STUDIES ON THE PROCUREMENT OF BLOOD COAGULATION FACTOR VIII

ALBERT FARRUGIA

Ph.D.

UNIVERSITY OF EDINBURGH

1984

"If you prick us, do we not bleed?"

WILLIAM SHAKESPEARE
The Merchant of Venice, Act III, Scene 1.

GHAL JOSEPHINE LI DEJJEM KIENET HEMM,

U GHAL DAVID LI ĠIE FL-AHHAR

DECLARATION

I hereby declare that the work described in this thesis was designed and carried out by myself, with the exception of the following:

1. The production of artificial substrate plasma for the factor VIII coagulation assay, which was done by Mrs. Brenda Griffin of the Scottish National Blood Transfusion Service Headquarters Unit Laboratory, Edinburgh.
2. The immunisation of rabbits with purified factor VIII related antigen and fibronectin to produce antisera to these proteins, which were done by Dr. Joan Dawes of the MRC/SNBTS Blood Components Assay Group, Edinburgh and by Mrs. Margaret Ferguson of the Glasgow and West of Scotland Blood Transfusion Service, respectively.
3. The viral inactivation studies on heat-treated factor VIII concentrate, which were done by the Research Department of the SNBTS Protein Fractionation Centre, Edinburgh.
4. The fibrinopeptide A assays which were done by Dr. Hagop Bessos at the MRC/SNBTS Blood Components Assay Group.

ACKNOWLEDGEMENTS

I would like to thank Dr. Christopher Prowse for his constant help and encouragement during the course of this work, which was performed in his laboratory at the Edinburgh and South-East Scotland Blood Transfusion Service. I am also grateful to my supervisor, Dr. Frank Boulton, for his support and advice.

My thanks are also due to Professor L.G. Whitby, Dean of Medicine at the University of Edinburgh for accepting me as a Ph.D. student in his department and for his constant help throughout, and to Dr. Brian McClelland, Director of the Edinburgh and South-East Scotland Blood Transfusion Service, for providing the facilities of his department for my research.

My thanks are also due to the following:
Dr. Duncan Pepper of the Scottish National Blood Transfusion Service Headquarters Unit Laboratory for friendship and stimulating advice throughout, and Mrs. Brenda Griffin for help in setting up immunoradiometric assays and supplying artificial substrate plasma for the factor VIII coagulation assay.
Dr. Peter Foster of the Protein Fractionation Centre, Edinburgh, for helpful discussions on various aspects of factor VIII fractionation and for permission to quote his data on viral inactivation in heat-treated concentrate.
Mr. Graeme MacKay of the Coagulation Laboratory of the Edinburgh Blood Transfusion Centre for instruction in

coagulation assays.

Mrs. Valerie Hornsey, my colleague in the Biochemistry Laboratory, for help in familiarisation with laboratory procedures and techniques.

Dr. Joan Dawes of the MRC/SNBTS Blood Components Assay Group, for instruction in the setting up and optimisation of immunoradiometric assays.

Mr. Adam MacGill of the Components Division and Sister Jean MacDonald of the Donor Area of the Transfusion Centre for organising supplies of fresh plasma for research purposes.

Mr. Sean Hanratty and Dr. J. O'Riordan of the Irish Blood Transfusion Service Board, Dublin, for allowing me to visit their Centre to observe their heparin-aided factor VIII production method.

Dr. Charles Rizza of the Oxford Haemophilia Centre for help in finding a place for my studies, and Dr. James Smith of the Oxford Plasma Fractionation Laboratory for allowing me to visit the P.F.L. and for interesting discussions on plasma fractionation.

Dr. Christopher Ludlam of the Haematology Department of the Royal Infirmary, Edinburgh, for medical advice and allowing me to reproduce the results of the heat-treated factor VIII concentrate infusion.

I am very grateful to the British Council and the Association of Commonwealth Universities for supporting and administering my studies in Scotland. I also thank the Department of Health and Environment in Malta for

allowing me study leave for the period of my studies.

I thank Mrs. Margaret Currie, who typed this thesis with patience and care.

Finally, I thank my wife, Josephine, for her constant love and encouragement for the whole period of our stay in Edinburgh.

CONTENTS

ABSTRACT

Coagulation factor VIII is needed for the treatment of patients with haemophilia. Requirements for factor VIII are important in determining the numbers of blood donations to be collected in Scotland. The final yield of factor VIII:C in products prepared from blood plasma, when prepared either in the Transfusion Centres or the National Fractionation Centre, is relatively low (30-40%). Improvements in these processes would have major implications for the Transfusion Service.

Present technology requires the freezing of plasma from blood donations prior to the preparation of cryoprecipitate; this may then be processed further to produce factor VIII concentrate. In this study, the effects of using different anticoagulants (citrate, heparin), of varying the rate of freezing and the duration of storage on the factor VIII content of plasma and subsequent products have been investigated. The influence of ABO blood group and of drug induced enhancement on levels of factor VIII in starting plasma were also studied.

Improvements in the mode of preparing cryoprecipitate from plasma have been made on the basis of the observed effects on processing plasma of different quality (collection procedure, age, etc.) and by various methods. Investigation of the mechanism of cryoprecipitation has led to the development of a high-yield method

for the preparation of factor VIII from unfrozen plasma by precipitation with hydrophilic polymers.

In the course of this work, methods were developed for the isolation of factor VIII and fibronectin and immunological assays for these and other proteins (fibrinogen), based on immunoprecipitation and immunoradiometric methods, were set up. The immunoradiometric methods were used to follow the recovery and survival of factor VIII components following infusion into patients deficient in this protein.

Based on the work described in this thesis, recommendations will be made on the possibilities for improved procurement of factor VIII from blood donations.

CHAPTER I

INTRODUCTION

(A) DEVELOPMENTS IN THE UNDERSTANDING OF HAEMOPHILIA

Writings from the 2nd century A.D. in the Jewish Talmud (Rosner 1969) mention rulings exempting a woman's third son from being circumcised if his two elder brothers had died of bleeding after circumcision. Similar rulings forbid a boy to be circumcised if the sons of his mother's sisters had died after circumcision (Rothschild 1882 qu. Bulloch and Fildes 1911). These features of fatal bleeding after minor surgery in brothers or in maternally related boy cousins are characteristic of the disorder called haemophilia. Down the years there are other scattered records of bleeding disorders more or less closely agreeing with the clinical picture of the disease (Ingram 1976). By the 19th century an extensive literature had developed, which noted the mode of inheritance of the disease as well as the nature of the bleeding disorder. The name 'haemophilia' - literally 'love of blood' - occurred first in the title of Hopff's treatise in 1828 (Ingram 1976). The characteristic bleeding and subsequent arthropathy in the joints was first described by König in 1890. The famous 'Royal haemophilia' (McKusick 1965) originated from Queen Victoria, who was a carrier of the disease. Two of her daughters proved to be carriers, and one of her sons, Leopold Duke of Albany, was a haemophiliac, dying of a cerebral haemorrhage at 31 after falling and hitting his head. History's most famous haemophiliac, Alex Tsarevich of Russia, was the son of

a grand-daughter of Queen Victoria, Alix of Hesse. The boy's tragic history of illness and near brushes with death led to the rise of Rasputin, whose influence rose after apparently resolving some of the boy's bleeding episodes. Thus, haemophilia might be said to have had a crucial role in the subsequent events which led to the overthrow of the Tsar in 1917 and brought about the modern Soviet Union.

The work of Addis (Addis 1911) showed that a component of the globulin fraction of normal plasma corrected the prolonged clotting time of haemophilic blood. Addis thought that this was prothrombin but because this factor was found to be normal in haemophilia (Govaerts and Gratia 1931), his important observation was not pursued. Although the classical blood coagulation theory of Morawitz only acknowledged four coagulation factors - prothrombin, thromboplastin, calcium ions and fibrinogen (Morawitz 1905) - the concept of an 'antihaemophilic globulin' essential for normal coagulation slowly evolved. The work of Patek and Taylor confirmed Addis' observation of a plasma fraction capable of correcting the coagulation defect (Patek and Taylor 1937) and Brinkhous (Brinkhous 1939) showed that it accelerated the conversion of prothrombin to thrombin in haemophilic blood. Development of better test systems such as the Prothrombin time and the Thrombo-plastin Generation Test led to the identification of numerous other components necessary for normal blood

coagulation (reviewed by Macfarlane 1976) which were organised in a series of clotting factors designated by Roman numerals - antihaemophilic globulin was named 'factor VIII'. In the classic 'cascade' scheme for blood coagulation reactions (Macfarlane 1964, Davie and Ratnoff 1964) factor VIII was assigned an enzymatic role, but subsequent work (Suomela et al 1977, Neal and Chavin 1979, van Dieijen et al 1981) has demonstrated a cofactor role of factor VIII in the enzymatic conversion of factor X to Xa by factor IXa (Figure 1-2).

Investigation of the defect in haemophilia was complicated in the 1950's by the demonstration that von Willebrand's disease, a haemostatic disorder characterised by autosomal inheritance and a prolonged bleeding time (seldom found in haemophilia) was also associated with a low level of factor VIII (Alexander and Goldstein 1953, Nilsson et al 1957). Developments in the immunological investigation of haemophilia revealed the presence of an antigen associated with purified factor VIII fractions that could be used to raise heterologous antisera after injection of such preparations in animals (Zimmerman et al 1971). Subsequent testing using the electroimmunoassay technique of Laurell (Laurell 1966) revealed normal levels of this antigen in the plasma of haemophiliacs but low levels in the plasma of patients with von Willebrand's disease. Thus it seemed that factor VIII was composed of two

TABLE 1-1 NOMENCLATURE AND BIOLOGICAL CHARACTERISTICS OF THE HUMAN FACTOR VIII/von WILLEBRAND FACTOR COMPLEXES

Name of Component (Abbreviation)	Characteristics and Physiological Function	Disease Associated With Deficiency	Methods of Detection / Assay
Factor VIII Procoagulant Activity (VIII:C)	Protein which corrects the coagulation abnormality in haemophilia A. $M_r \cong$ 200,000 (Weinstein et al 1981). Synthesised in liver (Stel et al 1983). Concentration in plasma 50-100 ng/ml. Cofactor in the activation of FX by FIXa (van Dieijen et al 1981)	Haemophilia A:sex linked recessive trait. Incidence about 5-10 per 100,000 population (Bloom 1982)	Coagulation assays using one-stage or two-stage methods (Rizza and Rhymes 1982)
Factor VIII Coagulant Antigen (VIII:CAg)	Antigenic expression of VIII:C	Haemophilia A. Mostly found as haemophilia A^- with total lack of VIII:CAg, about 10-20% of patients have detectable VIII:CAg-haemophilia A^+	a) Immunoradiometric assays using human antibodies to VIII:C; liquid (Lazarchick and Hoyer 1978) and solid phase assays (Peake 1982) can be used. b) Inhibition neutralisation assays (Denson 1967)
von Willebrand Factor Protein (vWF)	Protein which corrects the bleeding time abnormality in von Willebrand's disease. Multimeric protein $M_r \cong 8x10^5$-$12x10^6$, subunit size 220,000 (Hoyer 1981). Synthesised	von Willebrand's disease - various types due to deficiency and/or abnormality of vWF (Zimmerman and Ruggeri (1982). Usually autosomal dominant trait. Incidence about the same as	Prolongation of the bleeding time. Semi-quantitative methods - platelet retention in glass bead columns (Salzman 1963); platelet adhesion to perfused subendothelium

TABLE 1-1 (Cont) NOMENCLATURE AND BIOLOGICAL CHARACTERISTICS OF THE HUMAN FACTOR VIII/von WILLEBRAND FACTOR COMPLEXES

Name of Component (Abbreviation)	Characteristics and Physiological Function	Disease Associated With Deficiency	Methods of Detection / Assay
von Willebrand Factor Protein (vWF)	in vascular endothelium (Bloom et al 1973). Concentration in plasma 5-10 ug/ml. Mediation of platelet adhesion to sub-endothelium (Weiss et al 1978)	haemophilia A	(Sakariassen et al 1979)
Factor VIII Related Antigen (VIIIR:Ag)	Antigenic expression of von Willebrand Factor	von Willebrand's disease (V.W.D.)	a) Electroimmunoassay (Zimmerman et al 1975) b) Immunoradiometric assay liquid (Hoyer 1972) or solid phase (Peake 1982) using heterologous antisera c) Radioimmunoassay using radiolabelled VIIIR:Ag (Paulssen et al 1975)
Ristocetin Cofactor (VIIIR:RCF)	A property of von Willebrand Factor which promotes agglutination of platelets in the presence of the antibiotic risto-cetin. The equivalent property in bovine and porcine vWF agglutinates human platelets without ristocetin	von Willebrand's disease	VIIIR:RCF assay using fixed platelets and ristocetin (Macfarlane et al 1975); does not always correlate with assays for vWF activity (Ogata et al 1983)

entities - 'Factor VIII antigen' - measured by reaction with heterologous antisera raised against purified factor VIII fractions and needed for a normal bleeding time; and 'Factor VIII coagulant', measured by coagulation assay and needed for normal plasma clotting. The different mode of inheritance of the two disorders argued for the separate identity of the two components, but for many years the question as to whether they were one or separate molecules was vigorously debated (reviewed by Bloom 1977).

(B) THE FACTOR VIII/von WILLEBRAND FACTOR COMPLEX. STRUCTURE AND FUNCTION

Present knowledge about the various components of the factor VIII complex, together with the clinical conditions associated with their deficiency, is summarised in Table 1-1.

Factor VIII:C and von Willebrand factor usually circulate together in plasma and a number of techniques such as gel filtration point to their close association. This has prompted suggestions that they are properties of the same macromolecule (Ratnoff et al 1976, Switzer and McKee 1976). However, the existence of independent genetic control for the two proteins has long provided compelling evidence that they are distinct. In addition, the two proteins can be separated from one another by procedures that do not disrupt covalent bonds (Weiss and Hoyer 1973, Tuddenham et al 1979). von Willebrand Factor has been purified by various groups

FIGURE 1-1

DIAGRAMATIC REPRESENTATION OF THE ROLE OF FACTOR VIII-RELATED ANTIGEN/von WILLEBRAND FACTOR IN PLATELET AND VESSEL WALL INTERACTION.

AA = arachidonic acid

PGI_2 = prostacyclin

GP = glycoprotein

TXA_2 = thromboxane A_2

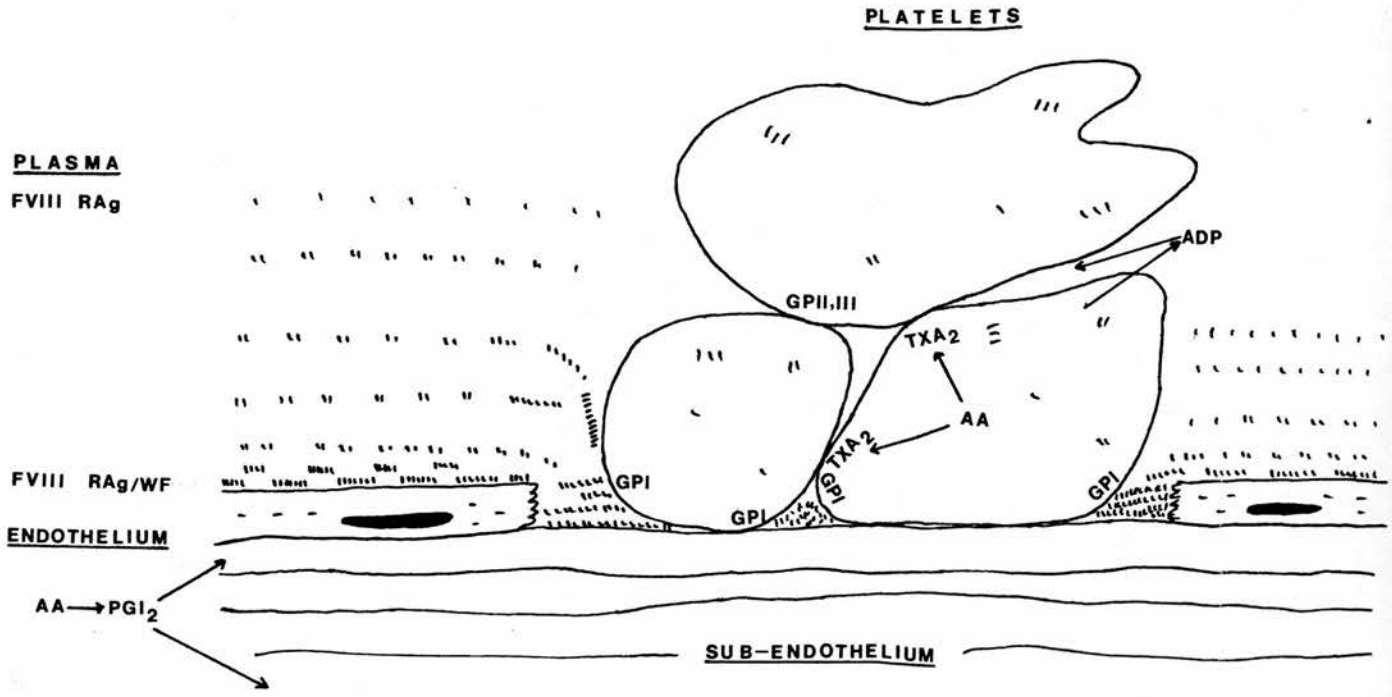
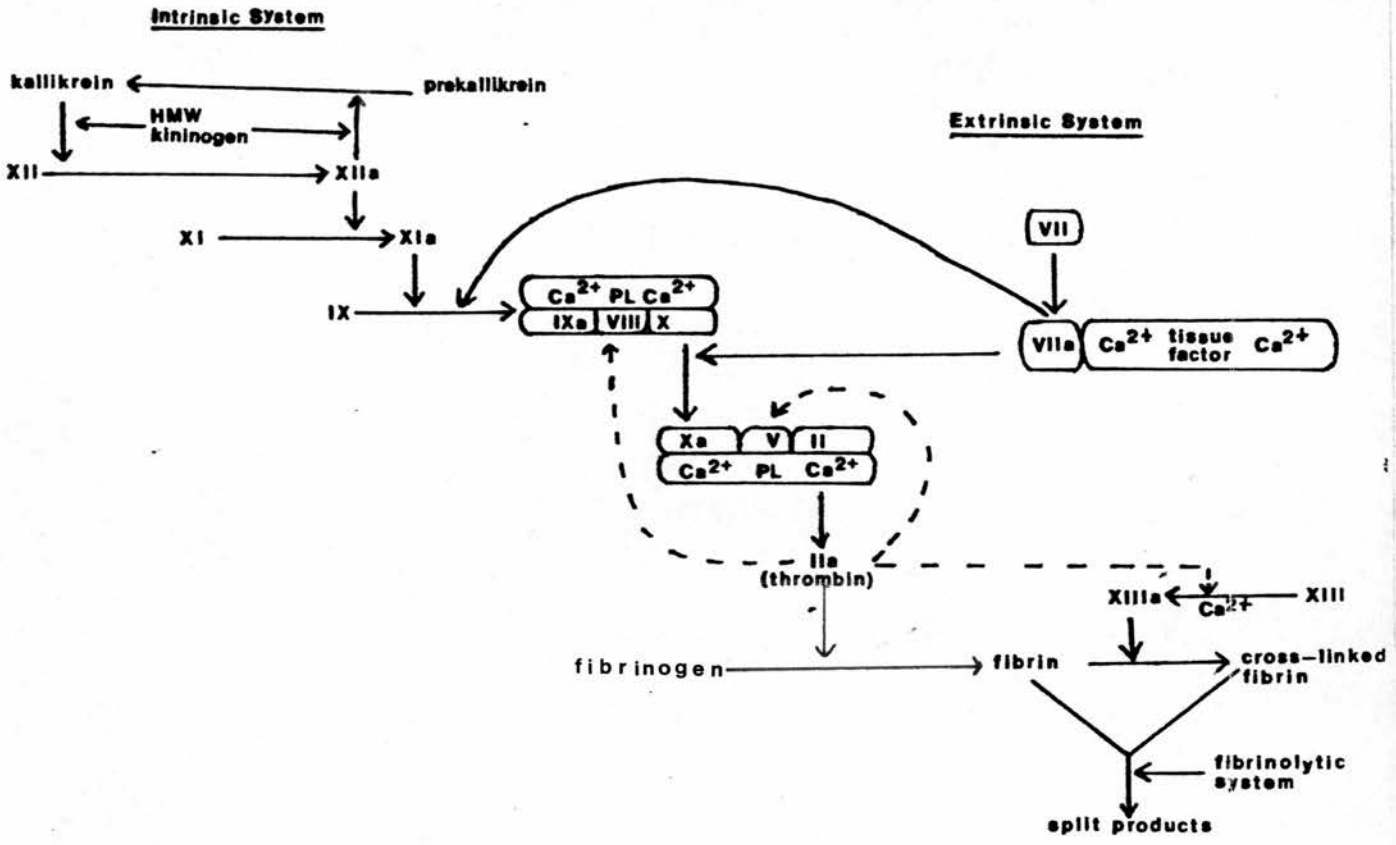

