and his associates (1946) have demonstrated scheme in which 85% of the fibrinogen is separated out of which 60% was fibrinogen of the total protein.

Fraction I was dissolved in citrate buffer. Further it was reprecipitated at pH 5.0-5.1. Ionic strength 0.3, temperature 0°C. and ethanol concentration 2% fraction IA is obtained in which 85% was fibrinogen. From this cold insoluble globulin was removed at pH 6.2, Ionic Strength 0.3, ethanol 0.5% and temperature 0°C. and the remaining fibrinogen was reprecipitated from the supernatant solution by raising the ethanol concentration to 8%. Such preparation contains less than 5% non-clottable protein and the yield of fibrinogen is about 40%.

Blomback and Blomback (1956) used glycine for extracting human plasma fraction I precipitated twice at 0°C. with citrate HCl buffer pH 6.0 Ionic Strength 0.3 containing 1M glycine and 6.5% ethanol. The undissolved material (fraction 1.0) which was 86-89% clottable, contained 95% of the initial fibrinogen.

Fraction I-0 was then dissolved in citrate HCl buffer pH 6.35, Ionic Strength 0.3 and cold insoluble globulin fraction I-1 was precipitated at 0°C., pH 6.35, Ionic Strength 0.3, glycine 0.12 M, ethanol 2% the fibrinogen fraction I-2 at 0°C., pH 6.5, Ionic Strength 0.09, glycine 0.5 M, ethanol 0.75% and from the supernatant solution fibrinogen I-4 was precipitated at 4°C., pH 6.5, Ionic Strength 0.08, glycine 0.45 M, ethanol 65%. This fibrinogen I-4 is 98-100% clottable and the overall yield about 40-45% relative to Fraction I.0.

Fraction I - Rework (R. Berg et.al. 1972)

Fraction I was suspended in 10 volumes of the following buffer; sodium chloride 0.234 M; sodium citrate 0.006 M; sodium acetate 0.060 M; glycine 1.0 M; ethanol 7% pH 6.0.

The suspension was prepared at 0 to -2°C and after stirring for 1 hour the washed precipitate was centrifuged in a refrigerated centrifuge at -2°C.

The precipitate, fraction Ia, was washed once more in the same buffer. The residue after the second

washing, fraction Ib, was dissolved at room temperature in 9 volumes of the following buffer: sodium chloride 0.21 M, sodium citrate 0.005 M; sodium acetate 0.005 M; pH 6.2.

To this solution prewashed BaSO_4 was added corresponding to a quantity of 2.5 gms./l. After stirring for 1 hour at room temperature the solution was cooled to 0°C . to precipitate the cold insoluble proteins. After 1 hour at 0°C . the precipitate was removed by centrifugation at 0°C . The supernatant containing about 1-2% fibrinogen was precipitated with half a volume of precooled acetone. The formed precipitate was allowed to sediment at -5°C . overnight and separated in a refrigerated centrifuge at -5°C . The sediment, consisting mainly of fibrinogen, was stored as a wet paste for 4-5 days at -5°C . to allow for a further possible inactivation of adventitious agents.

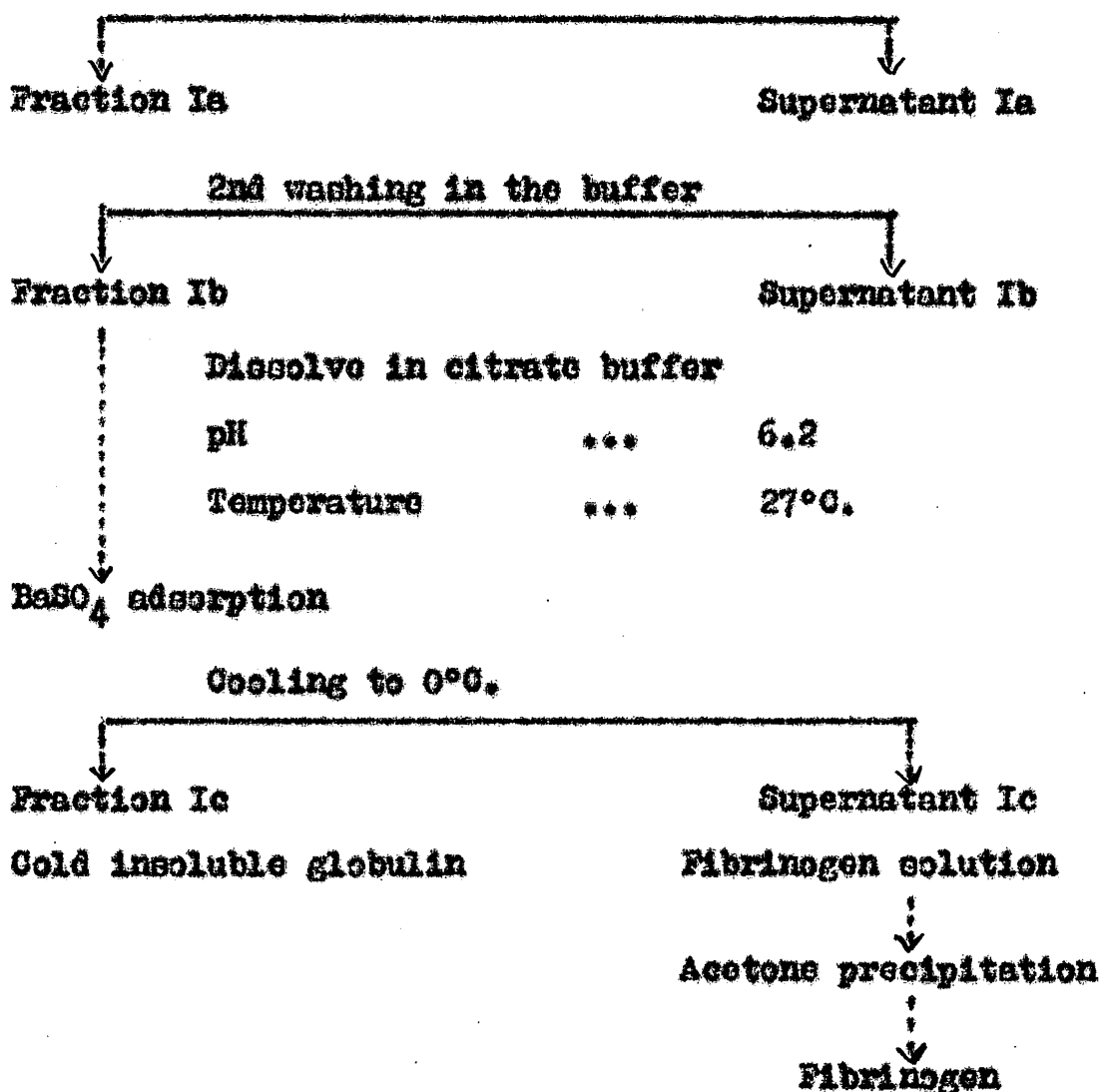
The fibrinogen was dissolved at room temperature in the above mentioned citrate buffer. The solution was filtered through seitz EKS sterilizing pads, dispensed in glass bottles, shall frozen and lyophilised.

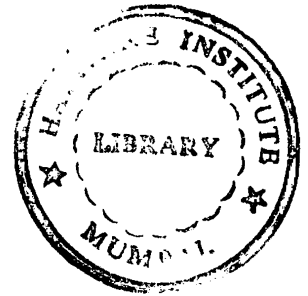


**Fraction I**

Suspended in citrate buffer

Glycine	...	1.0 M
Ethanol	...	7.0%
pH	...	6.0
Temperature	...	-2°C.

Diagrammatic representation of the Kabi fibrinogen methodFraction I Rework

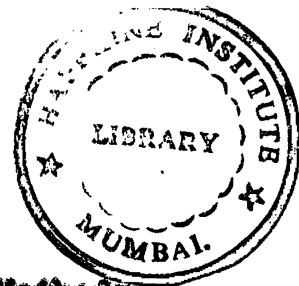


Preparation of antifibrinogen

2 rabbits (1.8 kg.) were injected weekly subcutaneously with 5 mg., 7 mg., and 10 mg. of purified fibrinogen and complete Freund's adjuvant. Ten days after the last injection the test bleeding was done. Last dose was repeated to obtain a suitable titre. The animals were bled after a week and serum was collected. The above sera were absorbed with normal human serum to obtain monospecific antisera.

Preparation of antihæmophilic globulin (AHF Factor VIII)

Patients suffering from hæmophilia A were initially treated with plasma or whole blood, often requiring excessive volumes to effect and maintain hæmostasis. Then it was discovered that Cohn fraction I obtained by ethanol fractionation (Cohn et.al. 1946) from fresh plasma possesses strong antihæmophilic activity (Minot et.al. 1945). Fraction I became widely used as clinical factor VIII concentrates (Baumgarten et.al. 1963, Blomback and Blomback 1956, Surgenor et.al. 1960). These preparations, however have low purity and potency factor VIII. Developments were towards the high purity and potency factor VIII such as cryoprecipitation



contained 50% of the original plasma activity. Methods were made available for the preparation of high potency and high concentrate which can be administered in small amounts. The stable fractions were prepared which can be lyophilised by using low potency material.

Some of the methods of preparation of factor VIII are discussed here -

- 1) Ether precipitation (Kokwick and Mackay 1954)
- 2) Slow freezing method (Pool and Shannon 1965)
- 3) Rapid freezing method (Brown et.al. 1967)
- 4) Preparation of high purity AHF with polyethylene glycol (Johnson et.al. 1969)
- 5) Alcohol precipitation method (Hoyer 1972)
- 6) Sodium chloride method followed by polyethylene glycol (Polson and Reiss Bravo 1972)

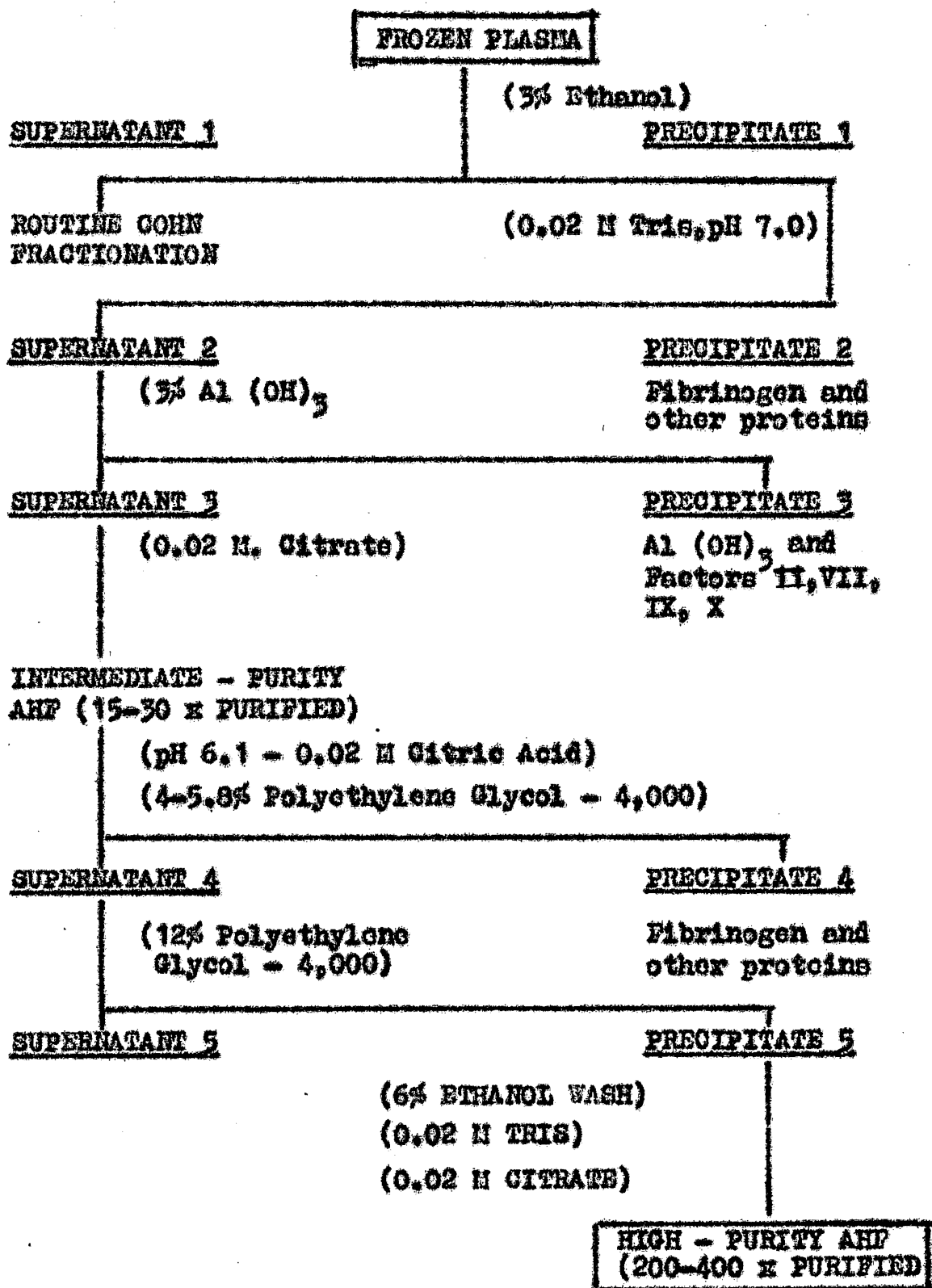
1. Ether precipitation : Fresh human plasma is used in this method. The addition of ether was carried out on the same day. 11% volume of ether was added to plasma at temperature 0°C. The precipitate was collected and suspended in 20% of the original volume of plasma in citrate saline. To which 8% volume of ether was added and again the precipitate is collected. The precipitate was dissolved in citrate saline 20% of the original volume. This preparation contains 50% of the total fibrinogen and AHG also.



2. Slow freezing method: Blood was collected in acid citrate dextrose as anticoagulant. The blood pack was then rapidly cooled to 2-4°C. in a waterbath containing melting crushed ice, and kept at this temperature until they could be transferred to a precooled centrifuge at 4°C. The pack was then centrifuged for 30 minutes at 1,600 g. to obtain the maximum yield of platelet poor plasma. The plasma was separated and placed in a cardboard carton and immersed in a mixture of solid CO₂ and ethanol at about -70°C. The plasma was allowed to remain in the freezing mixture for at least ten minutes, and then was placed in a 4°C. refrigerator and allowed to thaw for 18 to 24 hours. As soon as the thawing was completed, it was centrifuged for twenty minutes at 4°C. and 1,600 g. Supernatant plasma was taken out keeping 5-15 ml. of supernatant in the bottle, immediately stored at -30°C. and lyophilized.
3. Rapid freezing method : Blood was collected in acid citrate dextrose as anticoagulant and centrifuged as in the slow method, described above, to obtain platelet poor plasma. Plasma was separated and frozen at about -70°C. The plasma was allowed to remain in the freezing mixture for six minutes, and was then transferred to a waterbath at 6-8°C. Complete thawing was evenly and rapidly done within 90 minutes. The pack was then centrifuge at 1,600 g. for 15 to 20 minutes and supernatant plasma was removed, leaving 5-15 ml. in the pack with the cryoprecipitate. The cryoprecipitate was snap frozen at -70°C. before storing at -30°C. until use.