PLATELETS
PLASMA
FVIII RAg
ADP
GPII,III
TXA2
AA
TXA2
FVIII RAg/WF
GPI
GPI
GPI
GPI
ENDOTHELIUM
AA → PGI2
SUB-ENDOTHELIUM

FIGURE 1-2

SIMPLIFIED SCHEME OF THE COAGULATION SEQUENCE.
For the sake of this simplicity important control systems such as feed-back mechanisms and humoral inactivators and inhibitors, for example, protein C and antithrombin III, are omitted. The 'extrinsic' and 'intrinsic' systems are separated for descriptive purposes and their many possible points of interaction are omitted.
The dashed lines indicate the activation activities of thrombin considered to be the most important at the present time.

PL = phospholipid


Intrinsic System
kallikrein
prekallikrein
HMW kininogen
XII
XIIa
XI
XIa
IX
Ca2+ PL Ca2+
IXa VIII X
Extrinsic System
VII
VIIa
Ca2+ tissue factor Ca2+
Xa V II
Ca2+ PL Ca2+
IIa (thrombin)
XIIIa
Ca2+
XIII
fibrinogen
fibrin
cross-linked fibrin
fibrinolytic system
split products

(Legaz et al 1973, Olson et al 1977, Beck et al 1979) and has been well characterised as an oligomeric protein consisting of a series of multimers (Hoyer and Shainoff 1980). Factor VIII:C, which contributes about 1% to the mass of the VIII/VWF complex (Hoyer 1981) is less well characterised but present evidence points to it being a single chain polypeptide with M_r = 8 x 10^4-2 x 10^5 (Weinstein et al 1981, Fulcher and Zimmerman 1982, Fay et al 1982).

While Figure 1-1 shows the role of von Willebrand Factor in mediating platelet adhesion to exposed subendothelium, Figure 1-2 shows the role of factor VIII:C in the coagulation sequence, (both figures are taken from Bloom (1982)).

(C) EARLY DEVELOPMENTS IN THERAPY FOR THE FACTOR VIII DEFICIENCY DISORDERS

Current therapy has resulted in haemophiliacs having an almost normal life expectancy (Ikkala et al 1982), a situation in sharp contrast to the efforts at therapy outlined in the early literature (Birch 1937). The present position has developed over the past forty years but the modern treatment of replacing the missing component in blood dates from 1840 when the first blood transfusion for a case of haemophilic bleeding was given (Lane 1840). Rather fortuitously, no incompatibility problems were recorded, but further use of this form of treatment had to await Landsteiner's discovery of blood groups in 1901. Subsequent claims of benefit from the

use of a variety of measures, ranging from the administration of lime to the use of egg white (Ingram 1976) must today be viewed very sceptically.

Macfarlane's use of Russell's Viper venom (Macfarlane and Barnett 1934) as a topical application on wounds was one of the first efforts based on a scientific appreciation of the defect. By 1938 however, Macfarlane (Macfarlane 1938) had realised that only blood transfusion offered effective treatment for a bleeding episode, by replacing the missing essential component. Patek's group showed this to be present in the cell free plasma (Patek and Taylor 1937) and in Cohn's classic fractionation scheme it was shown to be in Fractions I and II of normal, but not haemophilic plasma (Minot and Taylor 1947). These fractions and similar fibrinogen-rich components from ether-fractionated plasma (Kekwick and Wolf 1957) were recognised as potentially therapeutic materials. These early products were relatively crude and insoluble due to their high fibrinogen content and tended to be unstable. The Blombäck modification of Cohn Fraction I (Blombäck 1958) led to a product that was much improved in stability and purity.

The introduction of cryoprecipitate as a therapeutic blood product by Pool and co-workers (Pool and Shannon 1965) was a big step forward. Although the gelatinous residue that remains undissolved when frozen plasma is allowed to thaw at a low temperature was known to be rich in fibrinogen and factor VIII (Ware et al 1947,

TABLE 1-2 SOME EARLY TYPES OF THERAPEUTIC MATERIALS FOR THE TREATMENT OF FACTOR VIII DEFICIENCY

Name of Preparation	Method of Production	Purification Over Plasma (X)	General Comments
Cohn Fraction I (F-I) (Cohn et al 1946)	Precipitation of plasma proteins with 8% ethanol at -3°C, pH 7 - most of the fibrinogen and factor VIII are precipitated	7-20	Careful technique allows factor VIII to be harvested at high yields; however, F-I is poorly soluble, rather unstable and cannot be sterile-filtered, and so has to be prepared by elaborate sterile techniques
FI-O (Blomback 1958)	Extraction of F-I at 0°C with a glycine-citrate buffer and resolution of the precipitate in isotonic saline	10-30	Extraction procedure improves greatly the solubility and stability of F-I; material still used extensively in Scandinavia
Ether-Fraction F-I (Kekwick & Wolf 1957)	Precipitation of plasma proteins with 11% ether at 0°C	25	Same characteristics and drawbacks as Cohn F-I
FI-O-Ta (Simonetti et al 1969)	Treatment of F-I-O with tannic acid to remove fibrinogen	40-160	Experience is with small batches; attempts to replicate initial findings produced widely varying results, probably due to wide variability in batches of tannic acid
Bentonite-F-I (Soulier 1959)	Treatment of F-I with bentonite to remove fibrinogen	40-160	As with FI-O-Ta
Glycine-Precipitated Fraction (Wagner et al 1964)	Addition of glycine to plasma to a concentration of 2.3 M at 0°C to precipitate factor VIII with some fibrinogen	20-30	Use of glycine at high concentrations expensive and wasteful as it precludes the use of the supernatant plasma - techniques still used to prepare 'high purity concentrates (Table 1-6A)

Brinkhous 1954) Pool developed this as a clinical product which could be produced by small centres. This made the widespread adequate treatment of haemophilia feasible.

Despite the benefits of cryoprecipitate, it has several limitations: it must be stored frozen, it has only about five times the potency of plasma, it is not standardised and it may induce reactions following administration. Cryoprecipitation was used by Johnson's group (Johnson et al 1969) in the bulk preparation of an intermediate purity factor VIII concentrate (classification of Smith and Bidwell 1979 - Table 1-5). This method, allowing production of a standardised bulk product, still forms the basis of most available factor VIII concentrates. Other methods, involving treatment of plasma or plasma fractions with chemicals, were used to prepare concentrates of varying quality. These are summarised in Table 1-2. Nowadays most of these products, with the exception of Fraction I-O, have been superseded by concentrates prepared by other techniques.

The production of animal factor VIII concentrate (Bidwell 1955) played a role in providing therapy when human products were scarce. Such products were associated with problems due to antigenicity and side reactions were common, as was thrombocytopenia from the Platelet Aggregating Factor associated with bovine and porcine factor VIII. Nowadays such products have been mostly superseded, although they still play a role in the

TABLE 1-3 VARIABLES INVOLVED IN CRYOPRECIPITATE PRODUCTION AND THEIR EFFECT ON PRODUCT QUALITY

(A) Anticoagulant	CPD gives higher yields than ACD (Regional Transfusion Directors' Committee 1978); some groups find no difference (Slichter et al 1976) or even better yields with ACD (Vermeer et al 1976)
(B) Plasma pH	Acidification reduces yield, optimal pH ≅ 6.9 (Pool 1967)
(C) Plasma Freezing	Most studies claim fast freezing improves yield and purity (Vermeer et al 1976)
(D) Plasma Thawing	Most studies claim rapid thawing improves yield (Brown et al 1967) and purity (Vermeer et al 1976)
(E) Blood-Group	Higher VIII:C in group A plasma is reflected in higher amounts in cryo (Regional Transfusion Directors' Committee 1978); one group claims a higher yield in cryo from group A plasma (Prowse et al 1982)
(F) Ionic Composition of Plasma	Maintenance of physiological Ca^{2+} levels is claimed to improve yield (Rock et al 1979)
(G) Additives in Plasma	Some workers claim increased yields by adding ethanol (Newman et al 1971) or polyethylene glycol (Johnson et al 1979) but others dispute this (Foster et al 1982). One group claims increased yields by adding heparin (Rock et al 1980)
(H) Conditions of Storage of Frozen Plasma	Most studies advocate storage below -30°C (Rock and Tittley 1979), the period of storage does not appear to be crucial (Kasper et al 1975)
(I) Storage of Blood Prior to Processing	General agreement that up to 6 hrs at room temperature has no effect on yield (Avoy et al 1978); longer periods result in lower yields according to some groups (Vermeer et al 1976), others claim no difference between blood processed at 6 and 18 hrs after donation (Regional Transfusion Directors' Committee 1978)

management of patients with inhibitors to human factor VIII (Rizza 1976b). Fractionation using 'polyelectrolyte' chromatography (see Table 1-6B) removes most of the Platelet Aggregating Factor from porcine factor VIII and lessens the risk of thrombocytopenia.

(D) SURVEY OF AVAILABLE PRODUCTS USED IN TREATMENT OF HAEMOPHILIA A AND von WILLEBRAND'S DISEASE

1. Cryoprecipitate ('cryo'):

Although being gradually superseded by freeze-dried concentrates, blood bank cryoprecipitate is still important due to its ease of preparation and the relatively high yield of factor VIII which is obtained. The original technique (Pool and Shannon 1965) was designed to allow the harvesting of cryoprecipitate while allowing the recovery of other blood components. Blood was collected into the primary bag of a double bag system, and after centrifugation platelet poor plasma was expressed into the secondary bag and frozen. Overnight thawing of the plasma was achieved in a normal blood bank cold room or refrigerator and the cryoprecipitate was then harvested by centrifugation. This allowed the supernatant plasma to be transferred back to the cells or used for further fractionation.

A large number of variables have been studied with respect to the yield and quality of cryoprecipitate and the results are summarised in Table 1-3. Table 1-4 lists some of the types of

TABLE 1-4 SOME TYPES OF CRYOPRECIPITATE PRODUCED BY VARIATIONS OF THE ORIGINAL TECHNIQUE

Name of Preparation	Method of Preparation	Improvements Over Conventional Product
Freeze-dried Cryoprecipitate (Milligan et al 1981)	Small numbers of individual cryos are pooled into stabilising buffer, aliquoted and lyophilised	Product can be stored at 4°C and can be subjected to more rigorous quality control
'Thaw-siphon' Cryoprecipitate (Mason 1978)	Cryoprecipitate produced by fast-thaw in 4°C water bath with continuous removal of thawed plasma supernatant by siphoning.	Marked improvements in yield and purity (Prowse and McGill 1979); however only two centres (Edinburgh and Brisbane) produce it routinely - careful attention to detail is required to produce good results.
'Cold-Insoluble Globulin' Cryoprecipitate (Smit-Sibinga et al 1981)	Cryoprecipitate harvested from plasma derived from blood collected in heparin; cryo is then subjected to a cold precipitation at 0°C, solubilised in buffer and lyophilised.	Collection in heparin claimed to greatly improve yields; the second (cold) precipitation doubles the specific activity of the original cryo. Material gave good clinical results (Smit-Sibinga et al 1983).

TABLE 1-5 CLASSIFICATION OF FACTOR VIII CONCENTRATES
(Smith and Bidwell 1979)

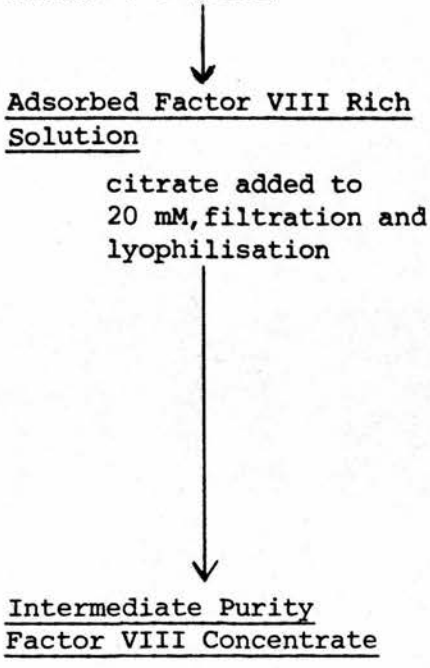
Group 1	Concentrates of low purity - < 0.2 i.u./mg protein soluble at ≅ 5 i.u./ml, contain 50-80% fibrinogen, obtained at a yield > 300 i.u./litre fresh frozen plasma
Group 2	Concentrates of intermediate purity - 0.2-0.5 i.u./mg protein soluble at 5-20 i.u./ml, contain 40-60% fibrinogen, obtained at a yield of 200-300 i.u./litre fresh frozen plasma
Group 3	Concentrates of high purity - > 0.5 i.u./mg protein soluble at > 20 i.u./ml, contain < 50% fibrinogen, obtained at a yield of less than 200 i.u./litre fresh frozen plasma

FIGURE 1-3

PRODUCTION OF INTERMEDIATE PURITY FACTOR VIII CONCENTRATE FROM FRESH FROZEN PLASMA (NEWMAN et al 1971). ALSO INCLUDED ARE MODIFICATIONS AT CERTAIN STEPS INTRODUCED BY SUBSEQUENT WORKERS.

NEWMAN PROCEDURE	SUBSEQUENT MODIFICATIONS
Crushed Frozen Plasma	
bulk thawed to slurry ethanol added 3% v/v cryoethanol precipitate harvested	1. Ethanol addition omitted as this compromised purity without improving yield (James and Wickerhauser 1972). 2. Plasma blocks mechanically crushed to 'snow' to increase surface area and rate of thawing (Foster et al 1982). 3. Continuous instead of bulk thawing to improve yield and purity (Foster et al 1982).
↓	
Cryoethanol Precipitate	
washed in 20 mM Tris/HCl pH 7 containing 8% ethanol, -2°C	Washing with 20 mM Tris/HCl pH 7 without ethanol improved purity and solubility (Wickerhauser et al 1978).
↓	
Washed Precipitate	
extracted in 2.5-10% plasma volume of 20 mM Tris/HCl pH 7 for 30 min at 25°C	1. Extraction into lower volume of buffer to increase potency (James and Wickerhauser 1972). 2. Cold precipitation at acid pH of contaminants to improve solubility and filterability (Smith et al 1979). 3. Extraction time optimised to improve purity (Smith et al 1979).
↓	
Factor VIII Rich Extract	
adsorbed with 5% v/v $Al(OH)_3$ gel for 5 min at 25°C; gel spun off and solution clarified by filtration	Proper pH control during adsorbtion improves filtrability and solubility of final solution (Liu et al 1980).
↓	

FIGURE 1-3 (Cont)



1. Addition of Ca^{2+} to prevent VIII:C lability due to citrate (Foster et al 1983b).

2. Adjustment of pH of final solution to 6.6 to improve stability (Liu et al 1980).

3. Addition of dextrose and celite to improve solubility and remove activation products (Margolis and Rhoades 1979).

cryoprecipitate produced.

2. Factor VIII Concentrates:

Following the classification of Smith and Bidwell (Smith and Bidwell 1979) these can be defined as in Table 1-5.

Group 1 concentrates include FI and cryoprecipitate, which have been discussed.

Group 2 concentrates are mostly derived from further processing of bulk cryoprecipitate. The process described by Newman et al (Newman et al 1971) is outlined in Figure 1-3 in some detail, as it is the most widely used procedure for manufacture of clinical concentrates at present.

The 'Newman' method and its modifications superseded nearly all previous techniques for producing factor VIII concentrates. Cryoprecipitation for large scale processing had the advantage that the fraction harvested was a by product and its production did not interfere with subsequent Cohn fractionation of the plasma to immunoglobulin and albumin. Also, the processing of frozen plasma to cryoprecipitate had an inherent advantage over the previous precipitation methods (alcohol, ether,etc.) which involved the use of plasma which had to be fairly fresh if a good product was to be obtained. This was because these precipitation techniques were only suitable for liquid plasma; processing of frozen-thawed plasma was not satisfactory. The use of freeze-thawing as a

TABLE 1-6A PRODUCTION OF HIGH-PURITY FACTOR VIII CONCENTRATES - PRECIPITATION METHODS

Starting Material	Fractionation Procedure	Product Characteristics and Yield
'Newman' Intermediate Type Concentrate	Fractional precipitation with Polyethylene Glycol (P.E.G.) (Newman et al 1971) - first precipitation at about 4% P.E.G. 4000 precipitates fibrinogen which is removed, factor VIII is precipitated and concentrated by increasing P.E.G. concentration to 11%	Depending on nature of starting material (plasma age, duration of storage etc.) product is 125-300 fold purified over plasma. Yield is usually < 20%.
Solubilised Cryoprecipitate	Fractional precipitation with P.E.G. to remove fibrinogen, followed by glycine precipitation in the cold (Shanbrom and Fekete 1971) to precipitate factor VIII. Heparin added in processing buffers to increase yield and purity (Fekete et al 1974)	Product is 110-380 fold purified over plasma, yield ≅ 17%.
'Newman' Intermediate Type Concentrate	Addition of Zn^{2+} to final [1mM] at pH 6.6 and 1 u/ml heparin precipitates about 90% of the fibrinogen and fibronectin and leaves factor VIII in the supernatant (Foster et al 1983a)	Product has roughly twice the specific activity of normal concentrate and is obtained at a yield of > 90% from cryoprecipitate. Product can be subjected to heat treatment to inactivate contaminating viruses (Macleod et al 1983)
Solubilised Cryoprecipitate	Precipitation with glycine at 30°C precipitates fibrinogen but leaves factor VIII in solution, from which it can be subsequently concentrated by precipitation with P.E.G. or NaCl (Blombäck and Thorell 1982)	Final product is obtained at a yield ≅ 60% from cryoprecipitate (Torma & Myllyla 1983) specific activity is between 3 and 7 u/mg depending on the final precipitating conditions (Thorell et al 1983). Product corrects the bleeding time defect in V.W.D. (Thorell et al 1983)

TABLE 1-6B PRODUCTION OF HIGH-PURITY FACTOR VIII CONCENTRATES - CHROMATOGRAPHIC METHODS

Starting Material	Fractionation Procedure	Product Characteristics and Yield
Plasma or Cryoprecipitate	Ion exchange chromatography on 'Polyelectrolyte' resin - VIII:C is adsorbed batchwise and eluted with high ionic strength buffers containing lysine (Johnson et al 1978). VIII:C is then concentrated by P.E.G.	'Polyelectrolyte' VIII:C is ≅ 50 fold purified over plasma and is almost free of von Willebrand Factor. The process yield over cryoprecipitate is ≅ 40%. The concentrate produced from porcine plasma has been used successfully to treat patients with VIII:C inhibitors (Kernoff et al 1981). The human product gave good clinical results in haemophilia A but poor results in V.W.D. (Tuddenham et al 1982)
Intermediate Purity Concentrate	Filtration on Controlled Pore Glass (C.P.G.) column (Margolis and Rhoades 1981). Fibrinogen is retained and subsequently adsorbed by the gel, while factor VIII is collected in the void volume	Concentrate is ≅ 90 fold purified over plasma and is obtained at ≅ 90% yield over the intermediate purity material. Clinical results are good
Intermediate Purity Concentrate	Column chromatography over aminohexyl Sepharose (Austen and Smith 1982) is claimed to remove much of the fibrinogen with possible reduction of hepatitis B virus and blood group isohaemagglutinins	Final concentrate is ≅ 90 fold purified over plasma at a yield of ≅ 43% of the starting material. No clinical use reported - still in small scale research stage
Solubilised Cryoprecipitate	Adsorbtion to and elution from Tris-Acryl matrix. Factor VIII is eluted at high ionic strength after low salt wash to remove fibrinogen (Levi et al 1982)	Final factor VIII fraction has a specific activity of > 5 u/mg and the yield over the initial cryo is ≅ 70%. No clinical use reported - still in small scale research stage

fractionation step without any reagent addition was thus much more suitable. A recent report describing the production (Casillas and Simonetti 1982) and clinical use (Simonetti et al 1981) of a concentrate produced by precipitation with the synthetic polymer polyvinylpyrrolidone indicates that this approach might be feasible on a small scale, provided that the plasma can be obtained fairly fresh.

Group 3 concentrates are usually produced by further fractionation of Group 2 materials. A variety of procedures are utilised, which are summarised in Table 1-6A.

A number of recent developments have utilised chromatographic techniques for the production of high purity concentrates. These are summarised in Table 1-6B.

It is worth noting that the term 'high-purity concentrates' is a misnomer, the specific activity of pure factor VIII:C being about 2000 u/mg (Tuddenham 1983). The only major advantage of high purity concentrates is that of high potency which may be required in clinical situations needing high doses and less fibrinogen to avoid associated 'dysfibrinogenaemia' with high dose therapy. Otherwise, the loss of factor VIII that is associated with the manufacture of high purity concentrates makes their production difficult to justify.

(E) BIOCHEMICAL ASPECTS OF FACTOR VIII FRACTIONATION

The development of more sophisticated assays to measure the different factor VIII related activities have led to several studies on the levels of these activities in therapeutic materials (Nilsson and Hedner 1977, Allain et al 1980, Nilsson et al 1980, Barrowcliffe et al 1981). Studies showed that all concentrates examined had higher levels of factor VIIIR:Ag than factor VIII:C (Yang and Duffy 1978, Nilsson et al 1980) suggesting a selective loss of clotting activity during fractionation. Furthermore, the VIIIR:Ag/VIII:C ratio was highest for the more purified products (Yang and Duffy 1978). Other studies confirmed these results, although an increase in VIIIR:Ag/VIII:C ratios for more purified material was not always found (Allain et al 1980).

Use of the VIII:CAg assay increased the scope of such investigations. The ratio VIII:CAg/VIII:C was found to be much closer to unity than VIIIR:Ag/VIII:C suggesting that rather than inactivation of clotting activity, mechanical loss of VIII:C molecules is occurring during fractionation (Barrowcliffe et al 1981). Other studies indicate that both inactivation and mechanical loss are occurring, as evinced by increasing VIII:CAg/ VIII:C and VIIIR:Ag/VIII:CAg ratios (Prowse et al 1981).

The development of more sophisticated assays for VIIIR:Ag led to greater understanding. Using immuno-radiometric assays (I.R.M.A's) the levels of VIIIR:Ag in concentrates were shown to be much lower than when measured

by electroimmunoassay (Laurell) (Nilsson et al 1980). The increased levels detected by Laurell assays were shown to be due to a higher electrophoretic mobility of the VIIIR:Ag in concentrates compared to that found in plasma, due to the loss of higher molecular weight VIIIR:Ag multimers. VIIIR:Ag in concentrates also had an abnormal antibody binding capacity in some I.R.M.A's (Nilsson et al 1980). A lack of large VIIIR:Ag multimers in concentrates was shown by the two-dimensional Laurell technique (Barrowcliffe et al 1981) and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (S.D.S.P.A.G.E.) (Jakab et al 1978). It seems that molecular changes during fractionation are generating a population of VIIIR:Ag molecules of lower molecular weight, by selective removal or/and degradation of higher molecular weight multimers.

The failure of certain high-purity concentrates to correct the bleeding time defect in V.W.D. had been previously noted (Blatt et al 1976). Since these concentrates also lacked higher VIIIR:Ag multimers, which had been shown to be associated with VIIIR:RCF activity, (Over et al 1978), such a lack was seen to be the reason for failing to correct the bleeding time in V.W.D. This explanation however assumes a direct relationship between the 'bleeding time factor' of V.W.F. and VIIIR:RCF. Other studies indicated that the question is more complex. It was shown that high VIIIR:RCF in some concentrates did not result in these materials correcting the defect in

V.W.D. (Nilsson et al 1980). One study indicated that the technique used for measuring VIIIR:RCF was of importance, a platelet counting technique giving lower values for most concentrates than the usual aggregometric method (Barrowcliffe et al 1981). Moreover, recent findings show that the VIIIR:RCF assay does not always correspond to von Willebrand activity (Ogata et al 1983). It is possible that V.W.F. activity and even VIIIR:RCF (Martin et al 1983) could be a function of some specific polypeptide chain rather than being solely dependent on molecular size. One study showed that concentrates cannot correct the abnormal retention to subendothelium of platelets in V.W.D. plasma, but no relationship to multimer size was shown (Sixma et al 1981). It thus seems that for concentrates, unlike cryoprecipitate, higher molecular weight VIIIR:Ag multimers are lost during production, and this loss leads to decreased VIIIR:RCF activity. However, the loss of V.W.F. activity and the inability to correct the bleeding time defect in V.W.D. is likely to be the result of an accompanying, less well characterised, molecular change.

Some reports have claimed that highly purified concentrates give in vivo recoveries and half-lives for VIII:C that are lower than for less purified fractions, suggesting a detrimental effect on the integrity of VIII:C upon further processing (Smith et al 1972, Nilsson and Hedner 1977). Other reports find no difference, however (Weiss et al 1976). One study claims a normal recovery

and half-life for VIII:C, but greatly reduced values for VIII:CAg (Holmberg et al 1981) but another study shows identical in vivo behaviour for VIII:C and VIII:CAg (McLellan et al 1982). Differences in assay techniques may account for these discrepancies.

(F) HAZARDS OF REPLACEMENT THERAPY

A life-long dependence on blood products almost inevitably results in some complications. These will be briefly reviewed below.

I. Viral Infections:

Liver disease in haemophiliacs.

Using sensitive radioimmunoassays the incidence for Hepatitis B virus (HBV) exposure in haemophiliacs approaches 100 percent as assessed by presence of antibodies to surface antigen (anti-HB_sAg) (Enck et al 1979). The prevalence of the antigen (HB_sAg) ranges from 4 to 9 percent (Self and Hoofnagle 1976). Lack of suitable markers renders difficult a detailed study on the exposure to non-A, non-B viruses (NANB), but strong evidence suggests that they are found in clotting factor concentrates and can be transmitted to haemophiliacs (Mannucci et al 1982, Tabor et al 1983).

In the light of these facts, the relatively low incidence (6-26 percent) of clinical hepatitis in haemophiliacs is surprising (Self and Hoofnagle 1976). However, a number of studies have shown a history of abnormal liver function tests and liver biopsy

histology in the majority of haemophiliacs (Hruby and Schauf 1978). A recent report (Mannucci et al 1982) indicates that in patients with chronic liver disease the disease was non-progressive in individuals having no evidence of hepatitis B or δ virus markers. This indicates that NANB might not pose as big a threat as these other agents.

Acquired immunodeficiency syndrome (AIDS) and haemophilia.

AIDS is a recently recognised and relatively poorly understood syndrome associated with abnormalities of immunoregulation and a profound susceptibility to opportunistic infections. It is eventually fatal in many patients (Marx 1982). One report has included three haemophiliacs among cases of the disease (Centers for Disease Control 1982) and the number has subsequently grown. More disturbingly, other reports (Lederman et al 1983, Menitove et al 1983) have reported abnormalities in the immune status of haemophiliacs as revealed by laboratory tests that are similar to but not of the same degree as found in AIDS patients.

These reports have shown abnormalities in lymphocyte function in patients receiving freeze-dried concentrate with normal values in patients receiving cryoprecipitate (Lederman et al 1983). The most commonly measured parameter is the ratio of helper to suppressor cells and as far as abnormalities in this

ratio are concerned, the plasma source - voluntary or commercial - does not seem to matter (Cable et al 1983, Froebel et al 1983). However, data from this rather non-specific test cannot be extrapolated to the actual clinical manifestation of AIDS and there seems little doubt that American concentrates now constitute a high-risk product. Although more patients need to be studied before definite conclusions can be drawn, it seems that AIDS poses a potentially major threat for haemophiliacs.

Measures to decrease the possibility of viral contamination of therapeutic products.

The dangers of hepatitis transmission and the new threat posed by AIDS, necessitate measures to decrease the possibility of viral contamination of factor VIII concentrates. Some recent studies cast doubts on certain long-held beliefs in this area. Although early reports suggested that plasma from paid donors exhibits a higher incidence of hepatitis transmission than plasma from voluntary donors (Sgouris and Wickerhauser 1973) a recent survey on Australian haemophiliacs cast some doubt on this assumption (Rickard et al 1982). All blood products in Australia are furnished from a voluntary donor system, yet liver abnormalities were found in 67% of severe and 45% of mild haemophiliacs. A study on Edinburgh haemophiliacs showed that the introduction of sensitive assays for HB_sAg for screening donor

plasma also had no effect on the prevalence of infection as determined by liver function tests (Ludlam et al 1982).

Heat treatment at 60°C is the traditional way of sterilising the main plasma protein fraction - albumin - but is not such a straightforward option with a protein as labile as factor VIII. Both VIII:C and VIII:CAg have been shown to be extremely labile at high temperatures (Furlong and Peake 1983) and for pasteurisation to work, ways of protecting the molecule must be found. The first heat-treated factor VIII concentrate had sucrose added to the solution to stabilise the factor VIII:C (Heimberger et al 1981). Yields achieved, however, are of the order of 5%. Some success has been claimed in heating lyophilised concentrate (Rubenstein 1981) with retention of activity. Infectivity studies with chimpanzees (the only suitable model for hepatitis as it is the only mammal in which the infection has been described) tend to support claims that these procedures result in reduction in infectivity. A new method involving sorbitol and glycine stabilisation of a fibrinogen-depleted cryoprecipitate extract has been described (Macleod et al 1983). This has allowed the standard pasteurisation of 60°C for 10 hours to be carried out with retention of 75% of the starting VIII:C, while inactivating several model viruses added to the system (Foster et al 1983c).

Alternatives to heat treatment include the addition of HB_sAg immunoglobulin to concentrates (Brummelhuis et al 1983) and combined treatment with ultraviolet irradiation and chemical agents and detergents (Prince et al 1983). The AIDS problem has highlighted the necessity for developing general ways of inactivating viruses rather than specific removal of one type of virus. The well-tried method of heat-treatment seems to be the best approach in this regard, but other less well validated methods may be acceptable provided they lead to a product with the same characteristics.

II. Other Hazards:

Development of VIII:C inhibitors.

It is still not known why about 10% of haemophiliacs develop inhibitors to factor VIII:C following replacement therapy (Deykin 1974). Development of such inhibitors constitutes an obvious therapeutic problem. The use of massive doses of conventional high-purity concentrate in an attempt to overcome inhibitors puts a strain on the supply of a limited resource, and such therapy often induces an anamnestic response (Rizza and Matthews 1982). The recent development of 'polyelectrolyte' porcine factor VIII:C (Hyate, Speywood Laboratories) has had some success while avoiding the problems previously associated with animal concentrates. Complications using the product have been reported however (Exner

and Rickard 1983).

The use of activated prothrombin complex concentrates first reported in 1972 (Fekete et al 1972) has led to intensive investigation of such materials for this purpose (Abildgaard et al 1980). Normal prothrombin complex concentrates have been shown to be equally effective (Lusher et al 1983) and this finding is difficult to reconcile with hypotheses linking the effectiveness of these products with their content of activated clotting enzymes. Recently it has been shown that the significant amounts of VIII:CAg found in these concentrates (Onder and Hoyer 1979) is bound to phospholipid at a site close or identical to the antibody binding site (Barrowcliffe et al 1983). This finding suggests a mechanism for the action of these products, and has implications in devising better concentrates for tackling this problem.

Blood group antibodies.

Haemolysis due to the presence of anti-A and anti-B in factor VIII preparations has been reported (Orringer et al 1976) but does not necessarily depend on the concentrations of these antibodies (Seeler 1976). Some companies supply 'isoagglutinin-free' or group-specific concentrates but such a policy adopted generally would be too restrictive.

It has been shown that prior mixing of multiple ungrouped donations of plasma resulted in

TABLE 1-7 AVAILABILITY OF FACTOR VIII PRODUCTS

Countries Reporting	Cryo-precipitate	Concentrate
Sufficient Products Available	25	19
Insufficient Products Available	16	18
Not Used	2	6
Total Number of Countries Reporting	43	43

TABLE 1-8 SOURCE OF FACTOR VIII FOR COUNTRIES SUFFICIENT IN CONCENTRATE

Source	Number of Countries
Domestic	6
Imported	3
Domestic and Imported	10
Total	19

Both tables taken from Britten (1983)

high concentrations of anti-A in the final concentrate, whereas processing of single donations to cryoprecitate resulted in much lower concentrations (Smith et al 1980). This suggests that formation of a macromolecular A substance - anti-A complex results in such a complex being concentrated in the final product, possibly by cryoprecipitation during fractionation. Resolution of the dried concentrate then dissociates the complex releasing the antibody. Processing of single donations or group specific pools avoids this problem.

(G) THE PRESENT STUDY

Despite the advances which have been described, there is a world-wide shortage of factor VIII products for haemophilia treatment. Tables 1-7 and 1-8 show the results of an investigation carried out by the World Federation of Haemophilia (Britten 1983). It can be seen that less than half the countries reported having sufficient factor VIII concentrate, and only 14% of countries reported producing enough concentrate themselves to meet their needs.

Moreover, 80% of the world's factor VIII concentrate is produced from plasma collected in the United States by plasmapheresis of paid donors. Countries which lack the technology to produce their own materials have to rely on importation of these expensive products. In the writer's own country of Malta a population of 25 haemophilia A patients and 3 von Willebrand's disease

patients is supplied with one million units of factor VIII yearly in the form of imported concentrate, at a cost of about 150,000 U.S. dollars. Such an outlay of scarce foreign currency on a small number of patients provides a heavy burden on the limited resources of underdeveloped nations. These financial considerations ignore the ethical problems associated with providing patients with materials derived from commercial plasma sources, problems which are once again highlighted by the AIDS problem.

The range of concentrates now available are usually manufactured by bulk processing of frozen plasma on the 1000 kg scale. After the preliminary extraction of factor VIII, such plasma is then further fractionated to immunoglobulin and albumin by industrial Cohn fractionation. These procedures are carried out by large industrial plants, both for commercial and state-funded concerns. Small scale processing is still largely made up of single donation or small pool cryoprecipitate made by blood banks. The disadvantages of cryoprecipitate have already been outlined. Some attempts have been made to overcome some of these by e.g. lyophilisation to enable more convenient storage and reprecipitation in the cold to improve purity (Milligan et al 1981, Smit-Sibinga et al 1981). However, freeze-dried concentrates in use by technologically unadvanced countries are still mainly supplied through large scale industrial fractionation in the developed countries, in the form of materials derived from commercially acquired plasma.

There thus exists a need to develop approaches for producing factor VIII concentrates that do not rely on present methods based on industrial fractionation of bulk plasma. These methods need to have the following characteristics:

(1) They have to be adapted to small scale processing (5-50 kg scale) such that they can be carried out in local blood banks.

(2) They must be able to produce factor VIII at a high yield, in order to make the most out of the limited plasma available.

(3) They must be based on technology that is simple and cheap to set up and maintain.

(4) They must fulfil all the pharmaceutical criteria for a good product, such that they are an acceptable substitute for commercially produced materials.

(5) They must allow use of the residual plasma after factor VIII extraction as a volume expander or possibly to be used to derive other products.