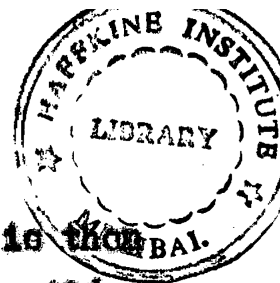


Preparation of high purity AHF with polyethylene glycol



FRACTIONATION OF INTERMEDIATE - AND HIGH PURITY AHF
FROM FRESH FROZEN PLASMA

5. Alcohol precipitation method : Blood was collected in siliconised bottles containing one-ninth volume of 3.8 per cent sodium citrate. Plasma was separated by centrifugation at 1,200 g. for 15 minutes at 4°C, and then respun at 1,6000 g. for 15 minutes at 4°C. 80% ethanol was added to bring the final ethanol concentration to 3% with gentle stirring of the plasma at -3°C, for 30 minutes. The mixture was centrifuged at 16,000 g. for 30 minutes at -3°C. The precipitate was dissolved in imadazol saline buffer using one-tenth the volume of the starting plasma.
6. Sodium chloride precipitation method followed by polyethylene glycol : Blood was collected in acid citrate dextrose as anticoagulant and the plasma was separated. The plasma is treated with 25 gmo. of sodium chloride/100 ml., a precipitate is produced, this precipitate has the same composition as a cryoprecipitate, but it contains fibrinogen. The precipitate is used as a starting material for the further purification. This precipitate is then dissolved in a volume of citrate phosphate buffer equal to 1/10 that of the original plasma and is once more precipitated with sodium chloride at 25 per cent concentration. The precipitate contains fibrinogen and AHF after resuspension it is then treated with 4-6% PEG (Molec. weight 4,000) following treatment



with $Al(OH)_3$ for adsorption of prothrombin. AHF is then precipitated by increasing the PEG concentration to 12%. Contaminating PEG is removed from the AHF fraction by using 25% sodium chloride and is then lyophilised and stored.

Purification of AHF

Purification of AHF was carried out by reprecipitation in the case of alcohol and other precipitation method. The reprecipitation was carried out twice or thrice to remove fibrinogen as much as possible. The AHF prepared by sodium chloride method, Johnson's method and Polson's method was purified by using PEG-4000. AHF prepared by slow and rapid freezing method were not purified.

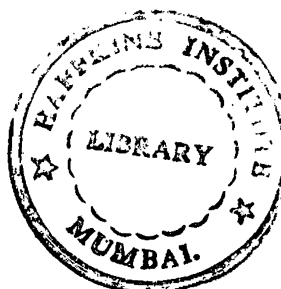
Preparation of anti-AHF

Two rabbits (1.8 kg.) were injected weekly subcutaneously with 10 μ g., 20 μ g. and 25 μ g. of AHF prepared by Hoyer's method, purified with reprecipitation and complete Freund's adjuvant. Ten days after the last injection the test bleeding was done. The last dose was repeated and the animals were bled after ten days and the serum was collected. The adsorption of anti-AHF serum

has been done by the method suggested by Hoyer 1972. The antiserum was tested against serum, fibrinogen, plasma and AHP. This antiserum formed a single line when tested by gel diffusion against normal human plasma and AHP.

Preparation of thrombin

- i) 100 ml. of plasma diluted to 1,000 ml. with distilled water. Adjusted pH 5.3 with 2% acetic acid. Centrifuged and precipitate was collected.
- ii) The precipitate was dissolved in 25 ml. of normal saline and pH adjusted to 7 with 2% Na_2CO_3 .
- iii) 3 ml. of 0.25 M CaCl_2 was added and coagulated fibrinogen was removed as it is formed.
- iv) Kept for 2 hours and equal volume of acetone was added at room temperature. The precipitate was separated by centrifugation.
- v) 25 ml. of normal saline was added to the precipitate and was shaken well. The mixture was centrifuged and precipitate was discarded. Supernatant contained thrombin (200 units/ml.).





CHAPTER - IV



RESULTS

Table I represents the electrophoretic analysis of different fractions by Cohn's cold ethanol method. Whole plasma contains fibrinogen 0.44% but in fraction I only 0.26% of fibrinogen is precipitated out. The major contaminants are β & γ -globulins. Fraction II+III contains major fractions as β and γ -globulin and some fibrinogen i.e. 0.16%. Fraction IV and V do not contain fibrinogen.

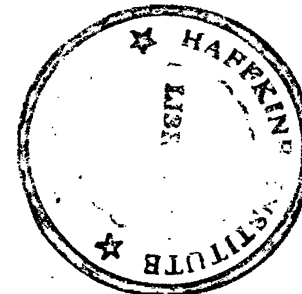
In the Table II the data of total protein content of fibrinogen fraction produced by different methods are tabulated. In ammonium sulphate method total protein estimated is 0.34 gm.% of which clottable and non-clottable proteins are 74% and 26%. In phosphate buffer method the fibrinogen content is 0.41 gm.% having clottable protein 30% and non-clottable protein 70%.

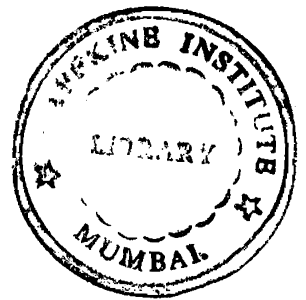
The precipitation by ethyl alcohol and ethyl ether method give more purified fibrinogen having protein 1.6 gm.% and 0.8 gm.% while clottable protein 80 and 49.8% and non-clottable protein 20% and 50.2% respectively. In sodium chloride method the contaminants are less still it gives clottable protein 40% and non-clottable 60%. When the supernatant of cryoprecipitate is used for the preparation of fibrinogen 10% alcohol is added (Grobelaar 1973) and when two bottles of fibrinogen are pooled together gives protein 1.8 gm.%. This fibrinogen

Table I

Plasma proteins in various fractions separated by ethanol method

Fractions	Total protein	Gms. proteins per 100 ml. of plasma				
		Albumin	Globulin	β -globulin	γ -globulin	Fibrinogen
Whole plasma	6.04	3.32	0.84	0.73	0.66	0.44
Fraction I	0.44	0.03	0.02	0.08	0.05	0.26
Fraction II+III	1.63	0.07	0.18	0.62	0.60	0.16
Fraction IV	0.97	0.10	0.54	0.31	0.02	-
Fraction V	2.96	2.90	0.06	-	-	-



Table II

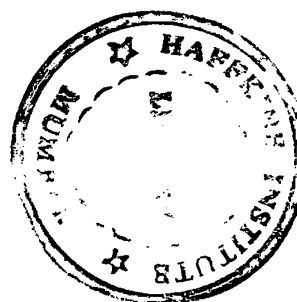
Clottable and non-clottable protein analysis of fibrinogen
isolated by different methods (Average of 30 batches)

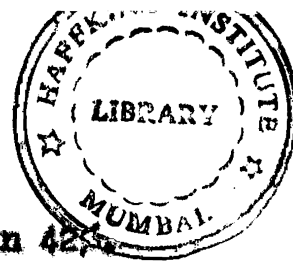
Sr. No.	Method	Total protein gm. %	Clottable protein %	Non-clottable protein %
1	Ammonium sulphate	0.34	74	26
2	Phosphate buffer	0.41	30	70
3	Ethyl ether	0.8	49.8	50.2
4	Sodium chloride	0.4	40	60
5	Ethyl alcohol (Absolute) 8%	1.6	80	20
6	Ethyl alcohol (Absolute) 10%	1.8	58	42
7	Highly purified fibrinogen	0.8	95	5
8	Ethyl alcohol (53.5%) 8%	2.0	70	30

Table III

Electrophoretic analysis and properties of fibrinogen isolated using ethyl ether and ethyl alcohol (Average of 30 batches)

Method	Electrophoretic Analysis				Clottable protein %	Non-clottable protein %
	Albumin	Alpha+beta	Gamma	Fibrinogen		
Ethyl ether (crude)	26.7	10.2	7.1	56.0	50.4	49.6
Reprecipitation of the above product	4.2	6.1	6.7	83.0	74.9	25.1
Ethyl alcohol (crude)	28.2	10.0	7.3	54.5	59.1	40.9
Reprecipitation of the above product	3.2	6.9	3.8	87.1	80.0	20.0





has clottable protein 58% and non-clottable protein 42%. When 53.5% alcohol is used for precipitation the fibrinogen content is 2.0 gm.% and clottable and non-clottable proteins are 70% and 30% respectively.

Highly purified fibrinogen was prepared by Blomback and Blomback method (1956) having total protein 0.8 gm.% and this fibrinogen has 95% clottable protein.

Electrophoretic analysis of two methods ethyl ether and ethyl alcohol (Table III) was carried out as the other proteins are precipitated with fraction I as contaminants. In ethyl ether method albumin is the major contaminant (26.7%) as compared to $\alpha + \beta$ and γ -globulin which are 10.2% and 7.1% respectively. The fibrinogen isolated is 56% which contains 50.4% clottable and 49.6% non-clottable. In ethyl alcohol method the crude fibrinogen contains 28.2% albumin, 10% $\alpha + \beta$ globulin and 7.3% γ -globulin, the fibrinogen content is 54.5% which is less than ethyl ether method but clottable protein is 59% and non-clottable is 41%. The reprecipitated fibrinogen contains more $\alpha + \beta$ -globulin 6.9% as compared to albumin and γ -globulin as 3.2% and 3.8%. The fibrinogen content is 87% and clottable protein is 80% and non-clottable 20%.

Table IV denotes the values of thrombin clotting time and clottability of the fibrinogen prepared by different methods. The minimum clotting time of purified fibrinogen by Blomback and Blomback method, ammonium

Table IV

The thrombin clotting time and clottability of fibrinogen
Isolated by different methods (Average of 30 batches)

Sr. No.	Methods	Thrombin clotting time in Sec.	Clottability %
1	Ammonium sulphate	7	70
2	Phosphate buffer	18	30
3	Ethyl ether	15	49.8
4	Sodium chloride	20	40
5	Ethyl alcohol (Absolute) 8%	9	80
6	Ethyl alcohol (Absolute) 10%	10	58
7	Highly purified	6	95
8	Ethyl alcohol (53.5%) 8%	13	70

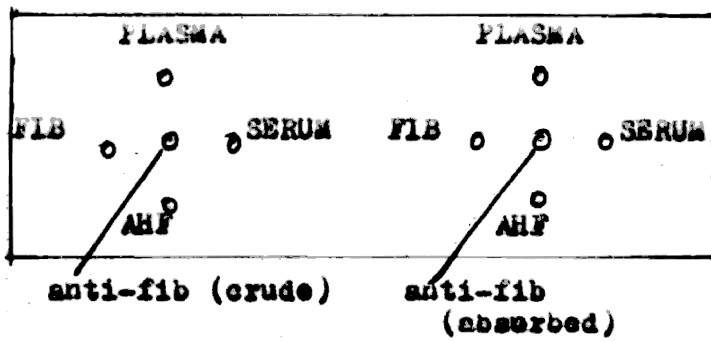
Table V

Comparison of three different methods of estimating clottable fibrinogen prepared by various methods (Average of 30 batches)

Sr. No.	Methods	Estimated clottable fibrinogen		
		Biuret method	Radial immuno- diffusion	Thrombin clotting time method
1	Ammonium sulphate	74%	66%	70%
2	Phosphate buffer	40%	26%	30%
3	Ethyl ether	50%	40%	49.8%
4	Sodium chloride	50%	35%	40%
5	Ethyl alcohol (Absolute) 8%	60%	75%	80%
6	Ethyl alcohol (Absolute) 10%	60%	55%	58%
7	Highly purified fibrinogen	90%	88%	95%
8	Ethyl alcohol (53.5%) 8%	75%	65%	70%



Fig. 7



sulphate method and ethyl alcohol method are 6, 7 and 9 seconds respectively. Whereas the clottability is 95%, 70% and 80%. It is found that the clotting time increases with the decrease in clottability. The decrease in clottability is due to denaturation of fibrinogen. The maximum clotting time observed in sodium chloride method which is 20 seconds and clottability is 40% but in phosphate buffer method though the clotting time is less the clottability is 30%.

The estimation of clottable fibrinogen by different methods was carried out (Table V). By Biuret method total protein is estimated. By thrombin clotting time the clottable fibrinogen by thrombin is estimated and by radial immunodiffusion method the clottable fibrinogen is estimated immunologically.

ANTI-FIBRINOGEN

Human fibrinogen prepared by Cohn's method was used to produce rabbit antihuman fibrinogen antiserum. The crude antiserum was absorbed with equal volume of human serum and was kept first for one hour at 37°C., then for 24 hours at 4°C. Immunoelectrophoresis showed that the antiserum reacted as a single line against human plasma and fibrinogen and did not react with human serum (Fig. 7).

Titration of antifibrinogen (crude)

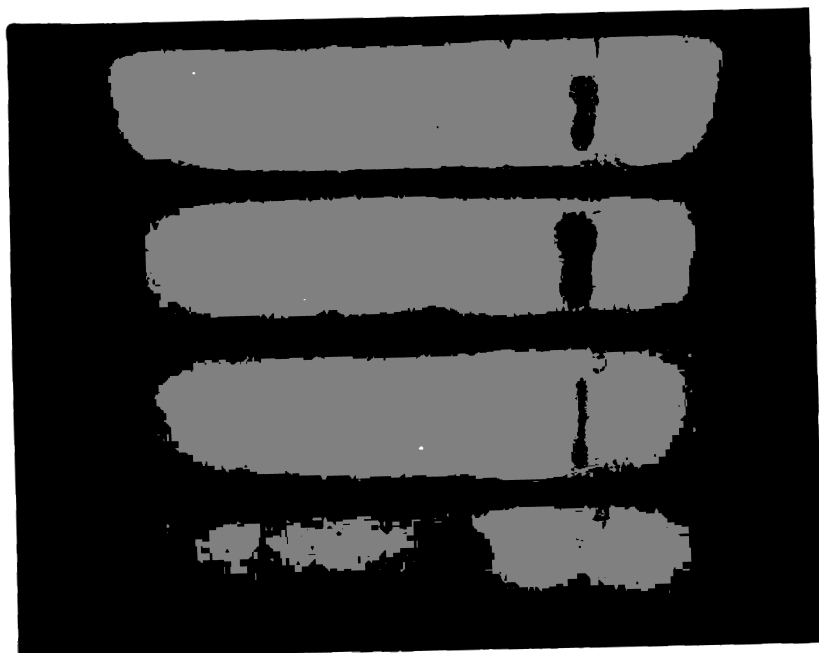
Routine titration of antibody was carried out. The tubes were incubated at room temperature for one hour. Supernatant was tested for antigen and antibody. This showed that there was no antigen or antibody left in the supernatant which was detectable by gel diffusion technique.

Titration of antifibrinogen (absorbed)

Routine titration was carried out and the supernatant was tested for antigen and antibody. This showed that no antibody was left which was detectable by gel-diffusion. The antigen was left till the titre 1:512 which can be detectable by gel-diffusion.

The titre of antisera

In this experiment fibrinogen was serially diluted and constant amount of antibody is added. The tubes were kept overnight and supernatant is tested. At dilution (1:32) of fibrinogen the amount of antibody is neutralized and can not be detectable by gel-diffusion. Only dilution upto 1:8 shows precipitation reaction against antibody. Further dilution does not show presence of antigen (left) in the reaction mixture.