It is the object of this study to examine the possibilities for improved procurement of factor VIII with the above considerations in mind, ie. to make possible a level of self-sufficiency in factor VIII concentrates for countries without access to high technology. In the course of this work, optimal conditions for processing plasma to cryoprecipitate have been derived after investigation of such variables as storage temperatures, storage periods, anticoagulants, freezing rates etc. The use of

alternatives to cryoprecipitation as a means of concentrating factor VIII have been examined and a new method involving the use of hydrophilic polymers to precipitate factor VIII from unfrozen plasma has been developed. Methods of further purification have also been investigated. The influence of ABO blood group and of drug induced enhancement on levels of factor VIII in starting plasma have also been studied.

Based on these findings, recommendations will be made for improved procurement of factor VIII from blood donations.

CHAPTER II

GENERAL MATERIALS AND METHODS

(A) PREPARATION OF NORMAL POOLED PLASMA STANDARD

The plasma was prepared for use as a routine coagulation control in the South-East Scotland Blood Transfusion Service. Each of 12 dry Fenwal double blood bags were injected with 50 ml of trisodium citrate/Hepes anticoagulant (106 mM citrate, 210 mM Hepes) on the morning of collection. 450 ml of blood were collected into each bag. Selection of donors was on the basis of blood group only; 6 group A and 6 group O were chosen. Plasma was separated at 4°C by centrifugation in a Damon (IEC) GPR 6000 centrifuge at 4,200 r.p.m. for 10 minutes. Plasma was expressed into the secondary packs which were then centrifuged at the same speed for 10 minutes to obtain platelet poor plasma. This was pooled into a 5 litre pack and mixed thoroughly. A platelet count was performed at this stage and was typically less than 5×10^9/litre. The plasma was aliquoted (0.8 ml) into prelabelled polypropylene test tubes using a Watson Marlow 10 channel peristaltic pump, silicone rubber tubing being used throughout.

Screw caps were fitted and all tubes placed in the vapour phase of a liquid nitrogen refrigerator. Several coagulation parameters including assays for factor VIII related activities were tested at least 5 times and a mean value was calculated. A supply of the material was placed in -40°C storage every week for routine use. A fresh preparation was made every 4 months.

(B) ASSAY OF FACTOR VIII COAGULANT ACTIVITY (VIII:C)

The assay used in this study was the one-stage assay based on the partial thromboplastin time (Hardisty and Macpherson 1962). In this assay system, dilutions of test material are used to clot samples of plasma totally deficient in VIII:C. The substrate plasma supplies all the factors required for the formation of fibrin except VIII:C which is supplied by the sample. The first stages of the coagulation sequence (see Figure 1-2) are initiated by addition of a negatively charged surface and phospholipid acts as a substitute for the platelet surface. Both these reagents were added in the form of the General Diagnostics APTT reagent, which provides these components as a mixture of micronised silica and cephalin. Calcium chloride was added to initiate the later stages of coagulation. Reagent addition and optical detection of the end point (fibrin formation) were done using a automated clot-detector, the Coag-a-pet (General Diagnostics). The assay can be summarised as follows:

*0.1 ml test dilution (in 50 mM Tris, 60 mM NaCl pH 7.4)

*0.1 ml substrate plasma

+0.1 ml activator (Silica / Cephalin)

↓ Incubated 5 minutes

+0.1 ml 33 mM $CaCl_2$

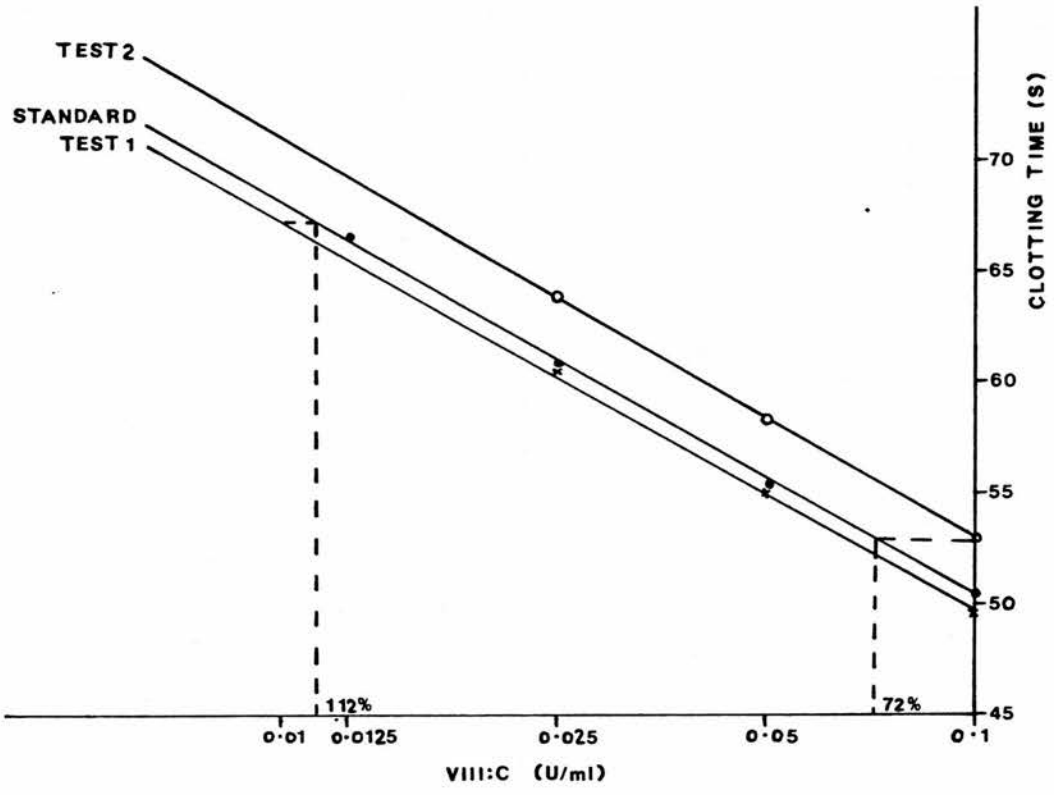
FIBRIN CLOT detected optically

* dispensed manually

\+ dispensed automatically

FIGURE 2-1

ESTIMATION OF VIII:C CONTENT IN COAGULATION ASSAY
Standard and test lines are shown, with intrapolation from test to standard to obtain test VIII:C content as a % of standard.


TEST 2
STANDARD
TEST 1
70
65
60
55
50
45
CLOTTING TIME (S)
112%
72%
0·01
0·0125
0·025
0·05
0·1
VIII:C (U/ml)

Calculation of sample potencies: A standard of known VIII:C content was assayed at 3 dilutions (1/10, 1/20 and 1/40) along with 3 dilutions of each sample. Clotting times obtained for each dilution were plotted on semi-log graph paper with log concentration being plotted against clotting time. Straight lines were drawn through the points for each sample. Lines for standard and test samples should be parallel - Figure 2-1.

By interpolation from test to standard lines, the potency of the test samples relative to the standard was estimated. As shown in Figure 2-1, this results in 112% for test 1 and 72% for test 2 samples. Using these and the assigned standard potency, the test potency was estimated e.g. if in Figure 2-1,

standard = 0.7 u/ml

then test 1 = 112% of standard = 0.78 u/ml

test 2 = 72% of standard = 0.5 u/ml

Dilutions of test samples were chosen so as to result in clotting times which overlap with those of the standard. Thus in samples containing low VIII:C levels, e.g. cryosupernatants, lower dilutions were used e.g. 1/5, 1/10, 1/20. The opposite was done with samples of high VIII:C content. When assaying concentrates, a concentrate standard supplied by the National Institute of Biological Standards and Control, London, was used. This was first diluted to 1 u/ml and then diluted as for plasma. Samples of concentrate for assay were also pre-diluted to about 1 u/ml.

Repeated assay of the same sample gave a between run geometric coefficient of variation for the assay of 5%.

Preparation of artificial factor VIII deficient substrate for use in the one-stage assay.

As congenitally deficient substrate plasma was not always available, assays were sometimes performed with artificial factor VIII deficient substrate. This material was generously provided by Mrs. Brenda Griffin of the Headquarters Unit Laboratory of the Scottish National Blood Transfusion Service and was prepared as follows:

Components

1. Serum: Serum was obtained from human blood donations and supplied by the Reagents Laboratory of the South-East Scotland Blood Transfusion Service. To each 100 ml of serum, 1 ml sodium azide (20% solution) was added to prevent bacterial growth. The serum was incubated at 37°C for 72 hours, citrated with 1 part 0.55 M trisodium citrate to 24 parts serum, and kept for a further 72 hours at 4°C. It was then centrifuged at 15,000 r.p.m. for 2 hours at 4°C to allow removal of lipid and the infranatant was collected. Tests found no detectable VIII:C in the material. Batches were also tested for prothrombin content, as a final level of > 20% average normal was necessary for satisfactory results in the VIII:C assay.
2. Factor V preparation: (Nyman 1970) Oxalated bovine

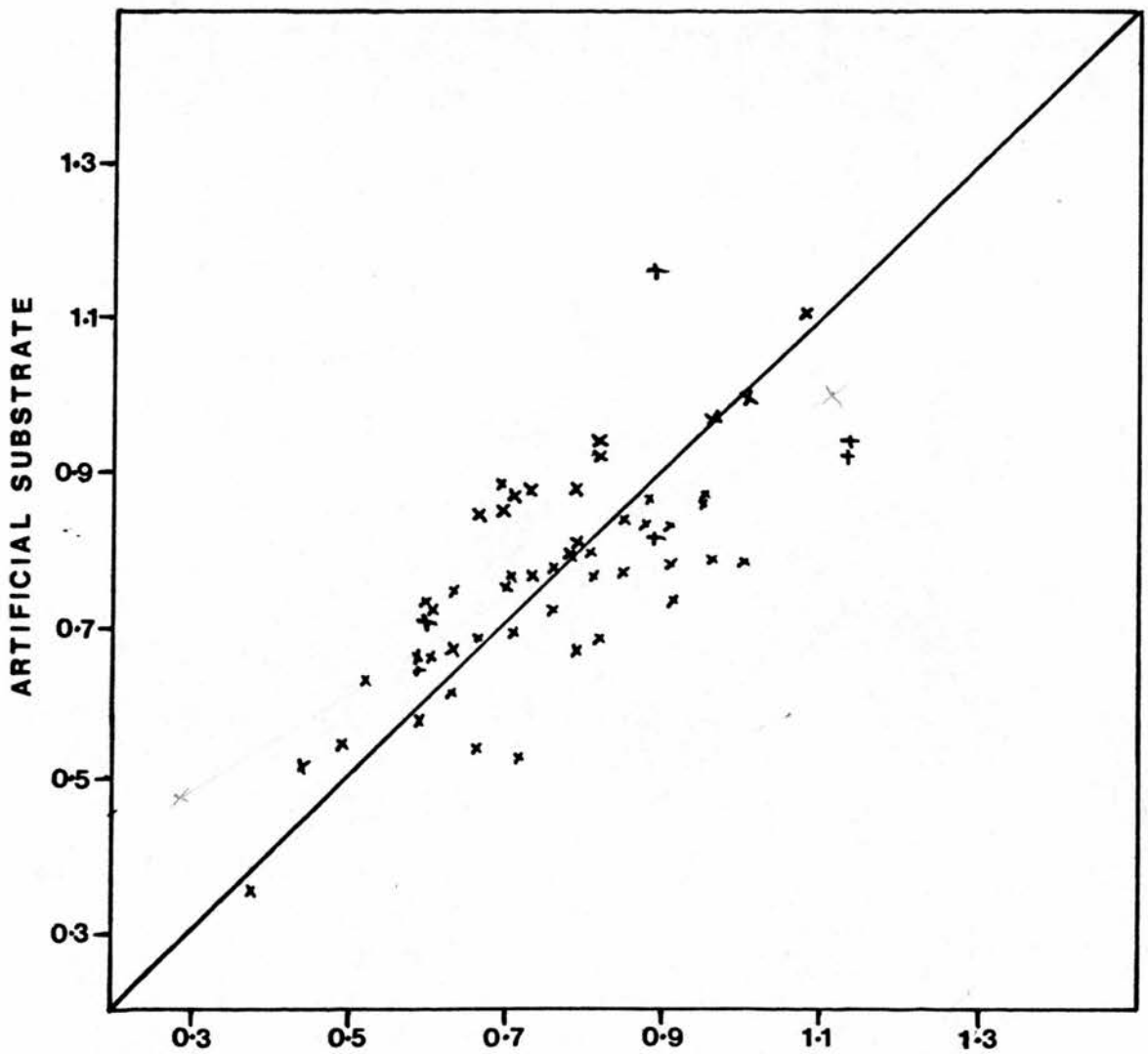
plasma was obtained from the West of Scotland Blood Transfusion Service or from the local abattoir. It was kept frozen at -40°C until used. The plasma was thawed at 37°C and recalcified with 1/4 of its volume of 0.1 M $CaCl_2$. It was incubated at 37°C for 4 hours. The clot was loosened from the sides of the bottle and removed by centrifugation at 13,000 r.p.m. for 10 minutes at 20°C. The serum was then adsorbed with barium sulphate (50 mg/ml) for 45 minutes at room temperature with constant stirring. The barium sulphate was removed by centrifugation at 13,000 r.p.m. for 10 minutes at 0°C. In later preparations, the barium sulphate step was omitted. The serum was immediately brought to 35% saturation with ammonium sulphate (i.e., 19.4 g (NH_4) SO_4 per 100 ml super-natant). Mixing was carried out for 30 minutes at 0°C (in ice-water mixture in a 4°C cold room) and the precipitate was then removed by centrifugation at 13,000 r.p.m. for 15 minutes at 0°C. The supernatant was brought to 55% saturation with ammonium sulphate (i.e., 11.8 g (NH_4) SO_4 per 100 ml 35% supernatant), mixed for 45 minutes at 0°C and centrifuged at 13,000 r.p.m. for 15 minutes at 0°C. The precipitate was dissolved in a minimum amount of 1 part physiological saline to 4 parts distilled water and dialysed for 24 hours at 4°C against 50 mM Tris, 150 mM NaCl pH 7.4. The factor V preparation was citrated with 1/24 of its volume of 0.55 M trisodium citrate and

FIGURE 2-2

COMPARISON OF ARTIFICIAL AND CONGENITAL SUBSTRATE PLASMA IN VIII:C ONE-STAGE ASSAY

Plasma samples were assayed against a known standard using both types of substrate.

Also shown is the statistical analysis of the data obtained.



n = 57 Congenital Mean = 0·767 ± 0·165 U/ml
Artificial Mean = 0·772 ± 0·142 U/ml

r = 0·7434 (p < 0·001) y = 0·642 x + 0·280

concentrated on an Amicon cell (YM 10 membrane) to 10 mls. Factor V estimation revealed that the preparation had about 34 times the potency of normal pooled plasma. Factor VIII:C content was about 7% of normal pooled plasma but as the preparation was diluted 1/50 in the final substrate, VIII:C content was only 0.14%. Assay for thrombin revealed levels of less than 0.01 u/ml.

3. Fibrinogen: Lyophilised human fibrinogen (Kabi Grade L) was dissolved in the serum preparation to give a concentration of 2 g/litre. The VIII:C level was 0.3%.

Preparation of substrate.

Fibrinogen was dissolved in serum and the mixture was centrifuged to deposit any undissolved material. 7 ml of the factor V preparation was added and the mixture was aliquoted, frozen at -40°C overnight and then cooled further in liquid nitrogen. The aliquots were then freeze-dried for 2 days. One sample of freeze-dried material was reconstituted with distilled water and had a VIII:C level of 0.75% of normal pooled plasma (assayed using congenitally deficient substrate) and a factor V level of 82%.

Validation of the VIII:C assay using artificial substrate.

Figure 2-2 shows the results of an exercise designed to assess the behaviour of the VIII:C assay using artificial substrate plasma. A good agreement with the assay using congenital substrate was obtained, showing the

TABLE 2-1 REMOVAL OF HEPARIN FROM PLASMA USING ECTEOLA** CHROMATOGRAPHY

Sample	n	Protein Content (mg/ml)		VIII:C (u/ml)	
		Pre-Chromato-graphy	Post-Chromato-graphy	Pre-Chromato-graphy	Post-Chromato-graphy
Plasma	11	59 ± 6	57 ± 4	0.92 ± 0.19	0.89 ± 0.23
Cryosupernatant	5	53 ± 7	51 ± 11	0.25 ± 0.06	0.22 ± 0.09
Concentrate*	3	12 ± 2.8	11 ± 3.5	5.7 ± 0.9	5.9 ± 1.4

Results show mean ± standard deviation. Samples were assayed for VIII:C and total protein, and heparin was then added to a final concentration of 10 u/ml. ECTEOLA chromatography was then performed as described, and samples were again assayed for VIII:C and total protein.

* Concentrate samples consisted of cold precipitates of cryoprecipitate prepared as in Chapter VI.

** Epichlorohydrin Triethanolamine Cellulose

suitability of the artificial substrate.

Assay of VIII:C in samples containing additives.

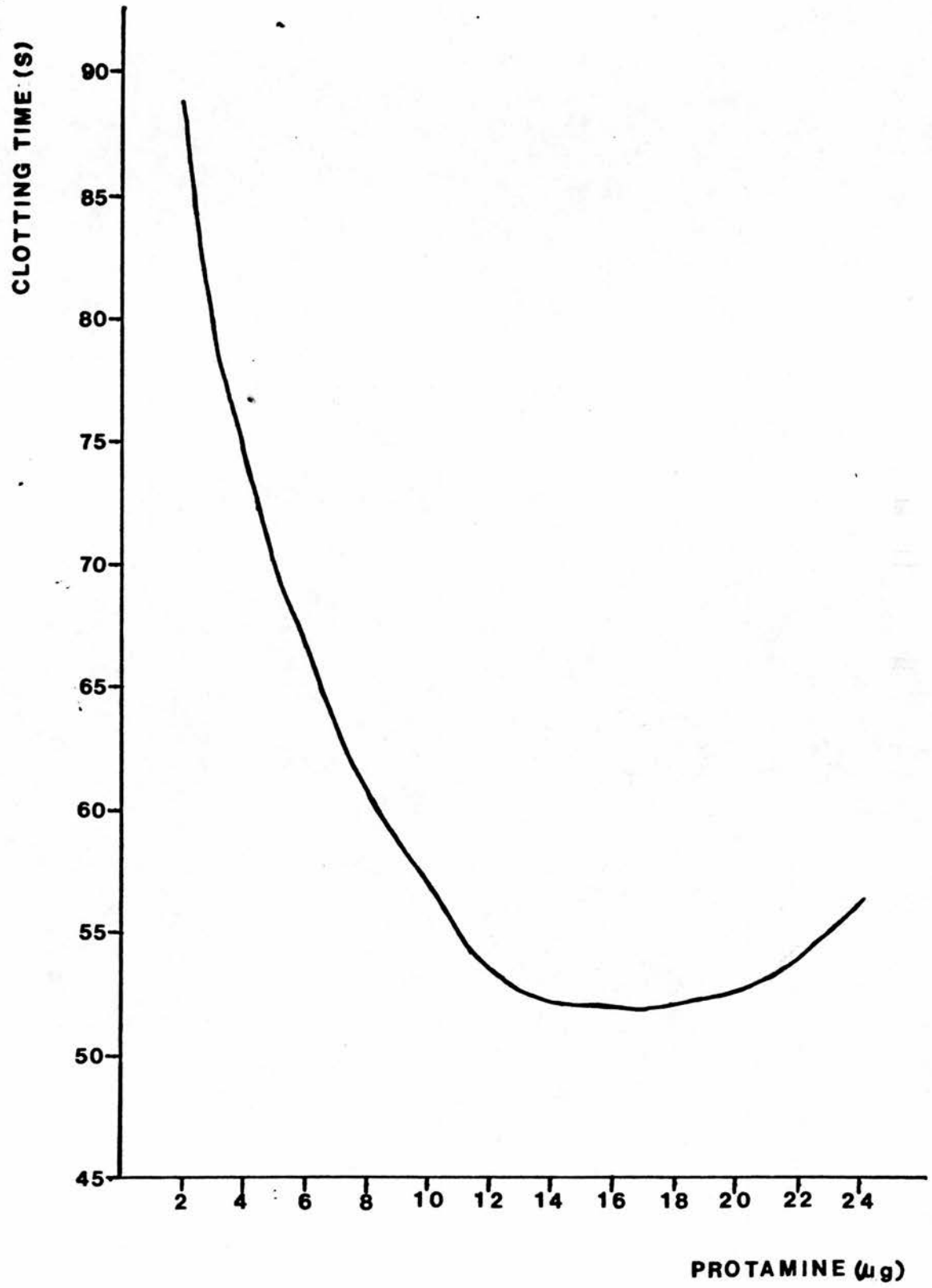
1. Heparin: In studies utilising heparin as a plasma anticoagulant or additive (Chapters III, V and VI) it was necessary to remove heparin from the samples prior to VIII:C assay. This was done using either of two methods.

   (a) ECTEOLA - cellulose chromatography (Thompson and Counts 1976): ECTELOA-cellulose (100 g, SIGMA) was suspended to form a slurry and then poured into a sintered glass funnel. It was washed alternately with 200 ml of 0.5 M NaOH, 0.5 M HCl and 0.5 M NaOH. Between these washes, one litre washes with distilled water were applied. Following the last water wash, the cellulose was equilibrated with 50 mM Tris, 150 mM NaCl pH 7.4 and stored at 4°C. For use in removing heparin from samples, the cellulose was packed into 3 ml plastic columns placed vertically into glass tubes and the buffer wash was allowed to flow into the bed. 1 ml samples containing heparin were then applied to the top of the cellulose and allowed to flow into the bed. When pigmented plasma was visible in the effluent, another 1 ml of the sample was applied. The first 1 ml of pigmented effluent was discarded, as it was found to be diluted with column buffer. The second 1 ml was collected and assayed for total protein and VIII:C. Table 2-1 shows that this method effectively removed heparin

FIGURE 2-3

TITRATION OF HEPARIN-CONTAINING PLASMA WITH PROTAMINE SULPHATE

Plasma contained 10 u/ml of sodium heparin and was titrated with a 1 mg/ml protamine solution as described in the text.


CLOTTING TIME (S)
90
85
80
75
70
65
60
55
50
45
2
4
6
8
10
12
14
16
18
20
22
24
PROTAMINE (µg)

from samples of varying VIII:C content. However, it was rather laborious and needed at least 2 mls of sample per assay. The method was furthermore unsuitable for samples containing high amounts of fibrinogen, which tended to adsorb to the cellulose and clog up the column. For assay of large numbers of samples, the method involving protamine neutralisation was found preferable.

(b) Protamine-titration: This was performed as described by Rock (1983a) with some modifications. Plasma samples containing heparin at concentrations of 2 to 12 u/ml were processed as follows: 0.1 ml aliquots of the plasma samples were placed in 2 ml plastic tubes. Amounts of 2 to 24 µl of a stock solution of 1 mg/ml protamine sulphate were added to 12 such tubes, so that tubes with 2, 4, 6.... 24 µl of protamine sulphate were thus made up. The contents were then mixed and made to 1 ml with 50 mM Tris, 60 mM NaCl pH 7.4. 0.1 ml of each of these mixtures was then assayed for VIII:C in duplicate as described above. For each sample, the 12 individual mixtures containing heparin and different amounts of protamine were assayed together, with a series of dilutions of standard plasma being included in the assay run. The clotting times shortened and reached a plateau with increasing amounts of protamine sulphate, excessive amounts tending to increase the clotting time (Figure 2-3). In general, the shortest

TABLE 2-2 ASSAY OF VIII:C IN PLASMA CONTAINING HEPARIN AND HEPARIN/CALCIUM

Sample	VIII:C (u/ml) No Heparin	VIII:C (u/ml) + 5 u/ml Heparin + Protamine Titration	VIII:C (u/ml) + 2 u/ml Heparin + 10 mM Ca^{2+} + Protamine Titration
Plasma Pool 1550	0.88	0.84	-
Plasma Pool 11	0.95	0.98	-
Plasma Pool 12	0.92	0.91	-
Plasma Pool 13	1.1	1.2	1.1
1st Scottish Plasma Standard	0.69	0.71	0.71
11th British Plasma Standard	0.73	0.72	0.74

clotting time was found at a heparin:protamine ratio of 1 u of heparin:15 µg of protamine. The shortest clotting time was used to derive the VIII:C value from the standard curve. Although this method utilised single point determinations instead of parallel line interpolation, assay of known plasma standards, to which heparin had been added, using this technique gave results corresponding closely with the assigned values (Table 2-2). The presence of added calcium, as well as heparin, did not affect the VIII:C determination.

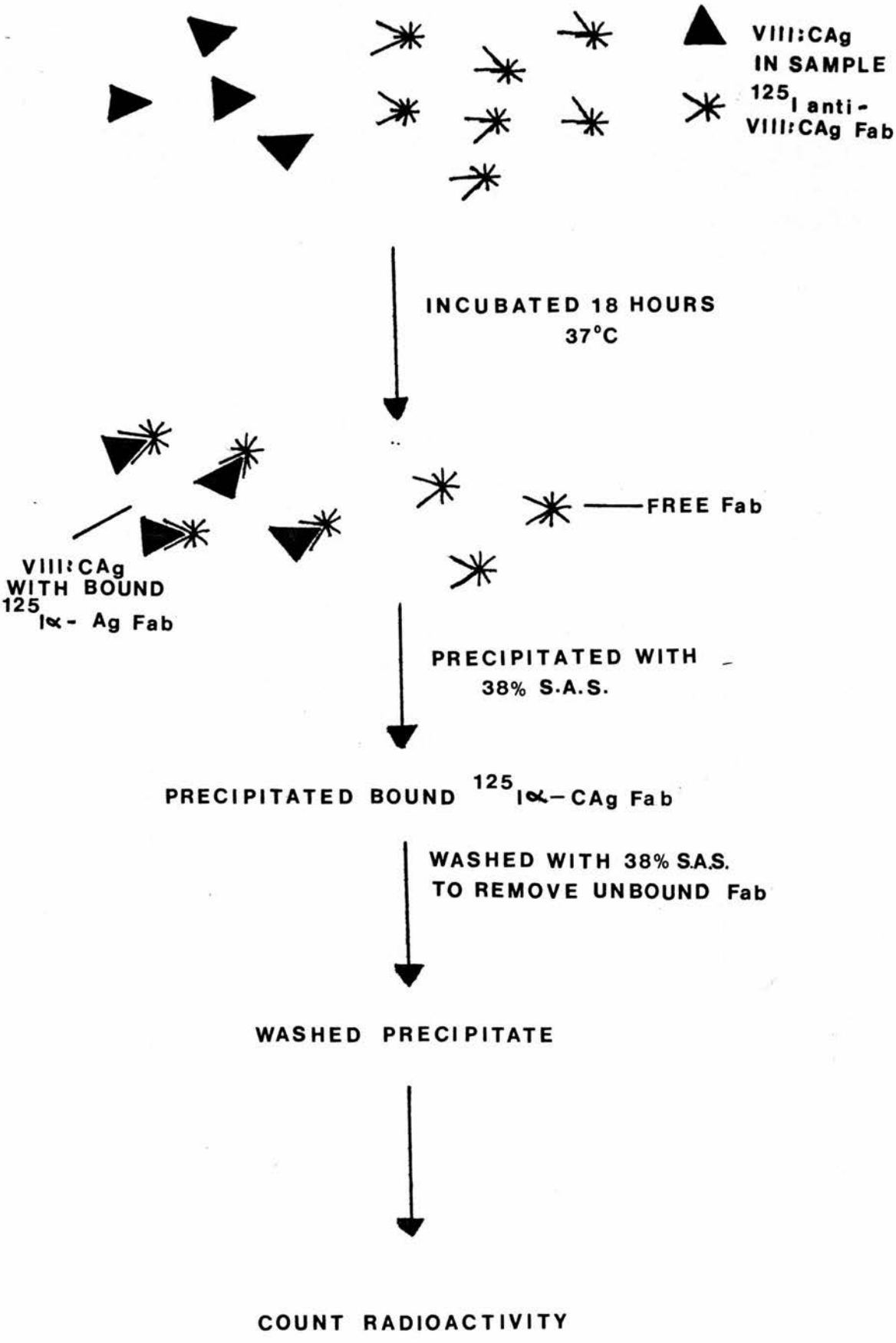
2. Zinc salts: In fractionation experiments using zinc ion precipitation (Chapter VI) it was important to determine whether the added zinc salts had any effect on the VIII:C assay. Addition of 1 mM zinc acetate to samples of concentrate of known potency had no effect on the VIII:C content as determined by the one-stage assay. Linearity and parellelism with respect to the standard curve were also unaffected. It should be noted that 1 mM zinc acetate in the sample was diluted about 600 fold as a result of the assay procedure.

(C) ASSAY OF FACTOR VIII COAGULANT ANTIGEN (VIII:CAg)

To measure VIII:CAg, a one-site fluid phase immunoradiometric assay (IRMA) was used, based on the method of Rotblat and Tuddenham (1981). The principle of the assay is shown in Figure 2-4.

Preparation of stock anti-VIII:CAg reagent: 2 ml of

Figure 2-4 : Principle of the VIII:CAg assay.



plasma from a patient with an inhibitor to VIII:C (2400 Bethesda units/ml) was clotted by incubation at 37°C for 18 hours with 1 u/ml of thrombin. The clot was removed and the serum was dialysed for one hour against 2 litres of 10 mM phosphate buffer, pH 6.5. 20 g of DEAE A25 Sephadex (Pharmacia) were swollen overnight in 0.5 M phosphate, packed into a column and washed with 10 mM phosphate pH 6.5 until the conductivity equalled that of the wash buffer. The serum was pumped through the column which was eluted with wash buffer. 2 ml fractions were collected and the absorbence at 280 nm was measured. Fractions containing unbound protein were pooled, and the protein content was estimated from absorbence at 280 nm, assuming an extinction coefficient of 14 for IgG. The pool was concentrated in an Amicon cell (YM 10 membrane). 4 ml of concentrate were dialysed against 0.1 M acetate buffer pH 4.2 for 3 hours. The IgG was then digested with pepsin at a concentration of 2 mg/100 mg IgG. This was added as a solution of 1 mg/ml in 0.1 M acetate buffer pH 4.2. Cysteine was then added to a final concentration of 10 mM. The mixture was incubated at 37°C for 22 hours after which it was neutralised with solid Tris and the protein content was estimated. The digest was mixed with Protein A Sepharose (Pharmacia) (1 ml packed gel in 50 mM phosphate pH 7.4) for 15 minutes at room temperature. The mixture was centrifuged to deposit the gel with the bound Fc fragments of the IgG. The supernatant protein represented 64% of the total

TABLE 2-3 PREPARATION OF STOCK ANTI-VIII:CAg Fab FROM INHIBITOR PLASMA

Stage of Purification	Volume ml)	Total Protein mg
1. Inhibitor Serum	2.9	112
2. DEAE Unbound Fraction	27.5	24.8
3. Concentrate of (2)	6.7	21.8
4. Pepsin Digest	4	13.8
5. Protein A Unbound Fraction	3.4	8.8
6. (5) Post Dialysis	12.6	8.8

digest, which is the proportion expected if the Fc was removed by protein A and the Fab was left in the supernatant. The supernatant was then dialysed against 50 mM phosphate pH 7.4. The stock Fab reagent thus prepared was stored frozen at -40°C in 20 µl aliquots. Table 2-3 summarises the various stages of the procedure.

Radiolabelling of stock Fab reagent: Labelling of Fab with ^{125}Iodine was done using the chloramine-T method (Greenwood et al 1963) as follows:

Reagents:

Chloramine-T - 50 mg in 10 ml of 50 mM phosphate pH 7.4

KI - 0.1 g in 10 ml of 50 mM phosphate pH 7.4

$Na_2S_2O_5$ - 160 mg in 10 ml of 50 mM phosphate pH 7.4, then 0.1 ml of this in 10 ml of 50 mM phosphate for use.

Method:

20 µl Fab reagent

20 µl ^{125}I (2 mCi)

10 µl Chloramine-T

↓ mixed for 10 seconds

add 0.85 ml $Na_2S_2O_5$

0.1 ml KI

Separate iodinated Fab by gel filtration (Figure 2-5a)

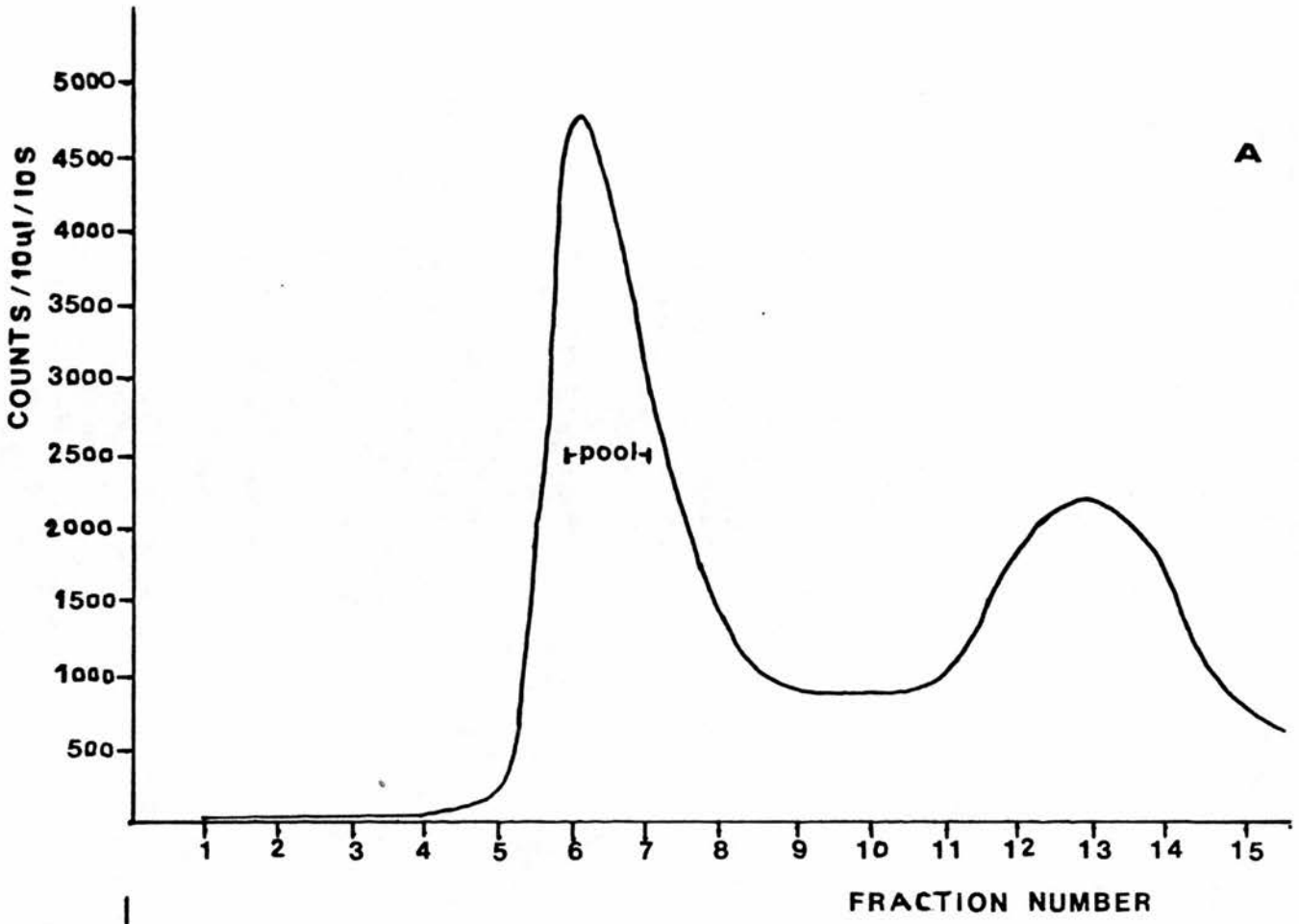
Purification of labelled Fab by immunoaffinity chromatography: 10 ml of intermediate purity factor VIII concentrate (15 u/ml VIII:C, Protein Fractionation Centre, Edinburgh) were coupled to 7.5 g of cyanogen bromide activated Sepharose 4B (Pharmacia). The beads were

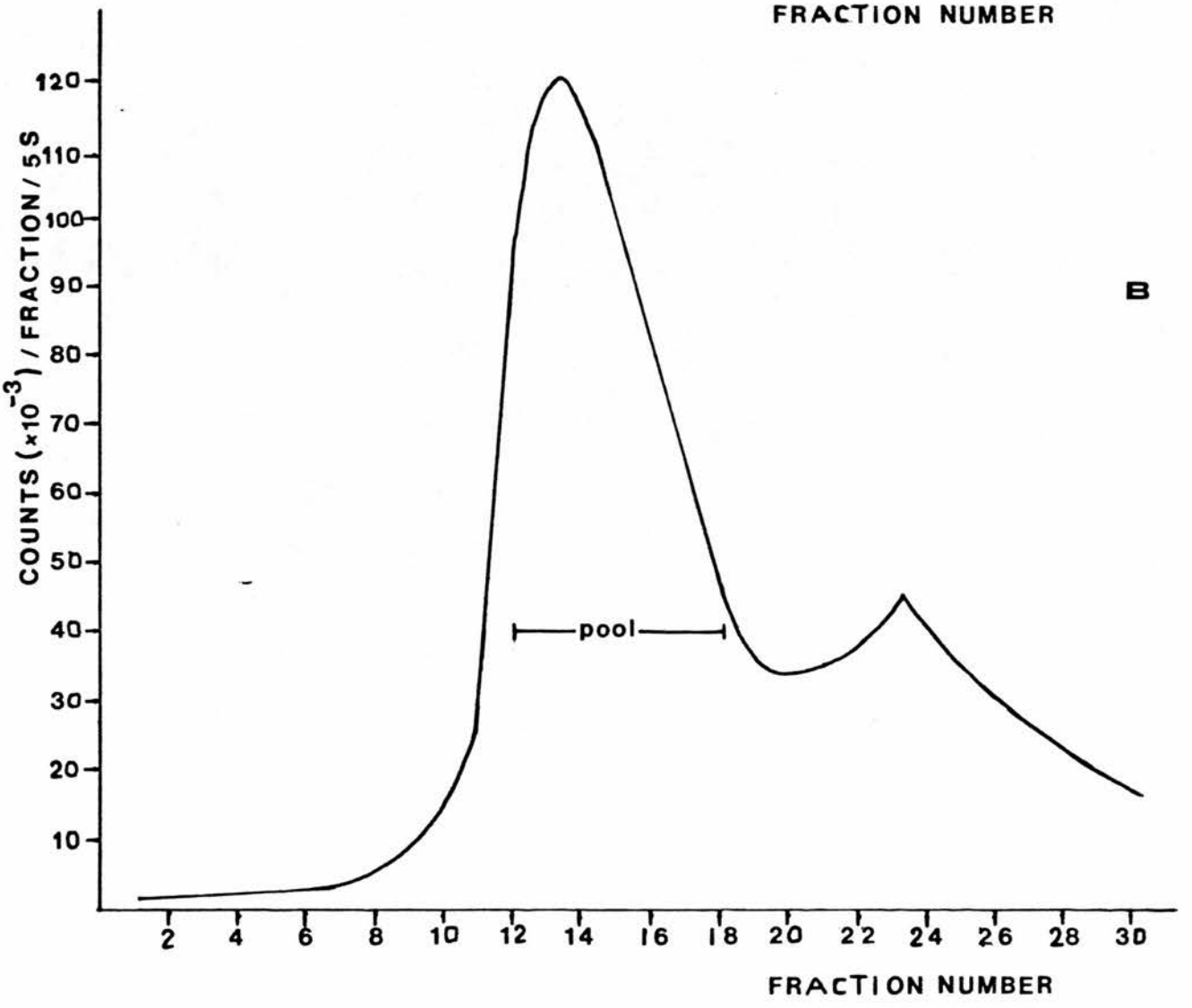
FIGURE 2-5

PREPARATION OF STOCK ^{125}I ANTI-VIII:CAg Fab

(A) shows the gel filtration of the mixture obtained after iodination. Fractions 6 and 7 were pooled and immunopurified as described in the text.