1. Ammonium sulphate

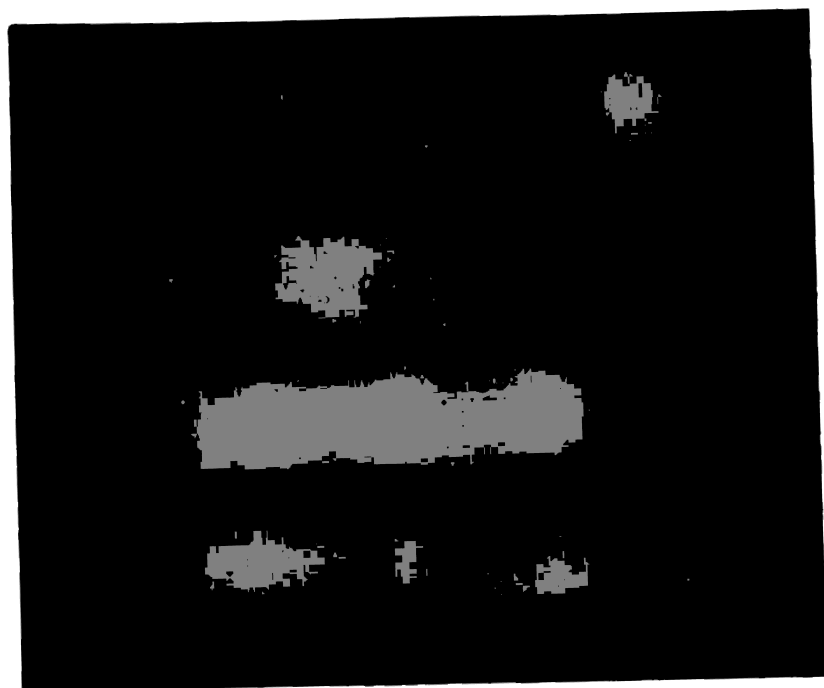
2. Phosphate buffer

3. Ethyl ether

4. Sodium chloride

Fig. 8

Paper Electrophoresis



5. Ethyl alcohol
(100%) 8%

6. Ethyl alcohol
(100%) 10%

7. Highly purified
Fibrinogen

8. Ethyl alcohol
(53.5%) 8%

Therefore 1:16 dilution of fibrinogen and constant amount of antibody (2 drops) gives complete neutralisation. Quantitation of fibrinogen was possible by this method.

Titration of fibrinogen

Titration was carried out by taking measured amount of fibrinogen (0.2 ml.) and measured amount of antibody serum. Supernatant was tested for antigen and antibody. Antibody was not present in the reaction mixture till dilution 1:32. Dilution 1:64 onwards the antibody remains in the reaction mixture and can be detectable by gel diffusion.

Dilutions 1:1 and 1:2 show the presence of antigen in the reaction mixture. Supernatant from dilution 1:4 to 1:16 shows no antigen left but in dilution 1:32 the presence of antibody is detectable.

Paper electrophoresis (Fig.8)

Fibrinogen precipitated by ammonium sulphate, phosphate buffer, sodium chloride, ethyl alcohol 53.9% contain traces of β -globulin along with fibrinogen. Whereas fibrinogen precipitated by Cohn's ethanol (8%) (10%) contains much impurities of α , β and albumin. Fibrinogen by ethyl ether (reprecipitated) and purified fibrinogen was comparatively pure.

Table VI

Quantitative estimation of fibrinogen in plasma

Sr. No.	Fibrinogen mg. %		
	Professional donors	Voluntary donors	Staff members
1	380	325	346
2	368	396	285
3	330	378	362
4	282	396	314
5	284	326	396
6	325	310	362
7	340	328	292
8	396	362	378
9	310	356	360
10	334	394	332
11	378	334	356
12	296	292	394
13	346	378	368
14	285	356	368
15	362	305	320
16	356	318	334
17	378	326	327
18	318	392	328
19	314	378	362
20	394	374	332
21	374	394	262
22	328	334	376
23	382	329	335





Fig. 9

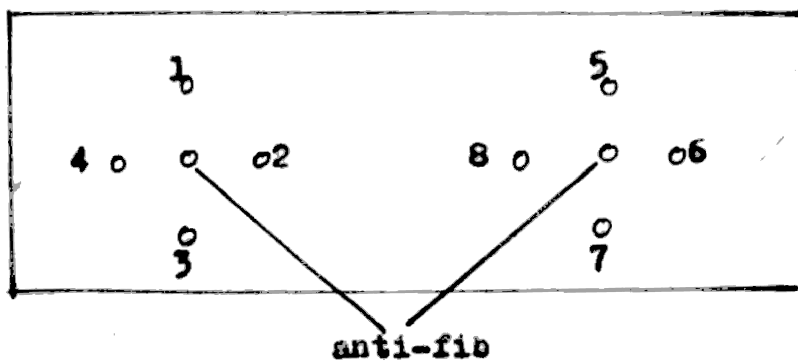
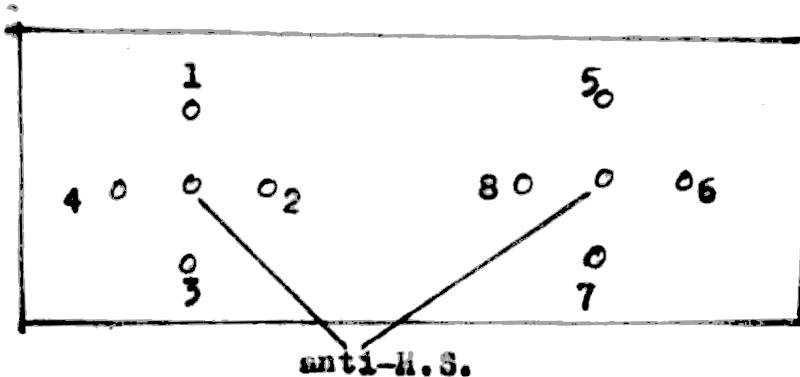


Fig. 10



- | | |
|----------------------|-------------------------|
| 1. Ammonium sulphate | 5. Ethyl alcohol |
| 2. Phosphate buffer | (100 %) 8% |
| 3. Ethyl ether | 6. Ethyl alcohol |
| 4. Sodium chloride | (100%) 10% |
| | 7. Highly purified fib. |
| | 8. Ethyl alcohol |
| | (53.5%) 8% |

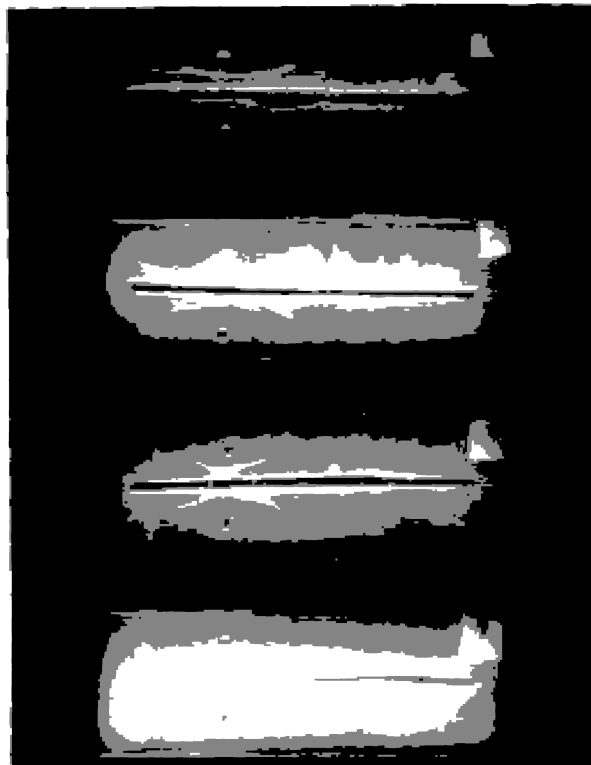


Fig. 11.

○ Ammonium sulphate	
○ Phosphate buffer	anti-fib
○ Ethyl ether	
○ Sodium chloride	anti-fib
○ E. alcohol (100%) 8%	
○ E. alcohol (100%) 10%	anti-fib
○ Highly purified fib	
○ E. alcohol (100%) 10%	anti-fib

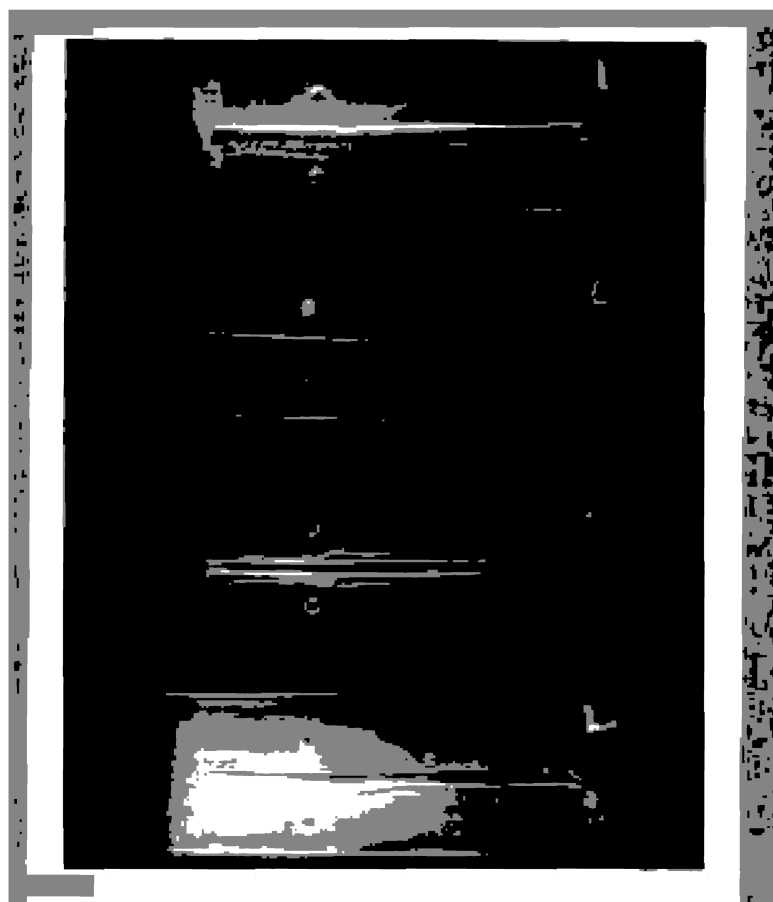


Fig. 12

○ Ammonium sulphate
 — anti-H.S.
 ○ [] ate buffer

○ Ethyl ether
 — anti-H.S.
 ○ Sodium chloride

○ E. alcohol (100%) 8%
 — anti-H.S.
 ○ E. alcohol (100%) 10%

Highly purified film

Radial immune-diffusion

Results obtained by radial immune-diffusion and thrombin clotting time are nearly same.

Gel diffusion

Fig. 9 shows the antigen antibody reaction of fibrinogen prepared by different methods and antifibrinogen which was produced in rabbits by injecting purified fibrinogen.

When the same samples were tested against antihuman serum immunologically, it was seen that fibrinogen by sodium chloride method and purified fibrinogen show no reaction against it (Fig. 10).

Immunoelectrophoresis

Fibrinogen prepared by different methods were studied immunoelectrophoretically using antifibrinogen (Fig. 11) and antihuman serum (Fig. 12), traces of γ -globulin and β -globulin ($<1\%$) were observed in nearly all the samples.

Table VII

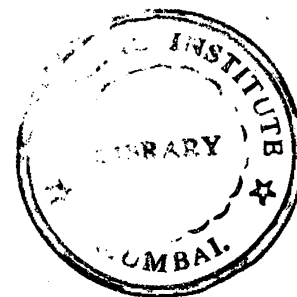
Estimation of protein and AHF activity of AHF prepared
by different methods (Average of 50 batches)

Sr. No.	Methods	Proteins % in gms.	AHF activity % of normal
1	Slow freezing method	3.1	260
2	Rapid freezing method	3.0	240
3	Hoyer's method	1.35	225
4	Johnson's method	1.2	200
5	Polson's method	1.3	210

Table VIII

AHF activity at different stages of storage
(Average of 50 batches)

<u>Sr. No.</u>	<u>Stage</u>	<u>Rapid freezing method</u>	<u>Slow freezing method</u>
1	Fresh AHF	240%	260%
2	Frozen AHF	160%	175%
3	Lyophilised AHF	145%	150%
4	Lyophilised one year old	80%	90%



Quantitative estimation of fibrinogen

Quantitative estimation of fibrinogen was carried out by radial immune-diffusion in three groups 1) professional donors 2) voluntary donors 3) staff members (Table VI). It is found that the mean values of each group are 302, 349, 341.8 mg.%.

Antihæmophilic globulin (AHF, Factor VIII)

Protein estimation of AHF prepared by different methods was done by Biuret method and AHF activity was also carried out by two stage thromboplastin generation test. Results are tabulated in Table VII indicate that highest activity was found in AHG prepared by slow freezing method and lowest activity was found in Johnson's method (200%).

Effect of storage on AHG

AHG prepared by two different methods was taken for this study. In rapid freezing method and slow freezing method when it is freshly prepared, AHF activity is 240 and 260% of the normal. In frozen state AHF activity decreases to 160% and 170% of the normal. When it is lyophilised it reduces to 145 and 150% and when estimated after one year in lyophilised form preserved at -30°C. shows AHF activity as 80% and 90% respectively (Table VIII).

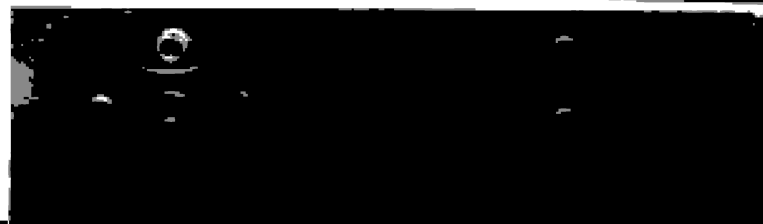


Fig. 13

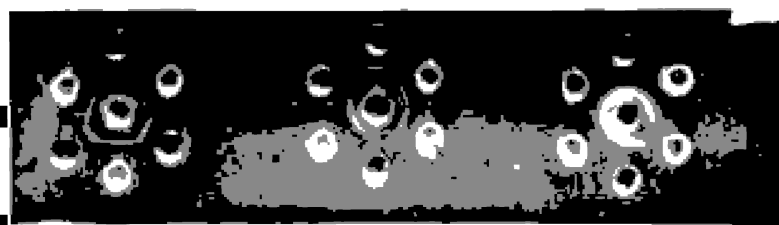
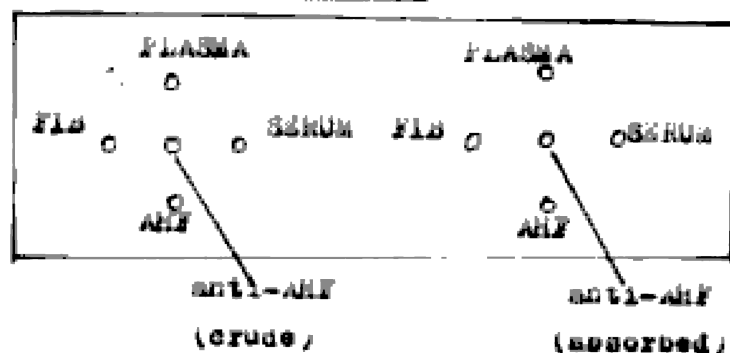
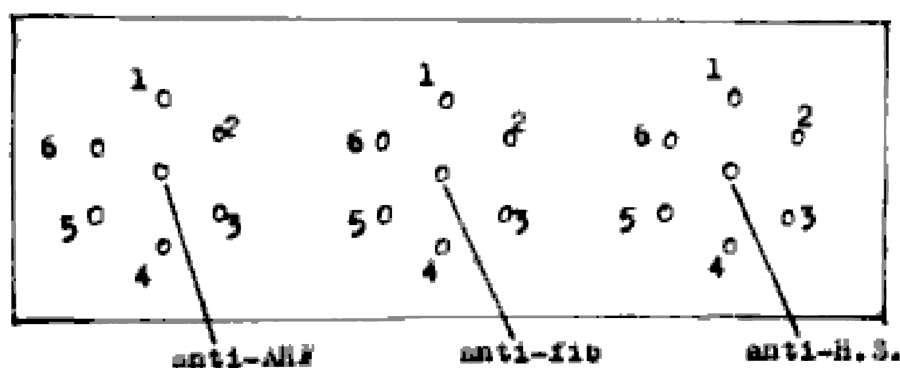


Fig. 14



1. PLASMA 2. AMF-H.F. 3. AMF-HOYER
4. AMF-POLSON 5. AMF-S.F. 6. AMF-JOHNSON

Anti-AHF

AHF prepared by Hoyer's method was used to produce anti-AHF. This crude anti-AHF contained anti-fibrinogen as the major impurity. This antiserum was absorbed with AHF poor absorbing antigen prepared by Hoyer's method by bringing the supernatant plasma from 3% ethanol -3°C , separation to a final concentration of 8% at -3°C . (Zimmerman et.al. 1971). To the precipitate was added the heated and calcium phosphate absorbed, rabbit antiserum in equal volume to one half the 3% ethanol supernatant used to obtain the absorbing antigen. Crude and absorbed anti-AHF was tested by gel diffusion technique against plasma, fibrinogen, normal human serum and AHF. The absorbed antiserum formed a single line against plasma and AHF (Fig. 13).

Gel diffusion

All the preparations of the AHF were tested immunologically by gel diffusion against antifibrinogen, antihuman serum and anti-AHF (Fig. 14).

In the first pattern precipitin lines against all the wells are seen. In the second pattern a faint line is seen against Johnson's method. It indicates that fibrinogen is totally absent. Other samples contain fibrinogen as impurity. In the third pattern precipitin lines are seen against all the wells. It concludes that other proteins are also present in the AHF preparation (Fig. 14).

Table IX

AHP was prepared with different methods from the plasma
of different groups and also from pooled plasma (P)

Sr. No.	Methods	Groups	<u>Plasma</u>		<u>AHP</u>	
			Anti-A	Anti-B	Anti-A	Anti-B
1	Slow freezing	A	-	1:4	-	1:2
		B	1:32	-	1:8	-
		O	1:8	1:8	1:4	1:2
		P	1:8	-	1:8	-
2	Rapid freezing	A	-	1:4	-	1:2
		B	1:8	-	1:8	-
		O	1:8	1:8	1:4	1:2
		P	-	1:16	-	-
3	Hoyer's method	A	-	1:4	-	-
		B	1:8	-	-	-
		O	1:32	1:32	-	-
		P	1:4	1:2	-	-
4	Johnson's method	A	-	1:2	-	-
		B	1:16	-	-	-
		O	1:32	1:4	-	-
		P	1:8	1:4	-	-
5	Polson's method	A	-	1:4	-	-
		B	1:16	-	-	-
		O	1:8	1:8	-	-
		P	1:8	1:8	-	-

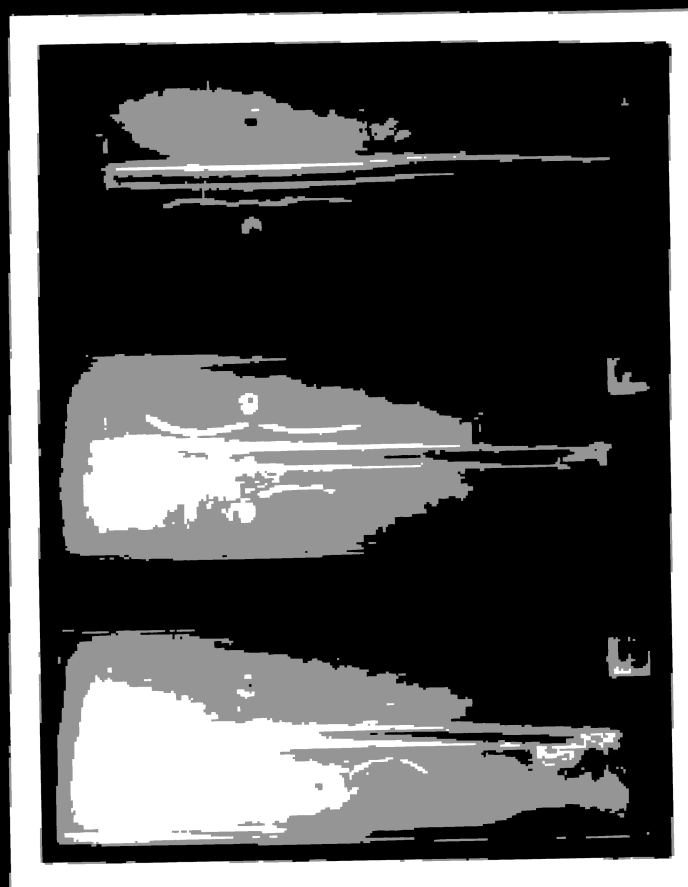
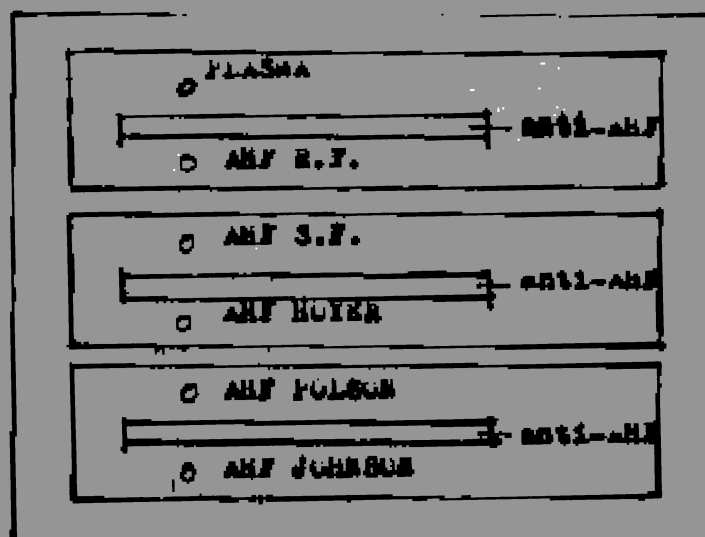


Fig. 15



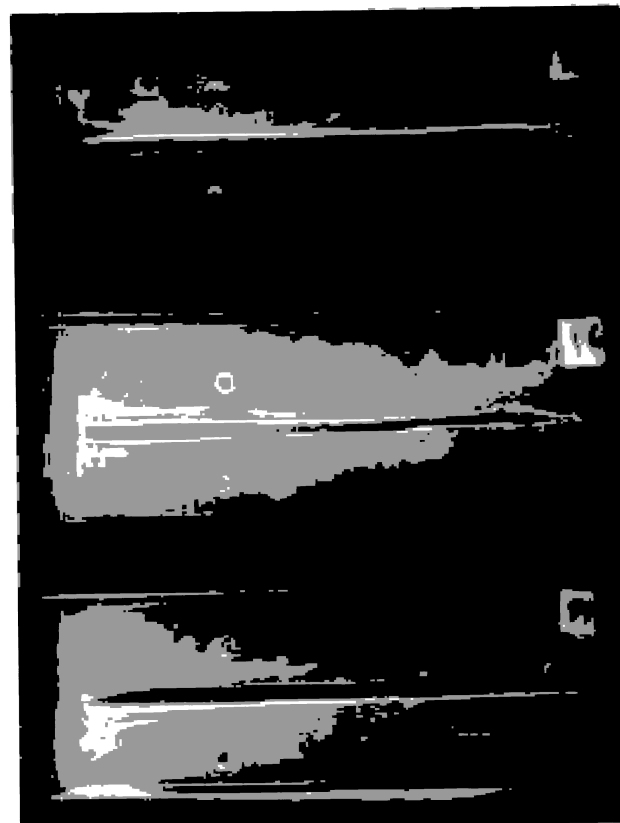
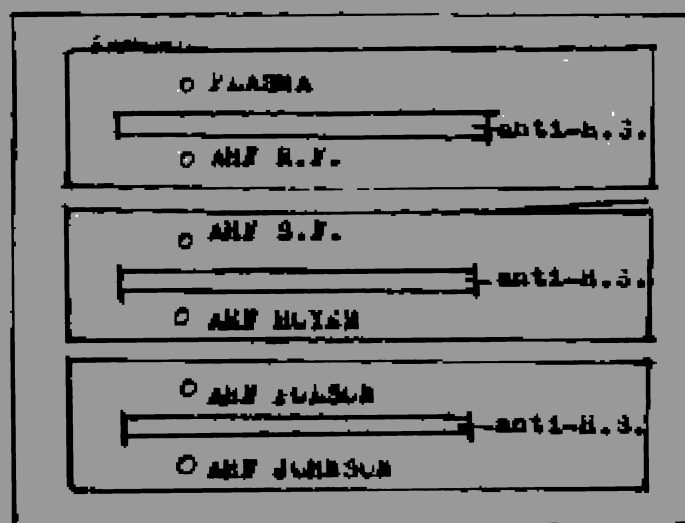


Fig. 10



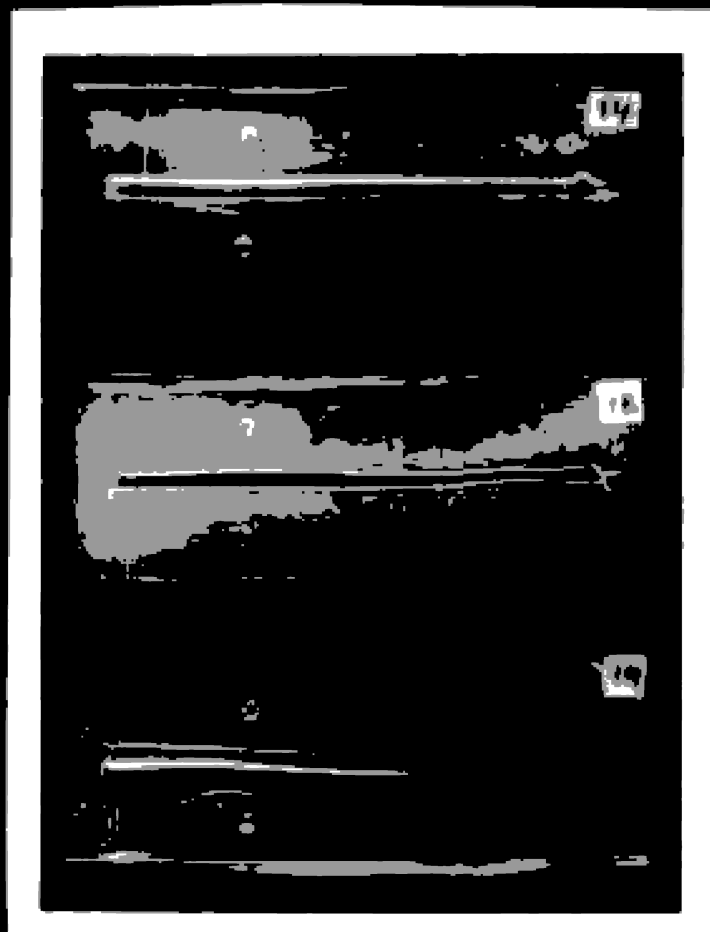
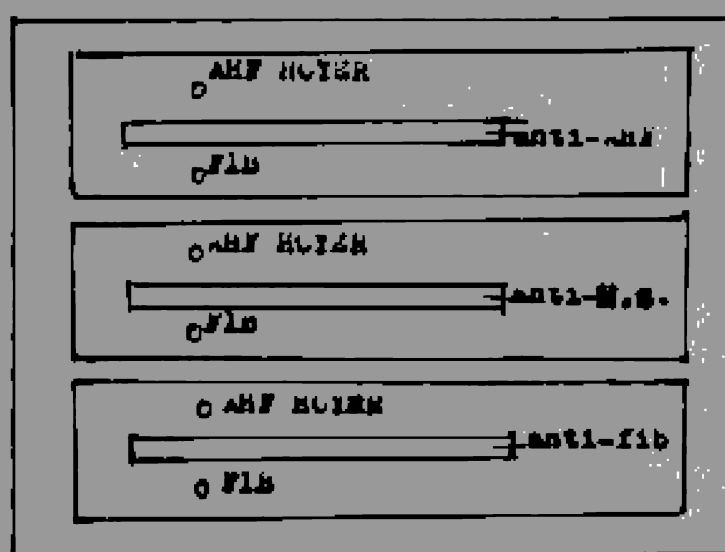


Fig. 17





Immunoelectrophoresis of AHP preparations

All the preparations have been tested against anti-AHP and compared with fresh plasma. It can be seen that the AHP prepared by all the methods contain AHP (Fig. 15).

The immunoelectrophoretic pattern of AHP prepared by different methods has been studied against anti-human serum, antifibrinogen and anti-AHP (Fig. 16 & 17).

The AHP prepared by Hoyer's method is highly purified. This preparation normally shows presence of fibrinogen with slight traces of globulin.

The AHP prepared by rapid freezing method and slow freezing method show some impurities when tested against antihuman serum. Johnson's and Polson's method also contain similar impurities.

Titration of antibody (anti-A and anti-B)

Antibody titration of AHP prepared from different groups plasma and pooled plasma was carried out. With different methods it was found that anti-A and anti-B titre of plasma and AHP prepared by the different methods is 1:8 and 1:2 respectively in the AHP prepared by A and B group plasma. There was no titre in the AHP prepared from pooled plasma (Table IX).

**Table X****Quantitative estimation of factor VIII levels**

Sr. No.	AHF activity in per cent			
	Male staff	Female staff	Voluntary donors	Professional donors
1	75	115	140	120
2	120	75	125	145
3	80	120	75	80
4	120	80	75	145
5	125	125	80	75
6	110	75	110	115
7	80	125	120	125
8	80	120	80	115
9	140	80	115	80
10	110	80	80	145
11	80	110	145	110
12	110	75	115	80
13	120	80	120	115
14	140	125	80	75
15	115	80	110	80
16	80	120	80	125
17	75	125	80	110
18	140	80	125	115
19	80	120	120	145
20	110	75	140	120
21	125	80	80	80
22	120	110	80	125
23	80	80	115	125
24	80	120	110	115
25	125	80	125	125

Table XI

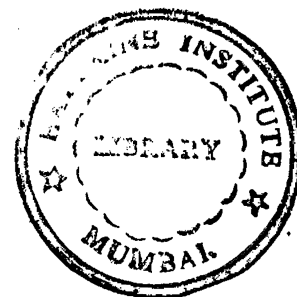
Factor VIII levels of cases of haemophiliae before and after
the transfusion of cryoprecipitate

Sr. No.	Age of the patient in years	AHF levels before cryoprecipitate administration	AHF levels after cryoprecipitate administration	Amount of cryoprecipitate given in I. Units *
1	38	10	50	800
2	14	10	50	800
3	23	15	65	800
4	3	15	95	1200
5	6	5	110	800
6	16	10	55	1600
7	11	15	105	2400
8	14	5	15 after 24 hrs.	800
9	8	15	75	800
10	4	25	120	1200

* I.U. of AHF = Amount in 1 ml. of normal plasma
(Ratnoff et.al. 1964)

Table XIIQuantitative estimation of AHG in plasma 37/2

Sr. No.	10/5		12/6		AHG mg.% 8	
	Male staff	Female staff	Voluntary donors	Professional donors	16/9	18/12
1	215	260	267	237		
2	222	237	322	215		
3	282	245	215	260		
4	245	297	337	282		
5	230	337	297	222		
6	252	252	337	237		
7	227	245	222	252		
8	267	290	330	267		
9	252	360	282	230		
10	337	227	360	282		
11	220	297	282	290		
12	260	282	262	282		
13	282	252	297	237		
14	245	267	330	222		
15	222	222	337	215		
16	360	260	215	237		
17	215	275	260	215		
18	337	297	245	260		
19	360	330	275	245		
20	337	352	237	360		
21	345	315	290	345		
22	297	360	360	275		
23	322	237	252	282		
24	315	297	322	275		
25	352	315	245	260		



Quantitative estimation of AHP activity

Quantitative estimation was carried out with four groups of healthy persons between 25 to 40 years. These four groups are (1) male staff members (2) female staff members (non-pregnants and not on oral contraceptives) (3) voluntary blood donors (4) professional blood donors. The test was performed with two stage thromboplastin generation test as discussed in methods. The samples were run in triplicate and AHP activity was found in terms of normal. It showed AHP levels between 50 to 150% of the normal (Table X).