(B) shows the elution of specific ^{125}I anti-VIII:CAg Fab off the immobilised factor VIII column, following application of the acidic buffer.


A
COUNTS / 10µl / 10S
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
pool
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
FRACTION NUMBER


B
COUNTS (×10⁻³) / FRACTION / 5S
120
110
100
90
80
70
60
50
40
30
20
10
pool
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER

swollen in 500 ml of 1 mM HCl, stirred at room temperature for 30 minutes and sedimented by centrifugation. The hydrated beads were resuspended in 1 M NaCl and the pH was adjusted to 7 with 0.1 M $NaHCO_3$. The factor VIII concentrate was added, and the pH was brought to 7.8. The gel was mixed end over end at room temperature for 24 hours and was then filtered and washed on a glass sintered funnel with normal saline. The gel was equilibrated in 0.5 M NaCl, 50 mM phosphate pH 7.5 prior to use in immunopurification.

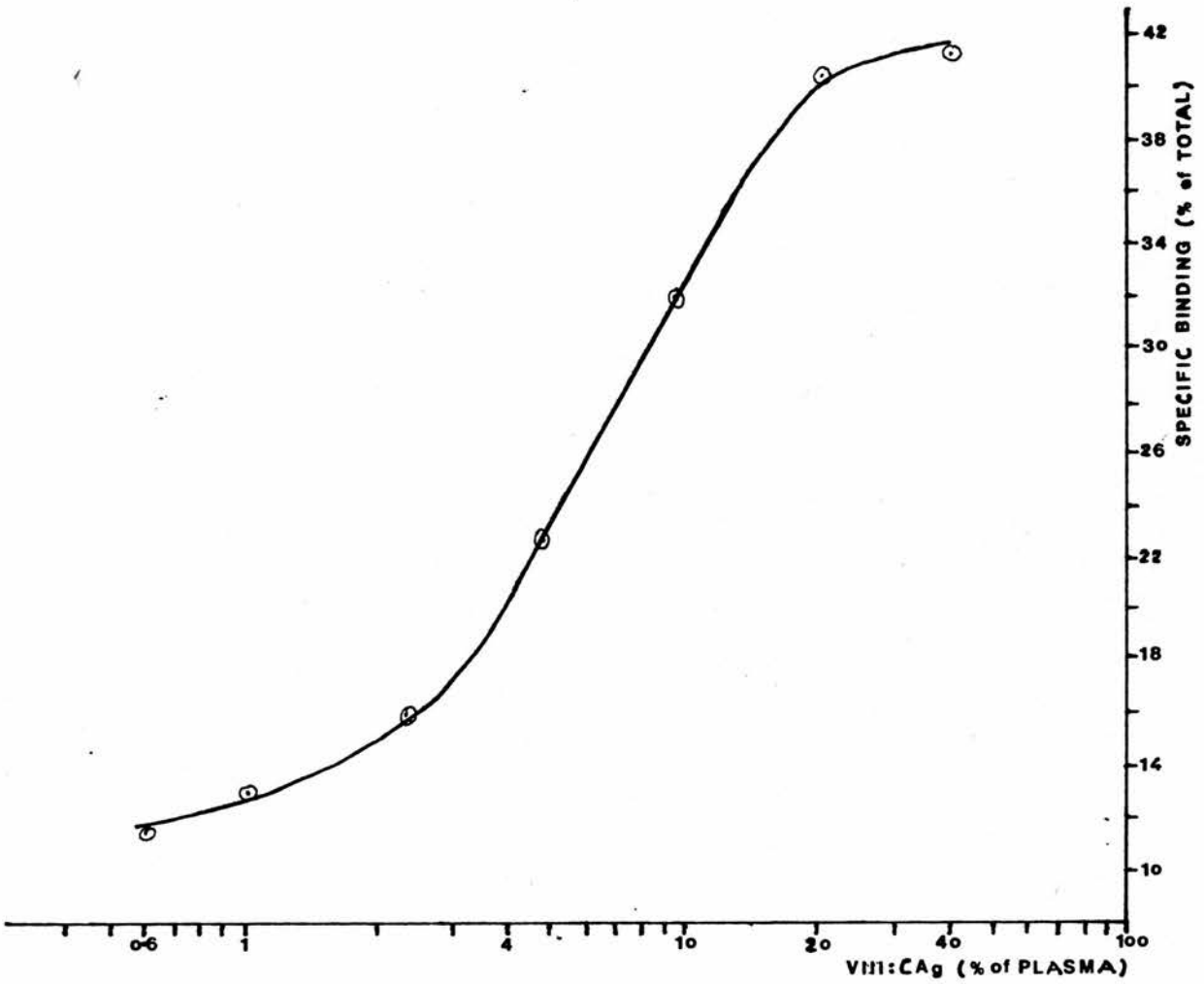
The pool containing the peak fractions of protein-bound radioactivity was mixed with 10 ml of Sepharose-coupled factor VIII concentrate for 2 hours at room temperature. The gel was then packed into a small column and washed with 0.5 M NaCl, 50 mM phosphate pH 7.5. Fractions were collected and the eluted radioactivity was monitored. When the radioactivity had reached a base-line, elution was continued with 50 ml of 0.1 M glycine-HCl pH 2.5. 1 ml fractions were collected. Fractions containing peak radioactivity were pooled (Figure 2-5b) and incubated at 37°C for 30 minutes. The pH of the pool was adjusted to 7.2 with solid $K_2 HPO_4$ and 1 ml of turkey serum was added. For use in the assay this stock ^{125}I anti-VIII:CAg Fab was diluted with 50 mM Tris, 150 mM NaCl 1% turkey serum pH 7.4 until a count of about 2000/100 µl/minute was registered in an NE 1600 gamma-counter.

Assay of factor VIII:CAg: The assay was performed as

FIGURE 2-6

STANDARD CURVE OF THE VIII:CAg IMMUNORADIOMETRIC ASSAY

To estimate sample potency, normal pooled plasma was assumed to contain 1 u of VIII:CAg per ml.


42
38
34
30
26
22
18
14
10
SPECIFIC BINDING (% of TOTAL)
0·6
1
4
10
20
40
100
VIII:CAg (% of PLASMA)

follows:

0.1 ml ^{125}I anti-VIII:CAg Fab

0.1 ml test sample

0.1 ml turkey serum

↓ incubated 18 hours at 37°C

↓ 0.3 ml 76% saturated $(NH_4)_2SO_4$ added

↓ mixture incubated 30 min. at 22°C, centrifuged for 36,000 g min.*

precipitates

↓ washed with 1 ml 38% saturated $(NH_4)_2SO_4$

↓ centrifuged for 35,000 g min.

count radioactivity of washed precipitate.

A standard curve was constructed using normal pooled plasma (Figure 2-6). In general, the assay was sensitive to levels of about 2% of the VIII:CAg level of normal plasma. In assaying samples, 2 dilutions were usually used, suitably estimated to fall on the linear part of the standard curve. The amount present in 1 ml of pooled plasma was taken as 1.U. The assay gave a between assay coefficient of variation of 21% when assaying repeatedly the same sample.

(D) ASSAY OF FACTOR VIII RELATED ANTIGEN - VIIIR:Ag

Initial assays in this study used the Laurell technique with a commercial antiserum to VIIIR:Ag (Hoechst). During the course of the work, an antiserum to VIIIR:Ag was raised and used to set up Laurell and IRMA assays for this protein.

Purification of VIIIR:Ag for use as an immunogen: Several

* Except where it is otherwise specified, in this thesis total centrifugal force is expressed as g min, being the centrifugation in g per minute multiplied by the time in minutes. Speeds and times ranged from 1200 to 5000 g per minute and 10 to 60 minutes respectively.

FIGURE 2-7

GEL-FILTRATION OF 4% FICOLL-70 PRECIPITATE

Gel filtration was performed on Sepharose CL-4B as described in the text.

Fractions containing VIIIR:Ag without detectable fibrinogen were pooled and used for immunisation.

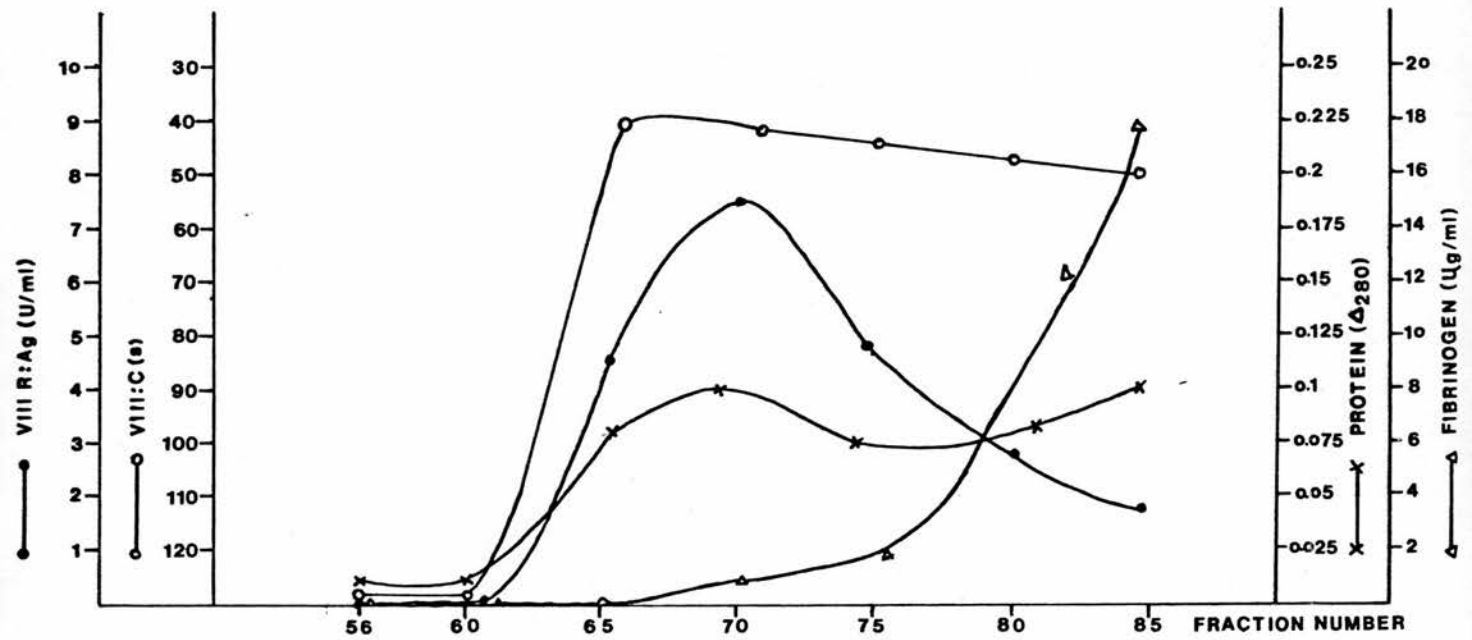

VIII R:Ag (U/ml)
VIII:C (s)
PROTEIN (Δ280)
FIBRINOGEN (μg/ml)
FRACTION NUMBER
56
60
65
70
75
80
85

TABLE 2-4 PURIFICATION OF VIIIR:Ag

Stage of Purification	Volume ml	VIII:C u/ml (Yield)	VIIIR:Ag u/ml (Yield)	Fibrinogen mg/ml (Yield)	Fibronectin mg/ml (Yield)	Protein mg/ml (Yield)
Pooled Plasma	1755	0.95 (100)	0.84 (100)	2.5 (100)	0.36 (100)	52.3 (100)
4% Ficoll Supernatant	1742	0.18 (20.3)	0	1.6 (65)	0.05 (13)	44.7 (92)
4% Ficoll Precipitate	45	28.9 (78)	31 (95)	25.3 (26)	11.2 (80)	45.4 (2.2)
V_o Peak From Gel Filtration	46	N.D.	3.5 (11)	< 0.02	< 0.004	0.04
Final Preparation	15	N.D.	7.5 (7.6)	< 0.02	< 0.004	0.09

N.D. = not determined

FIGURE 2-8

SDS-PAGE OF PURIFIED IMMUNOGENS

Factor VIIIR:Ag and fibronectin were purified as described in the text. Samples of each protein were electrophoresed in the presence of SDS. The preparations were reduced with 1% mercaptoethanol. The gels were 6% acrylamide.

The respective tracks from left to right were:

(1) Pharmacia low molecular weight marker proteins

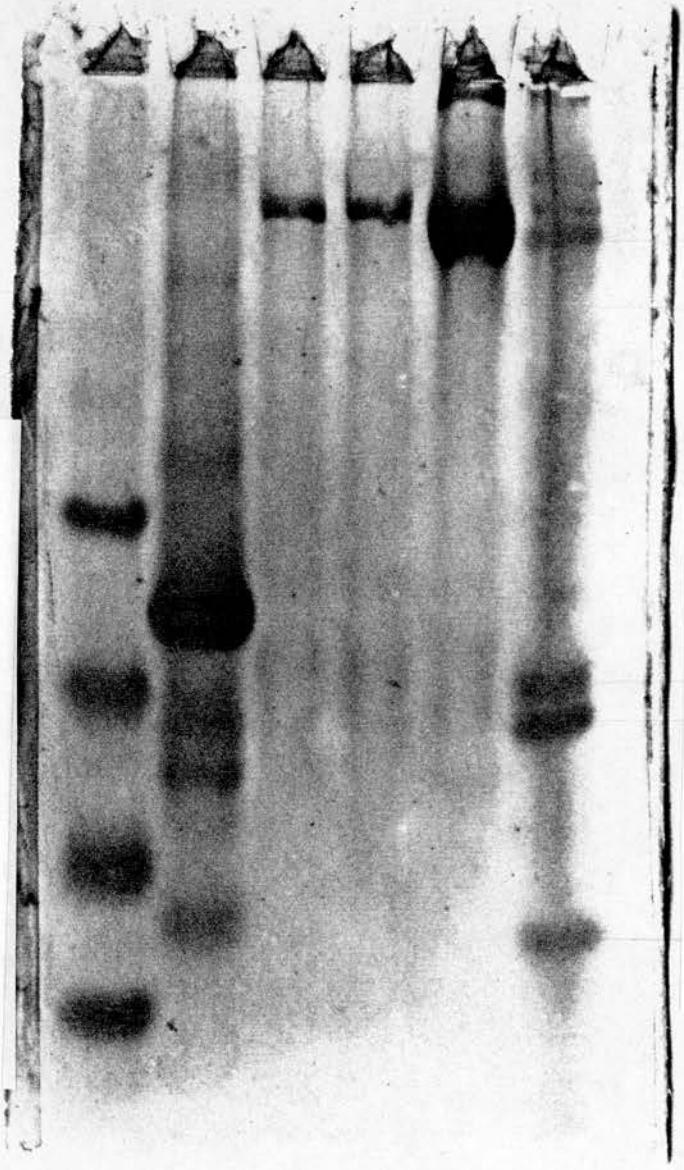
(2) Protein sample not relevant to the present study

(3) } Factor VIIIR:Ag

(4)

(5) Fibronectin

(6) Pharmacia high molecular weight marker proteins.



'HOSPHORYLASE B

LBUMIN

WALBUMIN

ARBONIC ANHYDRASE

FERRITIN (HALF-UNIT)

ALBUMIN

CATALASE

LACTATE
DEHYDROGENASE

preparations of VIIIR:Ag were made utilising cryo-precipitate or plasma as starting material. A typical preparation is described below:

Six donations of blood were taken from routine collection sessions and the plasma separated by centrifugation for 90,000 g min. at 4°C. The plasma was pooled into polycarbonate bottles and precipitated with 4% Ficoll 70 at 0°C for 2 hours (Chapter V). The resulting precipitate was harvested by centrifugation for 30,000 g min. at 0°C, dissolved in 35 ml of 15 mM citrate, 150 mM NaCl pH 6.9. The dissolved precipitate was applied to a column (2.5 x 65 cms) of Sepharose CL-4B (Pharmacia) equilibrated with citrate-saline-3 mM sodium azide (RATNOFF ET AL 1969). Figure 2-7 shows the elution profile for this column. Fractions were screened for VIII:C by coagulation assay, for VIIIR:Ag, fibrinogen and fibronectin by Laurell assay and for total protein by absorbence at 280 nm. Fractions containing VIIIR:Ag without detectable fibrinogen were pooled. The pool was concentrated by dialysis against 50% PEG 6000 in citrate-saline, and the concentrate was stored frozen at -40°C. Prior to immunisation, samples were dialysed against 150 mM NaCl, 50 mM phosphate pH 7.5 without any azide. Table 2-4 summarises the purification procedure. SDS-polyacrylamide gel electrophoresis in the presence of reducing agent confirmed that the VIIIR:Ag had a subunit molecular weight of 220,000 (Hoyer 1981) (Figure 2-8).

Production of antiserum: The purified VIIIR:Ag was used to immunise 6 New Zealand white rabbits. Immunisation

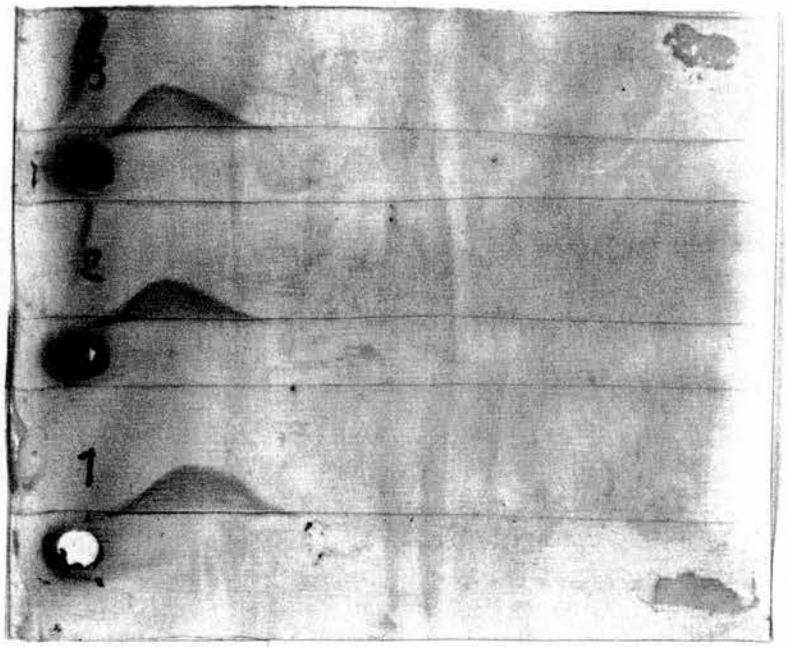
FIGURE 2-9

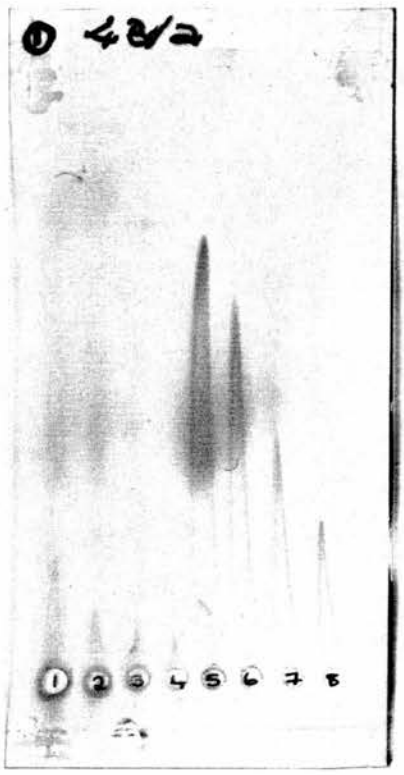
IMMUNOELECTROPHORESIS USING RABBIT ANTISERA TO VIIIR:Ag

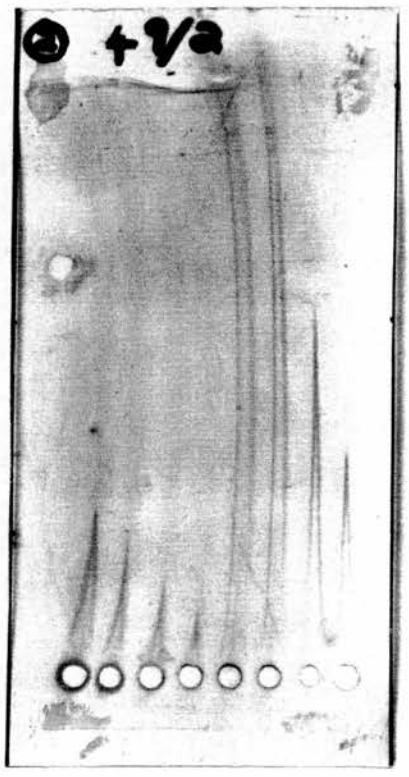
2DIEP and Laurell electrophoresis are shown for bleeds from three different rabbits.

In Laurell electrophoresis, samples were four dilutions each of normal plasma and factor VIII concentrate respectively.

Normal plasma was electrophoresed in 2DIEP.


3
2
1


① 4월2
1 2 3 4 5 6 7 8


② 7월2


③ 10월2

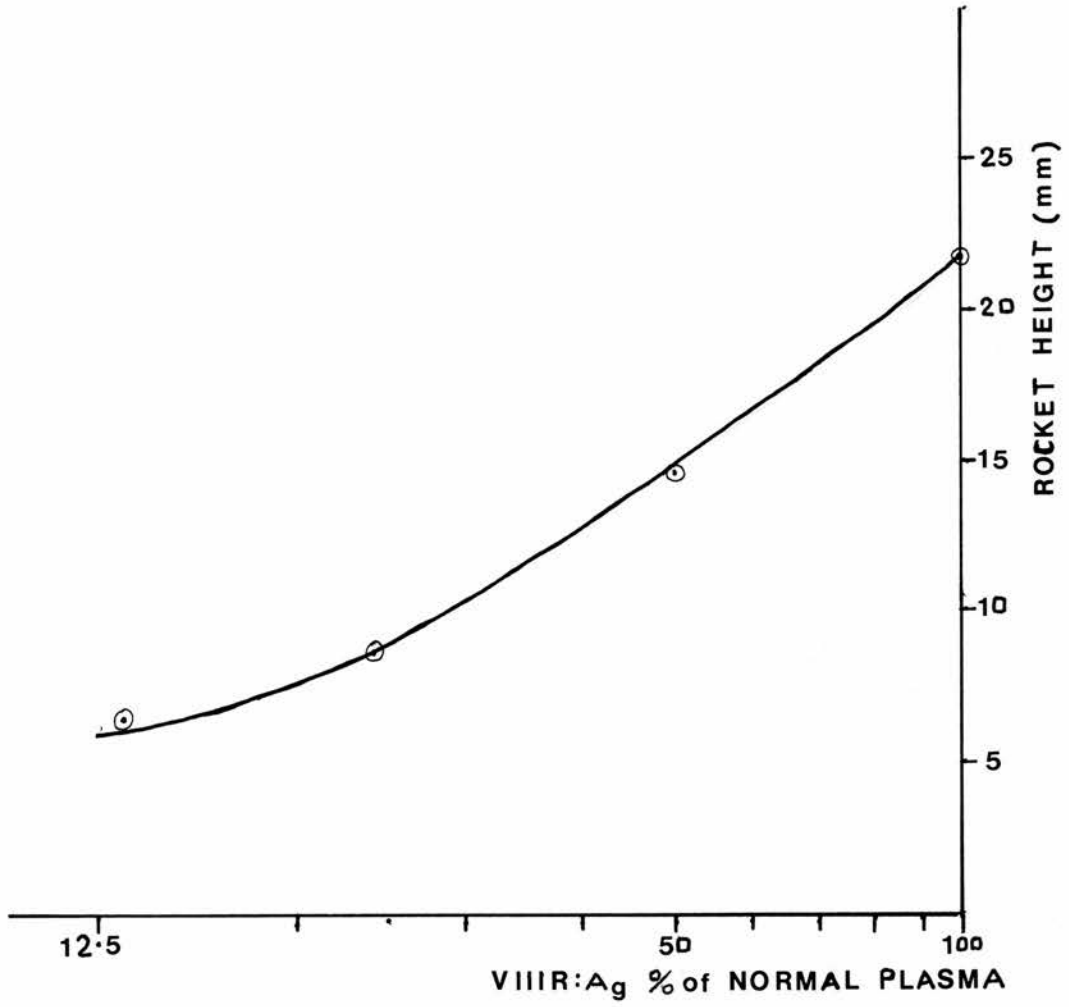
was by subcutaneous 4-site injections consisting of 150 µg of antigen in Freund's complete adjuvant. Boosts were given 2 months and 3 months after the primary injection and consisted of 65 µg of antigen in Freund's incomplete adjuvant. The rabbits were bled at intervals to test the sera for reactivity with VIIIR:Ag. This was done by incorporating the antisera in agarose and testing them in the Laurell technique with pooled plasma and factor VIII concentrate (Figure 2-9). Specificity was tested using two-dimensional immunoelectrophoresis (2DIEP) and immunodiffusion. In 2DIEP, the antisera produced only one precipitin arc against normal pooled plasma (Figure 2-9). Using immunodiffusion one or two faint precipitin lines were sometimes observed in addition to the main line due to VIIIR:Ag. However, a validation exercise carried out by the Coagulation Laboratory of the South-East Scotland Blood Transfusion Service demonstrated good agreement between Laurell assays using a commercial anti-VIIIR:Ag antiserum and the pooled antiserum from the rabbit bleeds (Mackay 1984, personal communication)*. The antisera were thus considered suitable for use. The pooled antiserum was heat inactivated at 56°C for 30 minutes, aliquoted and stored frozen at -40°C for use.

Assay for VIIIR:Ag - Laurell assay (ZIMMERMAN ET AL 1971): Commercial and later on locally produced antisera to VIIIR:Ag were used to set up an electroimmunoassay using the technique of Laurell (1966). Agarose (Bio-Rad) was dissolved to a final concentration of 1% in buffer consisting of 0.1 M Tris,

* In this exercise, the anti-serum failed to produce a precipitin 'rocket' with plasma from a patient with severe von Willebrand's disease, thus demonstrating specificity towards VIIIR:Ag.

FIGURE 2-10

STANDARD CURVE OF THE VIIIR:Ag LAURELL ASSAY


25
20
15
10
5
ROCKET HEIGHT (mm)
12·5
50
100
VIIIR:Ag % of NORMAL PLASMA

3.2 mM ethylenediaminetetraacetic acid (disodium salt) 15 mM boric acid pH 8.9, by heating to 80°C. The agarose was then cooled to 56°C and antiserum to VIIIR:Ag added and mixed. The volume of antiserum was 0.4% and 0.09% v/v of agarose for the commercial and locally produced antisera respectively. The agarose was poured onto a piece of Gel-Bond (Miles Laboratories) to a thickness of 2 mm and allowed to set. Wells were punched along one end of the plate and filled with 10 µl of sample. Electrophoresis was then carried out at 120 V for 18 hours using a Pharmacia 2000/300 power supply with the plate on a Pharmacia 3000 flat-bed support. Wicks at each end of the plate consisted of 3 thicknesses of Whatman No.1 filter paper and connected the plate to two 300 ml reservoirs of the buffer used for making up the agarose.

After electrophoresis, the plate was dried under a stream of warm air and stained using 0.25% Coomassie Blue R in 14% methanol, 7% acetic acid. The plate was then destained using 14% methanol, 7% acetic acid. The height of the 'rockets' (see Figure 2-9) were measured and a standard curve was plotted using dilutions of normal pooled plasma (100%, 50%, 25%, 12.5%). Rocket height was plotted against the logarithm of the concentration. The standard curve was linear for VIIIR:Ag concentrations of between 100% and 25% of normal pooled plasma (Figure 2-10). Samples for assay were pre-diluted to levels of about 1 u/ml and then assayed using

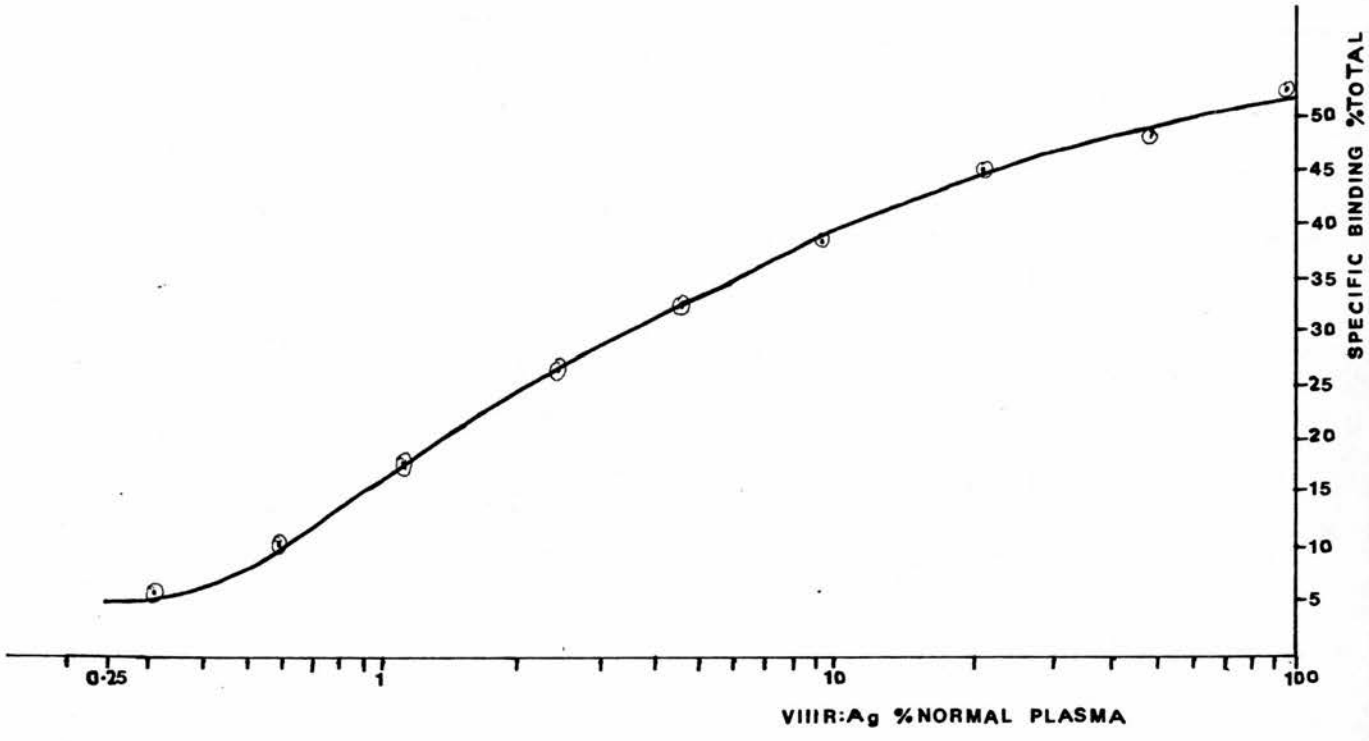
two separate dilutions. The coefficient of variation of the assay was 8%.

Two-dimensional immunoelectrophoresis (ZIMMERMAN ET AL 1976): An example of this is shown in Figure 2-9. Agarose was dissolved to a concentration of 1% in buffer composed of 22 mM Tris, 45 mM glycine, 28 mM barbitone, 15 mM sodium barbitone pH 8.6 and poured on Gel-Bond as in the Laurell assay. A 40 µl well was punched at one laterial edge of the plate and was filled with sample. The plate was then subjected to electrophoresis at 200 V until a 4% bovine serum albumin (BSA) - bromophenol blue marker had migrated 5 cms from the well. The flat-bed support was cooled internally with running tap water throughout the procedure. Electrophoresis was then discontinued and a strip of gel was removed from above the strip adjacent to the sample well. This was replaced by agarose into which antiserum to VIIIR:Ag had been incorporated. The anti-serum concentration was 2.5 fold that used in the Laurell assay. The plate was then electrophoresed for 18 hours at 120 V as for the normal technique, with the first dimension strip running along the cathode edge. The plate was then stained as described above.

IRMA: Rabbit antiserum to VIIIR:Ag (DAKO antibodies) was used to develop an IRMA for VIIIR:Ag using the same principle as for the VIII:CAg IRMA. Production of immuno-purified ^{125}I anti-VIIIR:Ag Fab fragments was exactly as for the production of the VIII:CAg reagent. The assay was also similar, except that ^{125}I anti-VIIIR:Ag

FIGURE 2-11

STANDARD CURVE OF THE VIIIR:Ag IMMUNORADIOMETRIC ASSAY


SPECIFIC BINDING %TOTAL
50
45
40
35
30
25
20
15
10
5
0·25
1
10
100
VIIIR:Ag %NORMAL PLASMA

Fab was used in the incubation mixture. The assay was sensitive to levels of VIIIR:Ag of 0.5% of normal plasma (Figure 2-11) and was thus 50 times more sensitive than the Laurell technique. Another advantage over the Laurell assay was the ability of the IRMA to measure VIIIR:Ag in samples which produced faint immunoprecipitates notably cryosupernatants.