Clinical trials

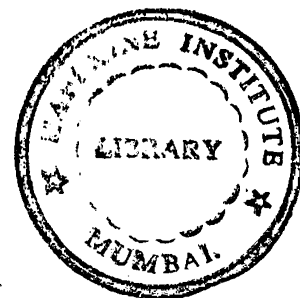
Cases of haemophilia were studied for AHP activity before and after the transfusion of AHP (Table XI). It is seen from the ten cases so far studied that minimum amount transfused to bring the AHP activity to normal is 800 units. In two cases 2,400 units and 1,600 units were transfused and other two cases were treated with 1,200 units of AHP.

Quantitative estimation of AHG in plasma by radial immuno-diffusion

Four groups were undertaken for this study (1) male staff (2) female staff (3) voluntary donors (4) professional donors. Quantitative estimation was carried out by radial immuno-diffusion (Table XII). It is found that AHG values are between 215 mg.% and 360 mg.%.



CHAPTER - V



DISCUSSION

Fractionation of plasma proteins by different methods such as ammonium sulphate, ethyl ether, phosphate buffer, sodium chloride and ethyl alcohol at different concentration (100% and 53.5%) has been carried out for the preparation of fibrinogen and antihæmophilic globulin.

In salting out method sodium sulphate, ammonium sulphate and sodium chloride have been widely used. The ammonium sulphate method is employed by many scientists. The advantage of this high salting out effect can be taken into account because of the great solubility of the ammonium sulphate in water. This permits the attainment of extremely high ionic strength. There are certain drawbacks which are counterbalanced by advantages of ammonium sulphate method. The direct use of nitrogen present in the form of ammonium ion is not useful to determine the protein concentration in the solution.

In analysis it is important that the salt must be removed by dialysis. Buffering action of ammonium sulphate is not satisfactory in the pH range below 8. The maintenance of the pH of the solution plays an important role in obtaining satisfactory results.

The large scale fractionation of proteins has been generally effected by salting out method which were given certain flexibility by the possible variation of pH and temperature. In protein separation neutral salt at concentration in the region of 2-3 M has disadvantages. The product must be freed from the precipitating salts by dialysis. The products used for clinical purposes required the process to carry out under aseptic conditions.

In solvent precipitating method there are five variables. These variable can be used to establish conditions such that a dried protein can be precipitated from protein mixtures in aqueous solution. These variables are pH, salt concentration, temperature, total protein concentration and solvent concentration.

The method indicated by Kekwick and Mackay (1946) for the precipitation of plasma proteins shows that the plasma proteins were more stable towards ethyl ether than to other water miscible solvents such as ethanol and acetone. The dielectric constant of ethyl ether is lower than that of ethanol and acetone. It was thought that this might be a further advantage in relation to its efficacy as a precipitant.

The disadvantages in ethyl ether method are that it is not completely miscible with water but reaches a limiting solubility. This limit is useful in decreasing the solubility of proteins in aqueous system.

The production of plasma proteins on a large scale, the low salt ethanol method has an advantage over the salting out procedure. In salting out procedure the high salt concentrations necessary for protein precipitation can be diminished by dialysis only. This procedure is time consuming and involves much work when a large volume of liquid is to be dialysed. The volume becomes favourable for bacterial growth, during the last step of dialysis when the salt concentration is diminished and the protein solution is likely to become contaminated. The ethanol may be readily removed when it is used as precipitant, instead of salt because of its volatility. The rapid freezing of the solution at very low temperature and subsequent drying from the frozen state in a vacuum appears to be the safest method for removal of alcohol. The cooling produced by evaporation maintains the frozen protein solution at low temperature through almost the whole process and the temperature begins to rise only when the protein is nearly dried. Under these circumstances most of the plasma proteins are found to be suitable in water or dilute salt solution say saline, after drying they may be preserved for a long time.

The ethanol method has got disadvantages along with the advantages. Variations in temperature often used for the separation of certain components. The limit of temperature employed in the system should be low in order to avoid denaturation of the protein. The necessity for the precautions in fractionation by low temperature, low salt, ethanol method makes it in many respects a more difficult process to carry out than in fractionation by salting out with ammonium sulphate.



On the basis of main property of fibrinogen i.e. clotting effect the alcohol method of Cohn (1946) is a suitable method. The clottable protein in various fibrinogen preparations when compared it is obvious that the maximum clotting effect is seen in the fraction I isolated by Cohn's method. In ether and alcohol methods identical contaminating proteins were found in similar proportions along with the precipitate of fraction I. When ethyl alcohol (absolute) 8% is used as precipitant 80% clottable fibrinogen is obtained but ethyl alcohol (53.5%) gives 70% clottable fibrinogen as the fibrinogen is lost in reprecipitation. In all other methods sterilisation is needed and in sodium chloride method removal of polyethylene glycol is most important. To sterilise the fibrinogen it is essential to have special type of filters which are not available in our country.

In electrophoretic analysis it is seen that in ethyl alcohol and ether method impurities of α , β and γ -globulin with albumin are seen as major contaminant. In Cohn's method it is difficult to remove these fractions under aseptic conditions. But purified fibrinogen can be prepared by Blomback's method which removes all the contaminants but there is the loss in protein concentration and sterility can not be maintained.

432

From the study of thrombin clotting time by batch method and clottability the clotting time is 9 to 13 seconds in the Cohn's method with clottability at 70 to 80%. Our results are in agreement with those of Godel and Kierulf (1972). They have shown that the clotting time of native fibrinogen is 18 seconds and clottability is 87%. But when the fibrinogen is denatured due to change in pH or with the action of heat or alkali clotting time increases to 37 seconds and clottability decreases to 70%. This shows that the products having low clotting time and high clottability are to be utilised for the clinical purpose.

Clottable fibrinogen can be estimated by Biuret method, radial immune-diffusion and thrombin clotting time method (Batch method). The results obtained by Biuret method differ from those obtained by other two methods. It is because other proteins get reacted with in Biuret method whereas in radial immune-diffusion only fibrinogen reacts with antifibrinogen giving precipitin rings and in thrombin clotting time method, clotting of fibrinogen takes place by adding thrombin. The results of radial immune-diffusion technique were compared with thrombin clotting time method of Farrell and Wolf (1973) and they are in agreement with our results (Table V).

Antisera to fibrinogen was prepared in rabbits. When crude fibrinogen was used antibodies against AHP and serum were produced therefore purified fibrinogen was used for immunization. Still this antifibrinogen contained some antibodies of serum proteins but they were absorbed with equal volume of serum. Brittin et.al. (1972) has prepared antisera against fibrinogen but he absorbed it with 1/10 volume of human sera. Our sera gave a titre of 1:32 where the total neutralisation of fibrinogen takes place. The antifibrinogen used for radial immunodiffusion by Brittin et.al. (1972) is 0.3 ml. per 10 ml. of agarose where as we have employed 0.8 ml. per 10 ml. of agarose, because in our experiment the antibody gets diluted as the absorption is done with equal volume of human serum.

In gel diffusion technique the fibrinogen (by different methods) give single precipitin line against antifibrinogen indicating that the preparations contain fibrinogen. The precipitin lines are also observed against antihuman serum as other proteins are present in the fibrinogen preparation. The fibrinogen prepared by sodium chloride method does not show any line against antihuman serum and a faint line is observed against antifibrinogen. It can be concluded that the fibrinogen is pure and free from other contaminants.



Fibrinogen prepared by different methods was tested immunoelectrophoretically using antifibrinogen and antihuman serum. Traces of γ -globulin and β -globulin were observed in mostly all the preparations. In precipitating fibrinogen proteins other than fibrinogen are also precipitated out along with fibrinogen as the dissociation constant of other protein is same as fibrinogen. This is also because of the physical adsorption of other proteins on fibrinogen molecules.

Purification of fibrinogen was carried out by Blomback and Blomback method (1956) and R. Berg (1972). The clottability of fibrinogen by Blomback method is 95% and R. Berg's method 94% but in R. Berg's method, Glycine is used which removes α -globulin and plasminogen from fibrinogen which is the advantage of this method. But acetone is used for the precipitation. Though pure fibrinogen is obtained it can not be used for clinical purpose.

AHF prepared by different methods and activity was estimated. It was found that the products of slow freezing and rapid freezing method have highest activity. It also contains other proteins. AHF prepared by Johnson's method shows lowest activity as the activity is lost during the processing.

AHF activity was studied at different stages. The temperature factor is most important. The activity remains till the freeze stage and decreases during lyophilization and also on long storage. Our results tally with those of Anstall et al. (1961), Preston et al. (1967) and Brown et al. (1967). Storage of cryoprecipitate in plastic bags at -25°C . does not result in any appreciable variations in the proportion of factor VIII activity after several months (Brown et al. 1967, Gombrisson et al. 1966). Plastic bags were not used in our laboratory. Freeze drying is done and the loss of AHF activity after drying is about 40%.

Clinical study implies that though the AHG prepared an year ago give the satisfactory results when transfused to the haemophilic patients. The clotting time decreased and bleeding is stopped.

The preparation of antisera is difficult in the case of factor VIII as AHG is associated with fibrinogen. AHG prepared by Hoyer's method was utilised after purification. The purification was carried out at -3°C . and the preparations were also tested against antifibrinogen. Thrice reprecipitation was done to remove the fibrinogen as much as possible and this purified AHF was used for immunisation. Some antifibrinogen was formed which was absorbed with absorbing antigen according to Hoyer (1972).

Single precipitin line was obtained against plasma and AHF and not against fibrinogen and serum.

The antisera were used for radial immunodiffusion. Estimation was carried out by using 0.6 ml. of anti-AHF in 10 ml. agarose. The results are expressed in gm.% and not in international units because while reprecipitation some loss of AHF takes place with fibrinogen. Quantitative estimation of AHF level in plasma was done in professional donors, voluntary donors, male and female staff. AHF values are between 215 mg.% and 360 mg.% (Table XII).

From gel diffusion and immunoelectrophoretic studies it is found that AHF by Hoyer's method contains some impurities of fibrinogen and γ -globulin because the dissociation constant of these proteins is similar to AHF. Johnson's and Polson's method though reported to be pure, our preparations contain some impurities. Slow freezing and rapid freezing method show impurities as the plasma is not removed completely and aseptic precautions prevent us to do so.

Preparations of AHF from pooled plasma show no anti-A and anti-B titre while Johnson et al. (1968) have reported a titre of 1:50 per unit of AHF which is as high as normal plasma. When AHF was prepared from A group and B group plasma, 1:8 and 1:2 titre was obtained.

Two stage thromboplastin generation test was performed for AHP activity (Biggs and Macfarlane 1962) slight modification was done. Instead of human brain monkey brain was used for brain extract as monkey brain was easily available from Polio Department of our Institute. This gave same results as human brain. Study of AHP activity was undertaken between age 25 to 40 years in four categories. AHP activity was found between 50 and 150% of the normal. No abnormal case was found in this series (Table X).

Clinical study was undertaken in ten known cases of haemophilia. While treating the patients with AHP 800 units to 2,400 units of AHP was transfused to bring the normal level (Table XI).

It is understood from the above discussion that Cohn's method is superior over all the other methods as we get a purified product with comparatively high yield and 80% clottable protein. In this method also when 53.5% alcohol is used, the purity of the product is more than that of absolute alcohol. The maximum clotting effect is seen in the fraction I isolated by Cohn's cold ethanol method.

The cryoprecipitate method (slow and rapid) has several advantages over the other methods. It is less complicated and can be carried out in closed system, without risk of contamination. The preparation contains a higher concentration of factor VIII relative to its total protein content.

Though the methods of Polson and Johnson seemed to give pure AHF than that obtained by slow and rapid freezing method, it can not be used clinically because polyethylene glycol is toxic. Moreover these methods need sterilization which results in loss of activity.





CHAPTER - VI



SUMMARY AND CONCLUSIONS

In this thesis work, several methods for the preparation of fibrinogen and anti-haemophilic globulin have been studied. The properties viz. purity, clottability and activity of the above preparations have been established. The quantitative estimation of fibrinogen and AHF has also been done by developing the specific antisera in rabbits and using radial immuno-diffusion technique. The results obtained are discussed in the Chapter IV.

The fibrinogen prepared by modified Cohn's cold ethanol method has been found to be efficacious in the cases of clotting disorders though this contains the impurities in trace amounts.

The AHF prepared by the slow and rapid freezing method has been found to be efficacious in cases of haemophilia.

The radial immuno-diffusion method has been used to estimate fibrinogen and AHF levels. This method is very simple less complicated and the results obtained are comparable with those obtained by other biochemical methods.



BIBLIOGRAPHY



B I B L I O G R A P H Y

Addis T.

The pathogenesis hereditary haemophilia.

J. Path. Bact. 15, 427, 1911.

Adelson E., Rheingold J.J., Parker O., Steiner M.
and Kirby J.C.

The survival of factor VIII (antihæmophilic globulin)
and factor IX (plasma thromboplastin component) in
normal humans.

J. Clin. Invest., 42, 1040, 1963.

Aggeler P.H., White S.G., Glendening M.B., Page E.W.,
Leake T.B. and Bates G.

Plasma thromboplastin component (PTC) deficiency a
new disease resembling haemophilia.

Proc. Soc. Exp. Bio. Med., 79, 692, 1952.

Alexander B.R., Goldstein L., Rich A.G., Bulloch L.E.,
Diamond L.K. and Borges W.

Congenital afibrinogenemia study of some basic aspects
of coagulation.

Blood : 9, 643, 1954.

Anstall, H.B., Grove-Rasmussen, M. and Shaw R.S.

Optimal conditions for storage of fresh frozen plasma.

Transfusion : 1, 87, 1961.

Astrup T. and Darling S.

Measurement and properties of antithrombin.

Acta Physiol. Scand. : 4, 293, 1942.

Bailey K., Bettelheim F.R., Lorand L. and Middlebrook U.R.

Action of thrombin in the clotting of fibrinogen.

Nature : (London), 167, 233, 1951.



Bailey K. and Bettelheim F.R.

The nature of fibrinogen thrombin reaction.

Brit. Med. Bull. II, 50, 1955.

Barrow E.H. and Graham J.B.

Kidney antihæmophilic factor. Partial purification and some properties.

Biochemistry : I, 3917, 1968.

Barkhan P., Lai H. and Stevenson M.

Antihæmophilic factor (factor VIII) an α_2 globulin.

Brit. J. Haematol. 9, 499, 1963.

Baumgarten W., Sanders B.E., Belkin B.D., Pagenkemper P.E.,

Albers W.G. and Giminera J.L.

Antihæmophilic factor, its presence in fibrinogen assay problems and the stability of lyophilised AHG fibrinogen preparations.

Thromb. Diath. Haemorrh. 9, 554, 1963.

Beck E.A., Characke P. and Jackson D.P.

A new inherited coagulation disorder caused by an abnormal fibrinogen (Fibrinogen Baltimore).

Nature : (London) 201, 143, 1965.

Boinert, H., Von Korff, R.W. and Green, D.E.

Unpublished : (A method of purification of co-enzyme A).

Quoted from J. Am. Chem. Soc. 74, 854, 1952.

Bettelheim F.R. and Bailey K.

The products of the action of thrombin on fibrinogen.

Biochemica et. Biophysica Acta : 9, 578, 1952.

Ball W.H. and Alton, H.G.

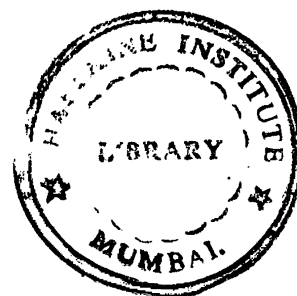
A brain - extract as a substitute for platelet suspensions in thromboplastin generation test.

Nature (London) : 174, 880, 1954.

Bettelheim F.R.

The clotting of fibrinogen II fractionation of the peptide liberated.

Biochemica et. Biophysica acta : 19, 121, 1956.



Bergna, Luis Jose.

A simple method for the assay of factor VIII.

Blood : 15, 637, 1960.

Berg R., Bjorling H., Bernatson K. and Bopmark A.

Recovery of Australia antigen from human plasma products separated by a modified Cohn fractionation.

Vox Sang. : 22, 1, 1972.

Berglund G.

Studies of the inhibitory activity of specific antisera to some clotting factors in human plasma.

Brit. J. Haematol. : 8, 204, 1962.

Berglund G.

Immunological studies of haemophilic plasma.

Int. Arch. Allergy and applied Immunology : 22, 1, 1963.

Biggs R.

Prothrombin deficiency.

Blackwell Scientific Publications, Oxford, 1951.

✓ Biggs R., Douglas A.S., Macfarlane R.G., Race J.V.,

Pitney W.R., Hershey C. and O'Brien J.R.

Christmas disease a condition previously mistaken for haemophilia.

Brit. Med. J. : 2, 1378, 1952.

Biggs R. and Douglas A.S.

The thromboplastin generation test.

J. Clin. Pathol. : 1, 15, 1953.

Biggs R., Eveling and Richards G.

The assay of antihæmophilic globulin activity.

Brit. J. Haematol. : 1, 20, 1955.

2 ✓ Biggs R. and Macfarlane R.G.

Normal values of antihæmophilic factor in human plasma.

Human blood coagulation and its disorder 3rd Edition,

1962, p. 73.

Blomback, B. and Blomback M.

Purification of human and bovine fibrinogen.

Arkiv for Kemi. : 10, 415, 1956.

Quoted from Annals of N.Y.Acad. of Sc. : 202, 77, 1972.

Blomback B.

The fibrinopeptides.

Thromb. et. diath. haemorrh. suppl. 20, 201, 1966.

Blomback B., Blomback M., Edman P. and Hessel B.

Human fibrinopeptides isolation, characterization and structure.

Biochemica^{et} Biophysica Acta : 115, 371, 1966.

Blomback M.

Purification of antihæmophilic globulin. I. Some studies on the stability of antihæmophilic globulin activity in fraction 10 and a method for purification for its partial separation from fibrinogen.

Arkiv for Kemi. : 12, 387, 1958.

Blomback B. and Vestermark A.

Isolation of fibrinopeptides by chromatography.

Arkiv for Kemi. : 12, 173, 1958.

Blomback B. and Yamashita I.

On the N-terminal aminoacids in fibrinogen and fibrin.

Arkiv for Kemi. : 12, 299, 1958.

Quoted from N.Y. Acad. Sci. : 202, 77, 1972.

Blomback B., Blomback M., Henschen A., Hessel B., Iwanga S. and Woods K.R.

N-terminal disulphide knot of human fibrinogen.

Nature (London) : 218, 130, 1968.

Blomback M., Blomback B., Hammon E.P., and Prasad A.S.

Fibrinogen Defect a molecular defect in the N-terminal disulphide knot of human fibrinogen.

Nature (London) : 218, 134, 1968.



Blomback B. and Blomback M. (1969).

The formation of the fibrin clot from fibrinogen.
In : Human blood coagulation.

Biochemistry Clinical Investigation and therapy.

(eds. Henker H.C., Lecliger E.A. and Velkamp J.J.)

Leiden University Press.

Roman H.G.

Chromatography of serum and some other proteins on
an anion exchange resin.

Nature : 175, 898, 1955.

Bordet J. and Delang L.

La coagulation du sang et. la genese de la thrombine.

Ann. Inst. Pasteur : 26, 655, 1912.

Bordet J. and Delang L.

Analyse et. synthese du processus de la coagulation.

Ann. Soc. Sci. Med. Nat. Brux. : 72, 87, 1914.

Bordet J.

Considerations sur les theories de la coagulation
du sang.

Ann. Inst. Pasteur : 34, 561, 1920.

Brandt N.J., Cohn J., Hilden M.

Controlled trial of oral contraceptives in haemophilia.

Scand. J. Haematol. : 11, 225, 1973.

Britten A. and Grove - Rasmussen M.

Stability of factor VIII in the frozen state.

Transfusion : 6, 230, 1966.

Brittin G.M., Rafinia H., Royal D., Werner M., Brown B.

Evaluation of single Radial Immunodiffusion for
quantitation of plasma fibrinogen.

Amer. J. Clin. Path. : 57, (1), 89, 1972.

5 ✓

Brown D.L., Hardisty R.M., Kosey M.H., Garolo Bracken.

Antihaemophilic globulin: Preparation by an improved cryoprecipitation method and clinical use.

Brit. Med. J. : 2, 79, 1967.

Bulloch W., and Fildes P. (1911).

Treasury of human inheritance parts V and VI section XIV a.

Haemophilia, Dulau & Co. Limited, London.

Caspary E.A. and Kekwick R.A.

Some physicochemical properties of human fibrinogen.

Biochem. J. : 67, 41, 1957.

Chan K.B., Rizza G.R. and Henderson M.P.

A study of the coagulant properties of Malayan pit Viper Venom.

Brit. J. Haematol. : II, 646, 1965.

Chick R. and Martin C.J.

The precipitation of egg-albumin by ammonium sulphate. A contribution to the theory of the "Salting out" of proteins.

Biochem. J. : 7, 380, 1913.

Clegg J.B. and Bailey K.

The separation and isolation of the peptide chains of fibrinogen.

Biochimica et. biophysica acta : 67, 525, 1962.

Cohn E.J., McNeekin, T.L., Oncley J.L., Newell, J.M. and Hughes W.L.JR.

Preparation and properties of serum and plasma proteins. I size and change of proteins separating upon equilibration across membranes with ammonium sulphate solutions of controlled pH, ionic strength and temperature.

J. Amer. Chem. Soc. : 62, 3386, 1940.



Cohn E.J.

Properties and functions of plasma proteins with consideration of methods for their separation and purification.

Chem. Revs. : 23, 395, 1941.

7 ✓ Cohn E.J., Strong L.E., Hughes W.L., Mulford D.J., Ashworth J.N., Melin M. and Taylor H.L.

A system for the separation into fractions of the proteins, lipoprotein components, biological tissue fluids.

J. Amer. Chem. Soc. : 68, 459, 1946.

Collingwood B.J. and Macfiehan M.T.

The anticoagulants in blood and serum.

J. Physiol. : 45, 119, 1912.

Combrissan A., Vergoz D.,

Champcommunal A. et. Andre R.

Propriétés de facteur antihémophilique VIII concentré a usage thérapeutique la chypoprotéine VIII.

Transfusion (Paris) : 2, 303, 1966.

Coolidge T.B.

A simple cataphoresis apparatus.

J. Biochem. : 127, 551, 1939.

Crowell E.B. Jr., Clatanoff D.V. and Kiekhoffer W.

The effect of oral contraceptives on factor VIII levels.

J. Lab. & Clin. Med. : 77, 551, 1971.

Dausset J.

Les anticoagulants circulants sont-ils des anticorps.

Sang. biologique et. Pathologie : 30, 428, 1959.

Denson K.W.E., Biggs R., Haddon M.E., Barrett R. and Cobb K.

Two types of Haemophilia (A⁺ and A⁻). A study of 48 cases.

Brit. J. of Haemat. : 17, 163, 1969.

Dodds, W.J.

Storage, release and synthesis of coagulation factors
in isolated perfused organs.

Amer.J. Physiol. : 217, 879, 1969.

Eagle H.

Studies on blood coagulation.I. The role of prothrombin
and of platelets in the formation of thrombin.

J. Gen. Physiol. : 18, 531, 1935.

Eagle, H. and Harris T.H.

Studies in blood coagulation.V. The coagulation of
blood by proteolytic enzymes, (trypsin, papain).

J. Gen. Physiol. : 20, 543, 1937.

Ellis D. (1969).

Personal communication.

Quoted from human blood coagulation, haemostasis and
thrombosis edited by Rosemary Biggs.

Blackwell Scientific Publications 1972.Oxford, London,
Edinburgh, Melbourne.

Endres G.P. and Shoraga H.A.

Equilibria in the fibrinogen - fibrin conversion VIII
polymerization of acceptor modified fibrin monomer.

Biochemistry : 7, 4219, 1968.

Fanconi G.

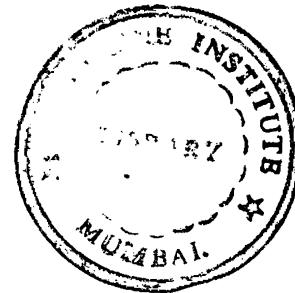
Fibrinaemia als ursache einer Schwere haemorrhagische
Diathese bei luis congenita.

Schweizerische medizinische Wochenschrift : 22, 255, 1944.

Farrell G.W. and Wolf P.

A batch method for estimating thrombin clottable
fibrinogen.

J. Clin. Pathol. : 26, 764, 1973.



Finlayson J.S. and Mosseson M.W.

Heterogeneity of human fibrinogen.

Biochemistry : 2, 42, 1963.

Fischer A.

Über die Identität des Lachet- und Blutthrombosin.

Biochem. Z. : 270, 275, 1934.

Quoted from human blood coagulation and disorders by
Biggs R. and Macfarlane R.G. (1962).

Fleischman J.B., Pain R.H. and Porter R.R.

Reduction of α -globulins.

Arch. Biochem. Suppl. : 1, 174, 1962.

Forman, U.B., Ratneff O.D. and Boyer M.H.

An inherited qualitative abnormality in plasma
fibrinogen: Fibrinogen "Cleveland".

J. Lab. & Clin. Med. : 72, 455, 1968.

Fold E. and Spiro K.

Beitr. Chem. Physiol. U. Path. 5, 171, 1904.

Ferguson J.H. (1949).

The present status of the platelets in coagulation
by A.J. Quick. Discussion and Table. In blood clotting
and Allied problems, 2nd Conference Josiah Macy
foundation, New York, as quoted by Biggs R. and
Macfarlane R.G. (1962) human blood coagulation and its
disorders 3rd ed. Blackwell scientific publications.

Gaffney P.J.

Heterogeneity of human fibrinogen.

Nature (New Biol.) : 230, 54, 1971.



Giri K.V.

Agar electrophoresis of serum proteins on cellophane and polyester films.

J. Lab. Clin. Med. : 48, 775, 1956.

✓ Gittlin D. and Berges W.H.

Studies on metabolism of fibrinogen in two patients with congenital afibrinogenemia.

Blood : 2, 679, 1953.

Gladner J.A. and Laki K.

The inhibition of thrombin by diisopropyl fluoridate.

Arch. of Biochem. & Biophys. : 52, 501, 1956.

Gladner J.A., Folk J.E., Laki K. and Carroll W.R.

Thrombin induced formation of co-fibrin.

J. Biol. Chem. : 232, 62, 1959.

Gedol H.C. and Kierulff P.

Thrombin clotting time a more sensitive indicator of denatured fibrinogen than clottability.

Hæmostasis : 1, 44, 1972.

Gordon A.H., Koll D., Sobotta K., Knosset O., Sorm, P.

Electrophoresis in agar gel collection of Czech Chem.

Comm. 15, 1, 1950.

Quoted from methods of Biochem. Analysis VII-1, 1959.

Grabar P. and William C.A.

Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Application au serum sanguin.

Biochem. et. Biophys. Acta : 10, 193, 1953.

432

Grabar P. and William G.A.

Methode immuno-electrophoretique d'analyse de
melanges de substance antigeniques.

Biochem et. Biophys. Acta.: 17, 67, 1955.

Graham J.B., Collins D.L. Jr., Godwin I.O. and Brinkhouse K.H.

Assay of plasma antihæmophilic activity in normal
heterozygous (hæmophilia) and prothrombinopenic dogs.

Proc. Soc. Expt. Biol. Med.: 77, 294, 1951.

Grabar Pierre

Immuno-electrophoretic analysis.

Methods of Biochem analysis VII, 1, 1959.

Grassol E., Bonifas V. and Pongratz E.

Rapid slide precipitin microreaction of poliomyelitis
antigen and antisera in agar.

Proc. Soc. Exp. Bio. Med. : 77, 72, 1958.

19 ✓ Grubbelaar, B.G. (1973).

The organisation of blood transfusion services.

Hæmophilia, page 95.

Proceedings of the VII congress of the world federation
of Hæmophilia May, 1971, Tehran.

Hall C.E., and Slayter H.S. (1962).

In : Proceedings of the Fifth International Congress
for Electron Microscopy.

(ed. Breese S.R.Jr.) : 2, 0-3, New York, Academic Press.

Hall C.E.

Electron microscopy of the fibrinogen molecule and
fibrin clot.

Laboratory Investigation : 12, 998, 1965.

Hardy, W.B. and Gardiner S.

J. Physiol.: 40, LXVIII, 1910.



Hardisty R.H. and Pinniger J.L.

Congenital afibrinogenemia further observations on the blood coagulation mechanism.

Brit. J. Haematol. : 2, 139, 1956.

Haschenmeyer A.E.V. and Tonoso I.J.R.

Charge distribution of fibrinogen as determined by transient electric birefringence studies.

Biochemistry : 1, 996, 1962.

Haschenmeyer A.E.V.

A polar intermediate in the conversion of fibrinogen to fibrin monomer.

Biochemistry : 2, 851, 1963.

Haschenmeyer, R.H. and Madenau, R.H.

Molecular structure of fibrinogen.

Biochem. and Biophys. Res. Commun. 11, 217, 1963.

Hammersten O.

Über das Fibrinogen.

Arch. Ges. Physiol. (Pflügers) : 19, 563, 1879.

Hammersten O.

The plasma proteins and other fractionation (Codium Chloride Method).

Arch. Ges. Physiol. (Pflügers) : 20, 431, 1880.

Haugard, C. and Kroner, T.D.

Partition chromatography of amino acids with applied Voltage.

J. Am. Chem. Soc. : 70, 2135, 1948.

Henschen A.

S-Sulfo-derivatives of fibrinogen and fibrin.

Preparation and general properties.

Arkiv for Kem. : 22, 1, 1964a.

Quoted from Human Blood Coagulation Haemostasis and Thrombosis page 40 Ed. by Rosemary Biggs, 1972 Edition.

Henschen A.

Number and reactivity of disulfide bonds in fibrinogen and fibrin.

Arkiv for Kemi.: 22, 355, 1964b.

Quoted from human blood coagulation, haemostasis and thrombosis page 40.

Henschen A.

Peptide chains in S-sulfo-fibrinogen and S-sulfo-fibrin isolation methods and general properties.

Arkiv for Kemi.: 22, 375, 1964c.

Hershgold E.J. and Sprawlo S.

Molecular properties of purified human, bovine and porcine antihæmophilic globulin (AHG).

Fed. Proc.: 25, 317, 1966.

Hershgold, E.J., Davison A.H. and Jansson, M.B.,

Isolation and some chemical properties of human factor VIII.

J. Lab. Clin. Med.: 77, 185, 1971.

Houder (1902).

Quoted from 'Antiseptic': 57, 960, 1960.

Hopff F. (1828).

Ueber die hæmophilie oder die erbliche Anlage zu tödtlichen Blutungen Innogeral - Dissertation Wurzburg.

Quoted Bulloch and Fildes 1911.

Quoted from human blood coagulation and its disorders by Biggs R. and Macfarlane R.G. (1962).

Howe, P.E.