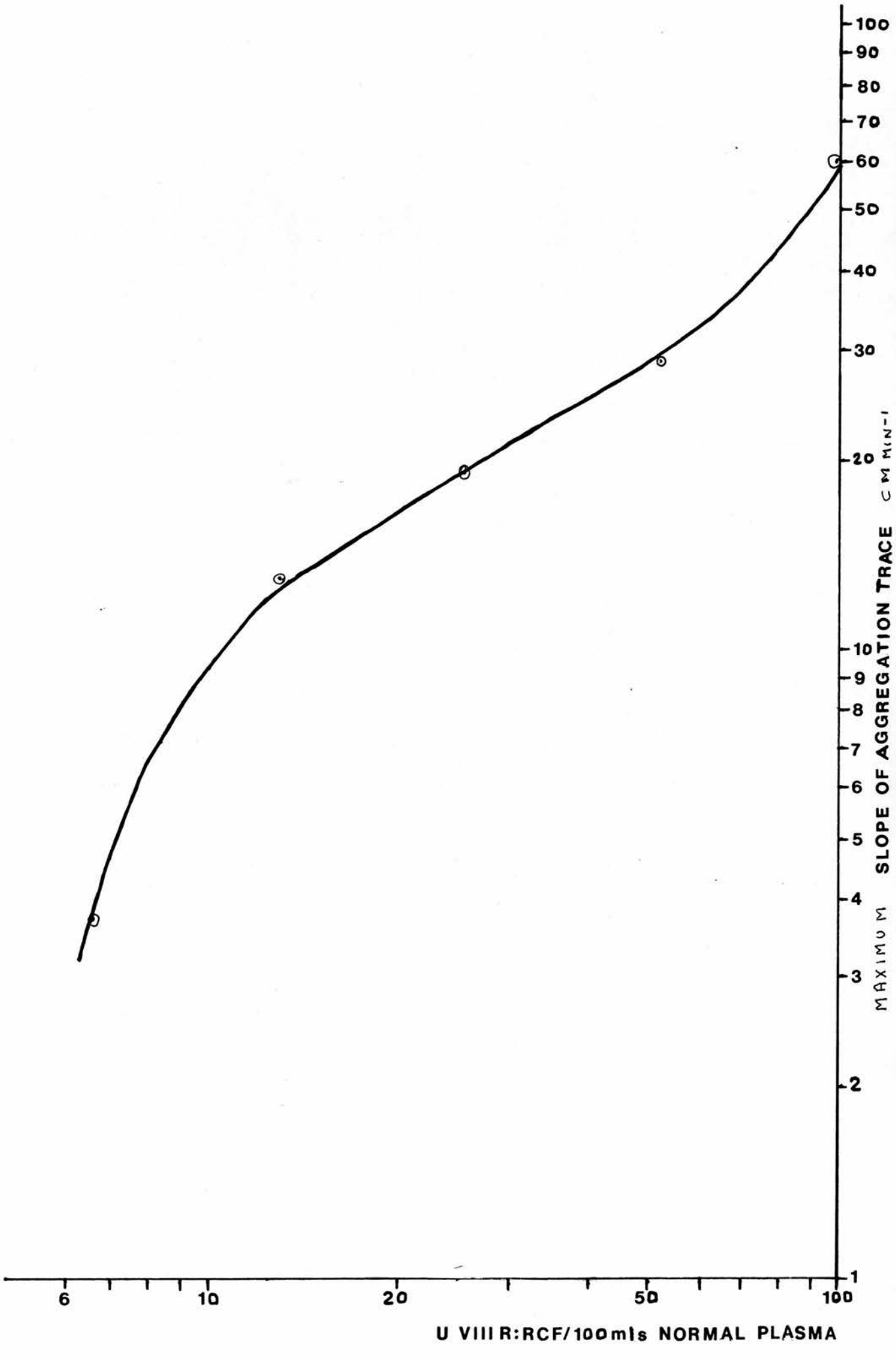
(E) ASSAY OF FACTOR VIII-RELATED RISTOCETIN COFACTOR (VIIIR:RCF)

The assay used was based on the method of Macfarlane et al (1975). Platelets fixed with formaldehyde are aggregated with ristocetin in the presence of factor VIII. The rate and extent of aggregation depend on the amount of VIIIR:RCF present.

Reagents - Fixed platelets: 72 hour old platelet concentrates were centrifuged for 6000 g min. at room temperature to remove residual red cells. The platelet concentrates were then incubated for one hour in plastic tubes at 37°C after which an equal volume of 2% formalin in 10 mM Tris, 1 mM EDTA, 150 mM NaCl pH 7.5 was added (giving a final formaldehyde concentration of 0.4%). The fixed platelets were stored at 4°C for 45 hours, after which they were washed 3 times in 0.15 M phosphate pH 7.3. After the third wash the platelets were resuspended in phosphate buffer at a concentration of 100 x 10^9/litre and an equal volume of 20% dimethyl sulfoxide (DMSO) in phosphate buffer was added. The suspension was kept at room temperature for one hour and was then frozen in 20 ml

FIGURE 2-12

STANDARD CURVE OF THE VIIIR:RCF ASSAY


100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
MAXIMUM SLOPE OF AGGREGATION TRACE C M MIN-1
6
10
20
50
100
U VIII R:RCF/100mls NORMAL PLASMA

aliquots in a -40°C freezer. For use, the platelets were thawed at 37°C, centrifuged for 27,000 g min. at room temperature and resuspended for 30 minutes in phosphate buffer. This procedure was repeated 4 times and the platelets were resuspended in Tris-EDTA-saline buffer at a concentration of 300 x 10^9/litre.

Ristocetin: A 30 mg/ml solution in normal saline was used.

Buffer for dilutions was Tris-EDTA-saline made 4% w/v with BSA.

The assay was performed as follows:

0.7 ml platelets

30 µl ristocetin

↓ stirred in 37°C heating-block

↓ transferred to aggregometer

↓ test sample added (0.1 ml)

aggregation recorded on Mallin aggregometer.

The initial rate of aggregation was calculated by determining the slope of the steepest portion of the recorded trace. This was plotted against concentration using a log-log plot. A standard curve consisting of doubling dilutions of normal plasma was constructed during each run of assays, (Figure 2-12) and sample potencies were estimated from the linear part of the standard curve. Samples were assayed at 2 or more dilutions. The assay coefficient of variation was 10%.

(F) ASSAY FOR FIBRINOGEN

In this study, two different fibrinogen assays

were used. Plasma samples could be assayed by the Ellis and Stransky (1961) method, based on the opacity of a clot generated by addition of thrombin. This technique was found to be unsuitable for samples containing low fibrinogen levels e.g. column fractions, which tended to produce wispy clots that could not be quantitated. Concentrates containing high fibrinogen levels also could not be assayed accurately by this method as spuriously low levels were obtained (see below). Therefore, in assessing fibrinogen distribution during fractionation, a Laurell assay using a commercially purchased antiserum was used throughout.

Ellis and Stransky method: In this technique, 0.5 ml of the sample being assayed was diluted in 5.5 ml of 28 mM sodium barbitone, 50 mM NaCl pH 7.2 and well mixed. 50 µl of calcium-thrombin reagent (0.56 M $CaCl_2$ and 5 u/ml thrombin) were then added to 3 ml of the mixture in a plastic cuvette and mixed by inversion, care being taken not to allow any air bubbles in the mixture. The residual 3 ml were placed in a plastic cuvette without any reagent. After 20 minutes at room temperature, the absorbence of the test mixture at 470 nm was read using a CECIL spectrophotometer. The control mixture was used as blank. The absorbence was multiplied by 8.63 to obtain the fibrinogen concentration in g/litre.

Electroimmunoassay: A commercial antiserum to human fibrinogen (Hoechst) was used to set up a Laurell assay as described previously for VIIIR:Ag. Antiserum

TABLE 2-5 COMPARISON OF FIBRINOGEN ESTIMATION METHODS

Sample Type	n	Ellis & Stransky mg/ml	Electroimmunoassay mg/ml
Plasma	13	2.8 ± 0.35 (100)*	2.6 ± 0.52 (100)
Cryosupernatant	5	2.2 ± 0.2 (70)	2.1 ± 0.4 (70)
Cryoprecipitate	5	3.7 ± 1.4 (16)	5.3 ± 1.8 (25)

Plasma was fractionated using the thaw-siphon technique.

Results show mean ± standard deviation.

* Figures in brackets show fibrinogen yield relative to starting plasma.

concentration was 0.53%. Standards were dilutions of 1/50, 1/100, 1/200 and 1/400 of normal pooled plasma. Samples were assayed at two dilutions.

Table 2-5 shows a comparison between the two fibrinogen estimation methods for assays on plasma fractions derived by cryoprecipitation (Chapter IV). Using the Ellis and Stransky technique tended to give low fibrinogen estimates in the cryoprecipitates, although good agreement between the two methods was observed for plasmas and cryosupernatants. In order to use the same method for each step of fractionation procedures, the Laurell assay was used throughout.*

(G) ASSAY FOR FIBRONECTIN

As with VIIIR:Ag, initial assays for fibronectin used a Laurell technique with a commercially purchased antiserum. During the course of the study, fibronectin was purified and used to raise an antiserum.

Preparation of purified fibronectin: The method used was based upon that of Hayashi and Yamada (1982) employing sequential affinity chromatography on immobilised gelatin and heparin. These were coupled to cyanogen-bromide activated Sepharose (see purification of ^{125}I anti-VIII:CAg Fab above). Several purifications were done during the course of the study, using plasma or fibronectin-rich fractions as starting material. A typical preparation is described as follows:

A litre of thaw-siphon cryoprecipitate prepared in the Components Division of the South-East Scotland Blood

* Although the antiserum used could have cross-reacted with fibrinolytic products of fibrinogen/fibrin, this was not considered a major handicap as (for the purpose of this thesis), such products had characteristics similar to fibrinogen.

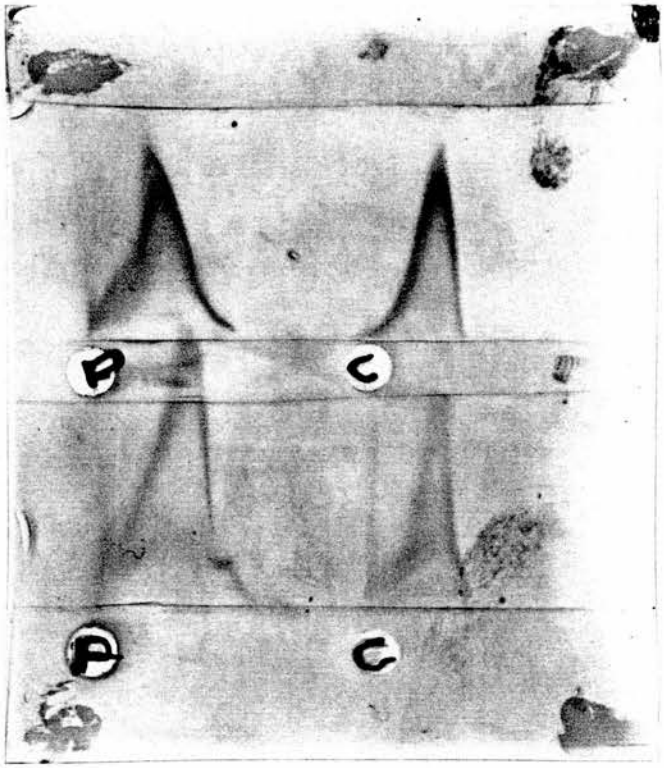
Transfusion Service was thawed and thoroughly mixed. PEG 6000 (SIGMA) was added, as solid flakes with constant stirring, to a final concentration of 5% w/v. Stirring was continued for 15 minutes at room temperature and the precipitate was harvested by centrifugation for 22,500 g min. at 20°C. The precipitate was dissolved at 37°C in 100 ml of 150 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5. Insoluble material was removed by centrifugation and the supernatant was applied to a column of gelatin-agarose (bed volume 90 ml) which had been pre-washed with 200 ml of 4 M urea, 10 mM Tris pH 7.5 followed by 500 ml of 150 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5. After the sample had passed through, the column was washed with 300 ml of 500 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5 and washing was continued with 150 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5 until the absorbance at 280 nm of the effluent had reached a constant basal level. The gelatin-agarose column was then eluted with 4 M urea, 10 mM Tris pH 7.5 and the eluted protein was applied to a heparin-agarose column (bed volume 170 ml) which had been pre-washed with 400 ml of 500 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5 followed by 400 ml of 150 mM NaCl, 10 mM Tris, 5 mM EDTA pH 7.5. Bound fibronectin was eluted off the column with 500 mM NaCl, 10 mM Tris pH 7.5, concentrated by addition of solid $(NH_4)_2SO_4$ to 40% saturation and dialysed against 150 mM NaCl, 10 mM Tris pH 7 for 18 hours at 4°C. It was stored in polypropylene (Nunc) tubes in liquid nitrogen. Yields were typically 55% from cryoprecipitate.

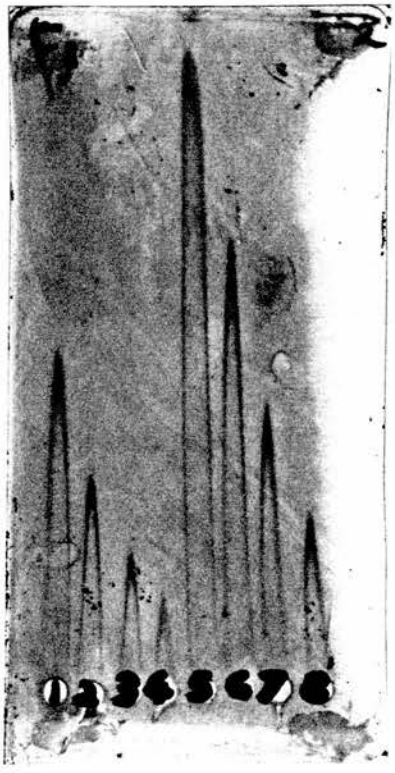
FIGURE 2-13

IMMUNOELECTROPHORESIS USING RABBIT ANTISERA TO FIBRONECTIN

2DIEP and Laurell electrophoresis were performed. In 2DIEP, plasma (P) and purified fibronectin (C) were electrophoresed with locally produced (upper strip) and commercial (lower strip) antisera to fibronectin incorporated in the second dimension.

In Laurell electrophoresis, samples were four dilutions each of normal plasma and clinical factor VIII concentrate respectively.


A
C
A
C


0 1 2 3 4 5 6 7 8

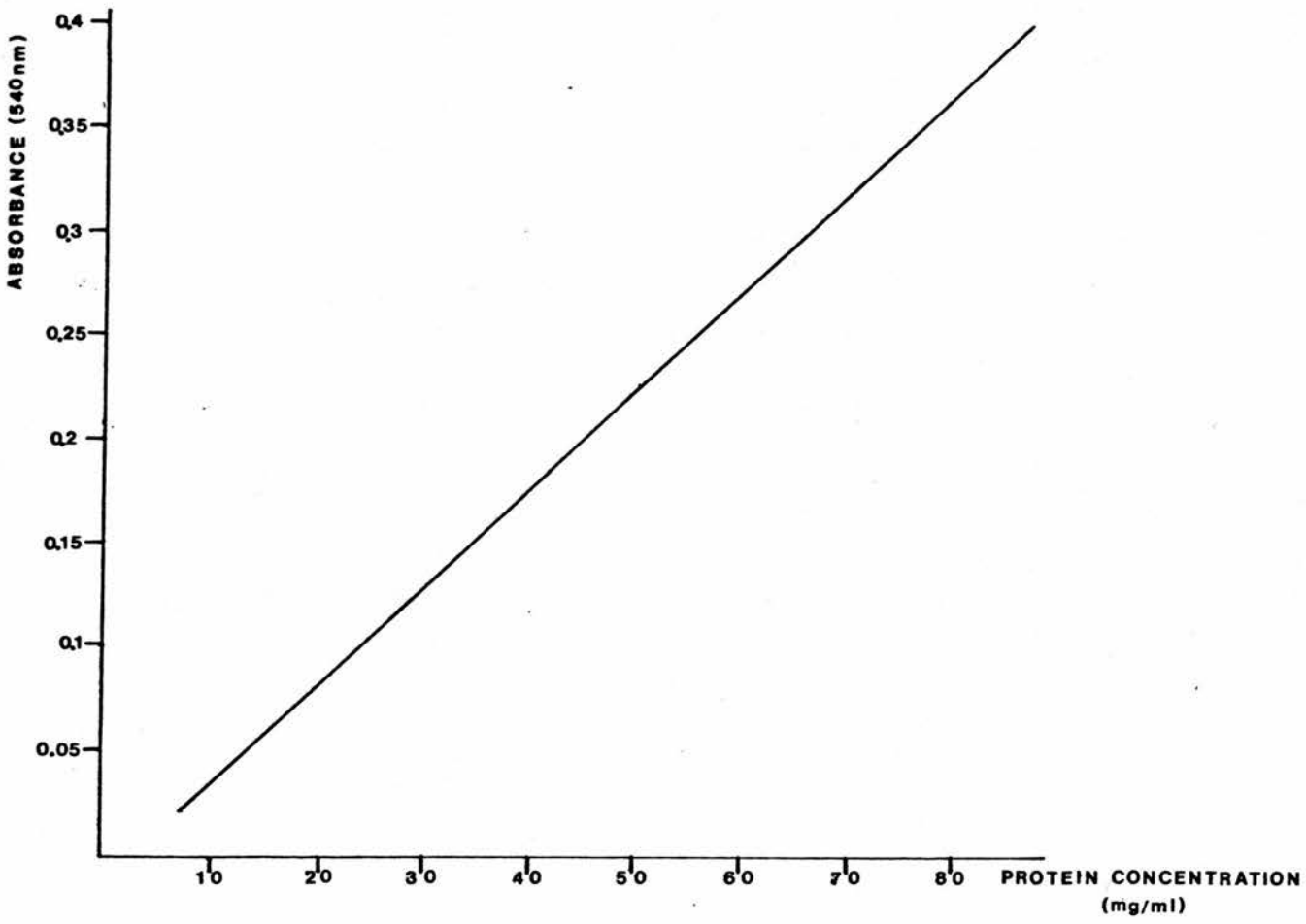
Lane 5 in Figure 2-8 shows the reduced protein in SDS-PAGE - a molecular weight of 205,000 was found which is in agreement with the accepted subunit size for fibronectin (Mosesson and Amrani 1980).

Assay for fibronectin: The purified protein was sent to the Scottish Antibody Production Unit, Carluke, where it was used to raise an antiserum in a rabbit. Upon analysis of the antiserum in immunoelectrophoresis against human plasma, multiple precipitin lines were obtained, indicating multiple specificity. This was presumably due to contaminating proteins in the immunogen. Further preparations of immunogen were therefore made and the resulting protein was adsorbed with immobilised antisera to human albumin, fibrinogen and immunoglobulin, which were thought to be the main potential impurities in the initial preparation. These antibodies were prepared by coupling sodium sulphate fractions of the appropriate antisera to cyanogen-bromide activated Sepharose 4B. The immunogen adsorbed with the immobilised antibodies was used to immunise further rabbits. Antisera produced proved to be monospecific in immunoelectrophoresis against human plasma. The antiserum also gave a single immunoprecipitin arc in 2DIEP against normal plasma and was found to be suitable for use in a Laurell assay at a concentration of 0.2% (Figure 2-13). In this assay, normal pooled plasma at dilutions of 1/10, 1/20, 1/40 and 1/80 was used as standard and was assumed to contain 0.33 mg/ml of fibronectin (Mosesson and Amrani 1980).*

* The antiserum did not give a precipitin 'rocket' with gelatin-absorbed plasma (fibronectin level < 1 μg/ml) indicating its specificity.

FIGURE 2-14

STANDARD CURVE OF THE BIURET PROTEIN ASSAY


ABSORBANCE (540nm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
PROTEIN CONCENTRATION
(mg/ml)

(H) ASSAY FOR TOTAL PROTEIN

The Biuret technique (Gornall et al 1949) was the main method used to assay protein in this study. In some measurements of low protein levels (less than 5 mg/ml) and in solutions containing dextran (Chapter V) which was found to cause precipitation of the Biuret reagent, absorbance at 280 nm was used to estimate protein content.

The Biuret assay: The reagent was prepared by dissolving 1.5 g of copper sulphate ($CuSO_4$ $5H_2O$), 6 g of potassium sodium tartrate and 300 ml of 10% w/v sodium hydroxide in a total volume of 1 litre of distilled water. The assay was performed as follows:

0.1 ml sample

5 ml reagent

↓ 10 minutes at room temperature

↓