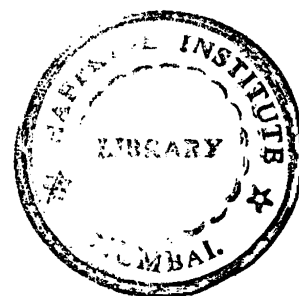
The determination of proteins in blood.: A micromethod.

J. Biol. Chem.: 42, 109, 1921.

Howell W.H. and Holt E.

Two new factors in blood coagulation Heparin and preantithrombin.

Amer. J. Physiol.: 47, 328, 1918.



✓ Howell W.H.

Theories of blood coagulation.

Physiol. Rev.: 15, 435, 1935.

Howell W.H. and Mellanby J.

Heparin and blood coagulation.

Proc. Roy Soc. Lond. B. : 116, 1, 1935.

Hoyer L.W. and Breckenridge R.T.

Immunologic studies of antihæmophilic factor (AHF,

Factor VIII) cross reacting material.

Blood : 32, 962, 1968.

✓ Hoyer L.W.

Immunologic studies of antihæmophilic factor (AHF, Factor VIII): III comparative binding properties of human and rabbit anti-AHF.

Blood : 39, (4), 481, 1972.

Ingram G.I.O.

Variations in the reactions between thrombin and fibrinogen and their effect on prothrombin time.

J. Clin. Pathol.: 8, 318, 1955.

✓ Jaques L.B.

Reaction of heparin with proteins and complex bases.

Biochem. J. : 27, 189, 1943.

Johnson P. and Mihalyi E.

Physicochemical studies of bovine fibrinogen.

Biochimica et. biophysica acta : 102, 467, 1965.

Johnson A.J., Newman J., Howell M.B. and Puskin S. (1966).

The preparation and some properties of a clinically useful high purity antihæmophilic factor (AHF).

XIth Congress of the International Society of Blood

Transfusion Abstracts of papers Sydney, Australia P.1109.

Johnson A.J., Karpaticin M.H. and Newman J. (1970).

Purification and clinical evaluation of a factor VIII concentrates.

Haemophilia and his world.

Proc. of 5th Congress of the world Federation of Haemophilia Montreal, 1968.

Bibliotheca Haematologica : 34, 30, Karger Basel, New York.

✓ Johnson A.J., Karpaticin M.H. and Newman J.

Preparation and clinical experience with antihæmophilic concentrates.

Thromb. et. diath. hæmorrhag. suppl. : 35, 49, 1969.

Kass, L., Ratnoff, O.D. and Loon M.A.

Studies on the purification of antihæmophilic factor (Factor VIII) I. preparation of antihæmophilic factor by Concanavalin A.

J. Clin. Invest. : 48, 351, 1969.

Kekwick R.A.

The serum proteins in multiple myelomatosis.

Biochem. J. : 34, 1248, 1940.

Kekwick R.A., Mackay, H.E. and Record, B.R.

Fractionation of human plasma with ether.

Nature (London) : 157, 629, 1946.

Kekwick R.A.

The purification of fibrinogen.

Thromb. et. diath. hæmorrhag. suppl. 13, 21, 1964.

✓ Kekwick R.A. and Mackay H.E.

The separation of protein fraction from human plasma with ether.

Special Report series Medical Research Council.

London : 286, 1954.

Kendy F.J., Lorand L. and Miller K.D.

Titration of active centers in thrombin solution standardization of the enzyme.

Biochemistry : 4, 2302, 1965.

Kunkel H.G.

Zone Electrophoresis.

Methods of Biochemical Analysis, New York : 1, 142, 1954.

Landaburu R.H. and Soegers W.H.

The acetylation of thrombin.

Canadian J. of Biochem. and Physiol. : 37, 1361, 1959.

Lano S.

Haemorrhagic diathesis successful transfusion of blood.

Lancet : 1, 185, 1940.

Langdell R.D., Wagner R.H. and Brinkhous K.M.

Effects of antihæmophilic factor on one stage clotting test.

J. Lab. Clin. Med. : 41, 631, 1953.

Legas M., Schmer G. and Davie E.

Characterization of human factor VIII (antihæmophilic factor).

Circulation : 46, Suppl. II, 206, 1972.

Lewis, J.H. Walters D., Didichon P. and Merchant V.R.

Application of continuous flow electrophoresis to the study of the blood coagulation proteins and the fibrinolytic enzyme system. I. normal human materials.

J. Clin. Invest. : 37, 1323, 1953.

Lorand L.

Fibrino peptide.

Biochem. J. : 52, 200, 1952.

Macfarlane R.G.

A boy with no fibrinogen.

Lancet. : 1, 30, 1938.

✓ Ladden, R.E. and Gould, R.G.

The turnover rate of plasma fibrinogen.

Ped. Proc. II, 252, 1952.

Mammen, E.F., Prasad A.S. and Au C.C.

Studies on a family with dysfibrinogenemia.

Proc. 16th Annual Symposium on Blood. College of
Medicine Wayne, State University. January 19-20,
p. 24, 1968.

Mammen E.F., Prasad A.S., Barnhart, H.J. and Au C.C.

Congenital dysfibrinogenemia. : Fibrinogen Detroit.
J. Clin. Invest. : 48, 235, 1969.

Mancini G., Carbonaro A.O. and Heremans J.F.

Immunochemical quantitation of antigen by single
radial immunodiffusion.

Immunochemistry : 2, 235, 1965.

Magnusson S.

Thrombin and prothrombin.

In : The enzymes Vol. III, 3rd Edition.

Ed. P.D. Boyer, Academic Press, 1971.

Merchosi S.G., Shulman H.R. and Gralnick H.R.

Studies on the purification and characterization of
human factor VIII.

J. Clin. Invest. : 51, 2151, 1972.

Marshall R. and Enoch M.P.

The effect of Ancistrodon rhodostoma venom in the dog.
Clin. Sci. : 32, 251, 1968.

Mekoe P.A.

Purification and electrophoretic analysis of human
antihaemophilic factor (factor VIII).

Fed. Proc. : 29, 647, 1970.

McLean J.

The thromboplastic action of cephalin.

Amer. J. Physiol. : 41, 250, 1916.

Melnicuk V. A.

The Royal haemophilia.

Sci. Am. : 213, 88, 1966.

Hollanby J.

Diphtheria antitoxin.

Proc. Roy. Soc. Lond. (B) : 80, 399, 1908.

Hollanby J.

The coagulation of blood.

J. Physiol. : 38, 28, 411, 1909.

Hollanby J.

Prothrombasc - Its preparation and properties.

Proc. Roy. Soc. Lond. (B), : 107, 271, 1930.

Monacho D.

Constitutional and familial abnormal fibrinogen.

In fibrinogen and fibrin turnover of clotting factors.

Thromb. et. diath. haemorrhagica suppl. 13, 173, 1964.

Mills G.A. and Guest G.M.

The role of tissue fibrinogen (thrombokinase) in
fibrin formation normal clotting.

Amer. J. Physiol. : 57, 305, 1921.

Mills D. and Karpatkin S.

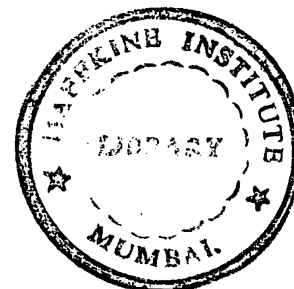
Heterogeneity of human fibrinogen : Possible relation
to proteolysis by thrombin and plasmin as studied by
S.D.S. Polyacrylamide gel electrophoresis.

Biochem. Biophys. Res. Commun. : 40, 206, 1970.

Minnot G.R., Davidson G.S., Lewis J.H., Tagnon H.J. and
Taylor F.H.L.

The coagulation defect in haemophilia, the effect in
haemophilia at the parenteral administration of a
fraction of the plasma globulins rich in fibrinogen.

J. Clin. Invest. 24, 704, 1945.





17 ✓ Moravits P.

Ergebn Physiol. 4, 307, 1905.

Quoted from "Antiseptic" Vol: 57, 1960.

Morrison P.R., Edsall J.T. and Miller S.O.

Preparation and properties of serum and plasma proteins XVIII. The separation of purified fibrinogen from fraction I of human plasma.

J. Amer. Chem. Soc. : 70, 3103, 1948.

Mosseson M.W., Alkjaersig N., Sweet B. and Sherry S.

Human fibrinogen of relatively high solubility comparative biophysical, biochemical and biological studies with fibrinogen of lower solubility.

Biochemistry : 6, 3279, 1967.

Mosseson M.W. and Unfloot R.A.

The cold insoluble globulin of human plasma.

J. Biol. Chem. : 245, 5728, 1970.

Mosseson M.W., Galanakis D., Budzynski, A. and Horder (1971).

Comparison of sub-unit structure of human fibrinogen fractions I₂ and I₃ with those derived by limited plasmin degradation of fraction I₄.

In Second Congress of International Soc. Thrombosis and haemostasis (Abstract 160) Oslo, Norway.

Mutt V., Hagmisson S., Jorpes J.E. and Dahl E.

Structure of porcine secretin I-degradation with trypsin and thrombin sequence of tryptic peptides.

The C-terminal residue. Biochemistry : 4, 2358, 1965.

18 ✓ Sahas, L., Denson, K.W.E. and Macfarlane, R.G.

A study of the coagulant action of eight snake venoms. Thrombosis et diathesis haemorrhagica : 12, 355, 1964.



Masse G.P.

Von einer erblichen Neigung Zu tödtlichen.
Blutungen.

Arch. Med. Erfahr. : 1, 385, 1820.

As quoted by Wintrobe H.H. in Clinical Haematology
6th ed. p. 936, 1967.

20 ✓ Neurath H., John E. Dees and Hilda Fox.

Preparation and properties of human fibrinogen
solutions.

J. Urology : 49, 497, 1943.

Nitschmann H.S., Kistler P. and Joss A.

Dried fraction I for clinical use from smallest
plasma pools, sterile without filtration.

Vox Sang. : 2, 100, 1957.

Nitschmann H., Rickli E. and Kistler P.

Fractionation of human plasma with polyphosphate.

Vox Sang. : 5, 232, 1960.

Helf P.

Contribution à l'étude de la coagulation du Sang Les
facteurs primordiaux leur origine.

Arch. internationales de physiologie : 6, 1, 1908a.

As quoted by Biggs and Macfarlane (1962), human blood
coagulation and its disorders 3rd Edn.

21 ✓ Helf P.

Contribution à l'étude de la coagulation du Sang La
fibrinolyse.

Arch. Int. Physiol. : 6, 306, 1908b.

Helf P.

The blood plasma in health and disease monograph of
medical and surgical science Heinemann London.

Quoted by Pickering J.W. 1928.

Lancet. : 2, 695, 1928.

Korman J.C., Lambillotte J.P., Kojima Y. and Sise H.S.

Antihæmophilic factor release by perfused liver and spleen relationship to hæmophilia.

Science : 152, 1060, 1967.

Otto J.C.

An account of an hæmorrhagic disposition existing in certain families.

Med. Reports : 6, 1, 1803.

Quoted from Physiol. Review : 54 (1), 23, 1974.

Owren P.A.

The coagulation of blood investigations of new clotting factors.

Acta Med. Scand. Suppl. : 19, 4, 1947.

28 ✓ Patch A.J. Jr. and Stetson R.P.

Hæmophilia I. The abnormal coagulation of blood and the relation of platelets.

J. Clin. Invest. : 15, 531, 1936.

23 ✓ Patch A.J. Jr. and Taylor F.H.L.

Hæmophilia II. Some properties of a substance obtained from normal plasma effective in accelerating the clotting of hæmophilic blood.

J. Clin. Invest. : 16, 113, 1937.

Ponick G.D.

Some factors that influence utilization of antihæmophilic activity during clotting.

Proc. Soc. Expt. Biol. Med. : 96, 277, 1957.

Pinniger J.L. and Prunty F.T.G.

Some observations on the blood clotting mechanism.

The role of fibrinogen and platelets with reference to a case of congenital afibrinogenæmia.

Brit. J. Exp. Pathol. : 27, 200, 1946.

Peterson E.A. and Sobor H.A.

Chromatography of proteins. I. cellulose ion exchange
adsorbents.

J. Amer. Chem. Soc. : 78, 751, 1956.

Pillemer L.

Recent advances in Chemistry of complement.

Chem. Revs. : 33, 1, 1943.

Piper V., Schreir H.H.

Über den immunologischen Nachweis Von Faktor-B Protein
in Blutor plasma and seine Bedeutung für das Verständnis
der Haemophilie A.

Thromb. Diath. Haemorrh. : 11, 423, 1964.

Polson A., Patgoiter G.H., Largier J.F., Hears, G.E.F.
and Jacobert F.J.

The fractionation of protein mixtures by linear
polymers of high molecular weight.

Biochimica et. Biophysica acta. : 32, 463, 1964.

23 ✓ Polson A. and Rues Bravo C.

Fractionation of plasma with polyethylene glycol.

Vox. Sang. : 23, 107, 1972.

24 ✓ Pool J.G. and Shanon A.E.

Production of high potency concentrates of AHG in a
closed bag system.

New Eng. J. Med. : 273, 1443, 1965.

Porath J. and Flodin P.

Gel filtration : A method for desalting and group
separation.

Nature (London) : 183, 1657, 1959.

Porath J.

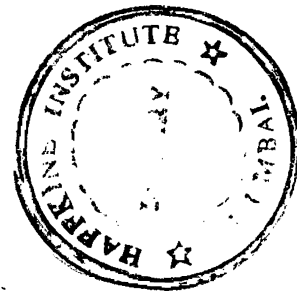
Gel filtration of proteins, peptides and amino acids.

Biochem. et. Biophys. Acta. : 39, 193, 1960.

Prentice A.I.D.

A case of congenital afibrinogenemia.

Lancet : II, 211, 1951.



✓ Preston A.E.

The factor VIII activity in fresh and stored plasma.

Brit. J. Haemat. : 13, 42, 1967.

Quick A.J.

The normal antithrombin of the blood and its relation to heparin.

Amer. J. Physiol. : 123, 712, 1958.

Rasmussen P.G.

Purification of thrombin by chromatography.

Biochemica et Biophysica acta, : 16, 157, 1955.

Ratnoff, O.D., Botti R.E., Brockenridge R.T. and Little A.S.

Some problems in the measurement of antihemophilic activity.

In Brinkhaus K.H. (Ed.), The Hemophilias. Chapel

Hill University of North Carolina Press, 1964, p.3.

Reid H.A., Chan K.E. and Thuan P.C.

Prolonged coagulation defect (Defibrination syndrome) in Malayan Viper bite.

Lancet : 1, 621, 1963.

Reid H.A.

Defibrination by Agkistrodon rhodostoma venom.

In animal toxin.

(Ed. Russel P.E. & Saunders P.E.) 323, 1967 Pergamon press, Oxford and New York.

Regoeczi E.

On the dual action of Agkistrodon rhodostoma venom in fibrinogen.

Zeitschrift für Tropen medizin und Parasitologie : 17, 144, 1966.

Regoeczi E., Gergely J. and Macfarlane A.S.

In vivo effects of Agkistrodon rhodostoma venom studies with fibrinogen 131^I.

J. Clin. Invest. 45, 1202, 1966.

Rinze, C.R. and Walker V.

Inactivation of antihæmophilic globulin by thrombin.

Nature : 180, 143, 1957.

Rosner, F.

Hæmophilia in the Talmud and rabbinic writings.

Ann. Int. Med.: 70, 833, 1969.

Rosenthal R.L. and Sloan E.

Elevated factor VIII (AHF) activity in acute leukemia.

Fed. Proc. : 26, 487, 1967.

Schmer G.E.P., Korby D.C., Teller and Davis E.W.

The isolation and characterisation of bovine factor VIII (antihæmophilic factor).

J. Biol. Chem. : 247, 2512, 1972.

28 ✓ Schmidt A.G.

Ueber den Faserstoff und die Ursachen seiner Gerinnung.

Arch. Anat. Physiol.: 1p2, p. 545, 1861.

Quoted by Biggs R. and Macfarlane R.G. (1962).

29 ✓ Schmidt A.G. (1892)

Quoted from Morawitz (1905).

Die chemie der Blutgerinnung.

Ergebn Physiol.: 4, 307, 1905.

Schulze H.E. and Heremans J.F.

Molecular Biology of human proteins.

Nature and Metabolism of Extra cellular proteins.

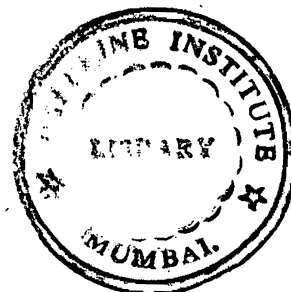
Amsterdam Elsevier I, 474, 1966.

Seegers W.H. and Landaburu R.H.

Purification of prothrombin and thrombin by chromatography on cellulose.

Canadian J. of Biochem. & Physiol.: 38, 1405, 1960.

432



Shanbrom E.

In discussion during the symposium on optimal use of human blood following the paper by verstrato.

Vox Sang. : 16, 382, 1969.

Shanberg, J.H. and Goro J.

Studies on the immunologic and physiologic activities of antihæmophilic factor (AHF).

J.Lab. Clin. Med. : 50, 945, 1957.

Sherga H.A. and Laskowski M.

The fibrinogen and fibrin conversion.

Advances in Protein Chemistry : 12, 1, 1957.

Shulman S.

The size and shape of bovine fibrinogen studies of sedimentation, diffusion and Viscosity.

J. Amer. Chem. Soc. : 75, 2846, 1953.

Shulman S. and Keesberg F.

Electron Microscope observations on intermediate polymers in conversion of fibrinogen to fibrin.

J. Biol. Chem. : 200, 293, 1953.

Shulman S. Landaburu R.H. and Seegers W.H.

Biophysical studies on platelets Cofactor I.

Thromb. Diath. Haemorrh. : 4, 336, 1960.

Simmonetti G., Casillas G. and Pavlovsky A. (1964).

Tannic acid purification of Factor VIII.

The hæmophilias (ed. Brinkhaus K.M.).

University of North Carolina Press, Chapel Hill.

Smiths G.

Zone electrophoresis in starch gels : Group variations in the serum proteins of normal human adults.

Biochem. J. : 61, 629, 1955.

Stein W.H. and Moore S.

Chromatography.

Ann. Revs. Biochem. : 21, 521, 1952.

Sorenson, S.P.L. (1925)

The plasma proteins and their fractionation.

Proteins, New York.

Quoted from Advances in Protein Chemistry,

Vol. III, 435, 1947.

Sorenson S.P.L. (1930)

The plasma proteins and their fractionation.

Quoted from "Advances in Protein Chemistry".

Acad. Press New York Vol. III, 435, 1947.

Stuber B. and Lang K. (1930)

Quoted from Howell W.H. (1935).

Theories of Blood Coagulation.

Physiol. Revs. : 15, 435, 1935.

Surgenor D.M. McMillan C.W., Diamond L.K. and Steele B.B.

Studies with AHF rich fibrinogen in classical hemophilia.

Vox Sang. : 5, 80, 1960.

Tegnon H.J., Davidson C.S. and Taylor P.H.L.

Studies on blood coagulation. A proteolytic enzyme prepared from calcium and platelet free normal human blood plasma.

J. Clin. Invest. : 21, 525, 1942.

Tegnon H.J.

The significance of fibrinolysis in mechanism of coagulation of blood.

J. Lab. Clin. Med. : 27, 1119, 1942.

Thorp J.M. (1967)

Personal Communication.

Quoted from J. Clin. Pathol. : 26, 764, 1973.

Tiselius A. and Flodin P.

Zone Electrophoresis.

Advances in Protein Chemistry. Acad. Press
New York : B, 461, 1953.

Tiselius A.

Electrophoresis of serum globulin. Electrophoretic
analysis of normal and immune sera.

Biochem. J. : 31, 1464, 1937.

Tocantins L.H.

Cephalin, Protamin and the antithromboplastic
activity of normal and haemophilic plasmas.

Proc. Soc. Exp. Bio. Med. N.Y. : 54, 94, 1943a.

Tocantins L.H.

Demonstration of antithromboplastic activity in normal
and haemophilic plasmas.

Amer. J. Physiol. : 139, 265, 1943b.

Tocantins L.H.

Anticephalin activity and prothrombin conversion
rate of normal and haemophilic plasmas.

Proc. Soc. Exp. Bio. Med. N.Y. : 55, 291, 1944a.

Tocantins L.H.

Estimation of the anticephalin activity of whole
blood.

Proc. Soc. Exp. Bio. Med. N.Y. : 57, 211, 1944b.

Tocantins L.H.

Influence of the contacting surface on the coagulability
and anticephalin activity of normal and haemophilic
plasma.

Amer. J. Physiol. : 143, 67, 1945.

Tocantins L.H. and Carroll R.T. (1949a)

Separation and assay of a lipid antithromboplastin from human brain blood plasma and plasma fractions. In blood clotting and allied problems, 2nd Conference Josiah Macy Foundation, New York, P. 11.

Tocantins L.H. and Carroll R.T.

Coagulation de-accelerating action of haemophilic plasma on normal plasma.

Fed. Proc. : 8, 157, 1949b.

Uszynski L.

Immunologic properties of factor VIII. I. studies on the inhibitory activity of specific antisera to human antihæmophilic globulin.

Thrombosis et. diathesis hæmorrhagica: 16, 559, 1966.

Von Felten A., Duckert P. and Friek P.G.

Familial disturbance of fibrin monomer aggregation.

Brit. J. Haematol. : 12, 667, 1966.

Wagner R.H., Molster W.D., Smith H. and Brinkhaus K.M.

Purification of antihæmophilic factor (factor VIII) by amino acid precipitation.

Thrombosis et. diathesis hæmorrhagica: II, 64, 1964.

Ware A.G., Guest M.H. and Seegers W.H.

Determination of fibrinogen in plasma by applying the Biuret reaction to the fibrin clot.

Arch. Biochem. : 13, 231, 1947.

Quoted from Clinical Chemistry, Principles and Techniques page 228, by R.J. Henry.

Weaver R.A., Gabriel D.A. and Langdell R.D.

Concentrated antihæmophilic factor from outdated blood. Transfusion : 7, 168, 1967.

Webster W.P., Roberto H.R., Tholin G.M., Wagner R.H.
and Brinichous K.M.

Clinical use of a new glycine precipitated
antihaemophilic fraction.

J. Med. Sci. : 250, 643, 1965.

Webster W.P., Roddick, R.L., Roberto H.R. and Ponick G.B.

Release of factor VIII (antihaemophilic factor) from
perfused organs and tissues.

Nature : 213, 1146, 1967.

Wooldridge L.O.

Further observation on the coagulation of the blood.

J. Physiol. : 4, 226, 1883.

As quoted by Biggs R. and Macfarlane R.G. (1962).

Wooldridge L.O.

The coagulation question.

J. Physiol. : 10, 329, 1889.

As quoted by Biggs R. and Macfarlane R.G. (1962).

Wright A.E.

On the method of determining the condition of blood
coagulability for clinical and experimental purposes
and on the effect of the administration of calcium
salts in haemophilia and actual or threatened haemorrhage.

Brit. Med. J. : 2, 223, 1893.

Quoted from Physiological Review Vol.: 54, P. 23, 1974.

Wright I.S.

Nomenclature of blood clotting factors.

Thromb. Diath. Haemorrhagica : 2, 435, 1959.

Quoted from Physiological Review Vol.: 54, P. 23, 1974.

✓ Zimmerman T.S., Ratnoff O.D. and Powell A.E.

Immunologic differentiation of classic haemophilia
(factor VIII deficiency) and Von Willebrand's disease.

J. Clin. Invest. : 50, 244, 1971.

S Y N O P S I S

Name of the candidate : P.R. Dichwalkar, M.Sc.
Degree : Ph.D. in Applied Biology
Guide : Dr. H.V. Sant, M.D., F.C.P.S.,
D.E. (Prague)
Place of work : Haffkine Institute, Parel,
Bombay - 400 012
Title : "Biochemical and immunological
study of human fibrinogen and
cryoprecipitate (AHF, Factor VIII)".

Blood clotting results from the conversion of a soluble plasma protein, fibrinogen (factor I) into fibrin, an insoluble network of fibres. The jolly-like appearance of blood clots is due to the entrapment of cells and serum within the meshes of network. The formation of fibrin is catalyzed physiologically by a hydrolytic plasma enzyme, thrombin. Thrombin is present in the form of prothrombin. Recognition of part played by calcium led to the development of the classical theory of the coagulation mechanism (Morawitz 1905), in which four factors were regarded as necessary thromboplastin, calcium, prothrombin and fibrinogen.

In modern concept of coagulation blood clotting is conceived as a dynamic process in which positive forces leading to coagulation are antagonised by negative contrary forces the latter including natural anticoagulants and agents which remove formed clot (Howell 1910, Nelf 1908).

There are three distinct phases in the clotting of blood namely the formation of thromboplastin, the formation of thrombin, and the production of fibrin. Modern theories of coagulation will be more easily understood if the various factors are considered first separately.

International Committee has agreed upon uniform terminology for various coagulation factors namely factor I (Fibrinogen), factor VIII (Antihæmophilic factor) etc.

There are some elements naturally occurring in the body which tend to keep the blood in liquid phase in vitro as well as in vivo. Several such inhibitors are antithrombin, heparin and antithromboplastin. Antithrombin removes excess of thrombin formed during coagulation process thereby prevents clotting in vivo. Heparin is a highly active anticoagulant.

Among the coagulation defects known so far, afibrinogenemia and hæmophilia A are considered here.

Congenital deficiency of factor I (afibrinogenemia)

This disease is extremely rare and is probably inherited as an autosomal recessive condition and affects both the sexes. Bleeding is more than normal. The plasma level of fibrinogen required for hæmostasis is thought to be in the region of 50-100 mg./100 ml. This level can

usually be easily obtained by transfusion of fresh or stored plasma or freeze dried fibrinogen concentrates. The most striking laboratory findings are the infinitely prolonged whole blood clotting time and one stage prothrombin time and the failure of the blood to clot on the addition of a strong solution of thrombin.

Congenital deficiency of factor VIII (haemophilia A)

The term haemophilia A is now used to describe the disorder when antihæmophilic globulin (AHF, Factor VIII) is deficient and haemophilia B is used to describe the disorder when plasma thromboplastin component is deficient. Haemophilia A is more common than haemophilia B. Both are inherited as sex linked recessive character because the genes for the disorder are carried by X-chromosome.

Object of the work

Fibrinogen derived from the plasma protein has been used for restoring plasma fibrinogen levels to normal in the treatment of hæmorrhagic condition. Fibrinogen is always associated with antihæmophilic globulin. Attempts are being made to separate it from antihæmophilic globulin. Biochemical and immunological properties have been studied by obtaining immunologically purified fibrinogen and antihæmophilic globulin.

Fibrinogen (Cohn Fraction I) has been isolated from fresh plasma by various methods using different salts and organic solvents as protein precipitants.

Methods used for the isolation of fibrinogen

1. Sodium sulphate method (Howe 1921)
2. Saturated ammonium sulphate method
(Hans Neurath et.al. 1943)
3. Phosphate buffer method (Jaques 1943)
4. Precipitation with ethyl ether (Kekwick and Mackay 1954)
5. Precipitation with glycine (Kazal 1963)
6. Precipitation with sodium chloride (Polson 1972)
7. Precipitation with ethyl alcohol (Cohn et.al. 1946)

Purification of the fibrinogen has been carried out by Blomback and Blomback method 1956, Rubeka Berg's method 1972 and also by gel filtration using sephadex G-200.

Clottable and non-clottable protein has been estimated by Biuret method. The purity of the above preparations has been determined by paper and immuno-electrophoresis. Antisera to fibrinogen have been prepared by immunising rabbits with purified fibrinogen. Having obtained a monospecific antiserum to fibrinogen, Single Radial Immunodiffusion Technique has been developed for

quantitation of plasma fibrinogen. The results obtained by the above method have been compared with those obtained by the other methods.

The antihæmophilic factor (AHF, Factor VIII) is found in association with euglobulins and fibrinogen of plasma. Factor VIII can be concentrated from plasma by precipitation with a variety of agents. These agents precipitate large amounts of fibrinogen in addition to factor VIII. Partial removal of fibrinogen can be achieved by adsorption with Fuller's earth, but it may remove significant amount of factor VIII activity.

Methods used for the isolation of antihæmophilic globulin (AHF, Factor VIII).

1. Ether precipitation (Kekwick and Mackay 1954)
2. Slow freezing method (Pool and Shannon 1965)
3. Rapid freezing method (Brown et.al. 1967)
4. Preparation of high purity AHF with polyethylene glycol (Johnson et.al. 1969)
5. Alcohol precipitation method (Hoyer 1972)
6. Sodium chloride method followed by polyethylene glycol (Polson and Ruiz Bravo 1972)

Purification of the products prepared by the above mentioned methods has been carried out with reprecipitation, by using polyethylene glycol. Purity of the product has been determined by protein estimation, by electrophoresis



and immuno-electrophoresis. Nonspecific antiserum against anti-haemophilic globulin has been prepared by immunising rabbits with purified anti-haemophilic factor. Single Radial Immunodiffusion Technique has been developed to find out the association of fibrinogen and antihemophilic globulin quantitatively. Anti-haemophilic factor activity has been determined of the products with two stage thromboplastin generation test. The study of AHF activity of normal subjects has been carried out which included professional donors, voluntary donors and staff members. Ten patients of haemophilia were treated with cryoprecipitate. The AHG level was determined before and after the transfusion of cryoprecipitate.

Results : Cohn's method is advantageous over all the other methods as we get the value of fibrinogen 87% and clottable protein 90%. In case of clottable protein the maximum clotting effect is seen in the fraction I isolated by Cohn's ethanol method. Fibrinogen values are found to be low in Single Radial Immunodiffusion as this method estimates only fibrinogen and not other proteins.

AHF prepared by rapid freezing method shows impurities as the other plasma fractions are not removed totally. Hoyer's method also gives purer AHF with twice reprecipitation. Cryoprecipitate method is advantageous over all other methods. It is also less complicated and can be carried out in closed system without risk of

contamination. The preparation contains a higher concentration of factor VIII, relative to its protein content.

Quantitative estimation of normal subjects showed AHG levels between 50% to 150%.

The clinical trials show that though the cryoprecipitates are old (about one year) and having AHF activity of 80% to 90% the clotting time of the patient is improved and bleeding was immediately stopped.

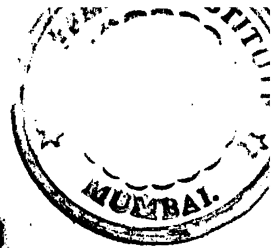
Name and signature
the candidate

P. R. Dichwalkar
(P. R. Dichwalkar, M.Sc.)

Name and signature of the
Research Guide

M. V. Sant
(Dr. M. V. Sant)
M.D., F.C.P.S., D.E. (Prague).





STATEMENT UNDER UNIVERSITY RULE NO.0.770 & 0.771

As required by the University Rule No.0.770 & 0.771
I, state that the work presented in this Thesis which
I intend to submit is of original nature and so far, has
not been submitted for any degree of this or any other
University, wherever reference has been made to the work
of others, it has been clearly indicated along with the
source of information in the "Bibliography".

P.R. Dichwalwar

Signature of the Candidate

Certified that the above statement made by the
Candidate is correct.

W. Sant.

Signature of the Research Guide.

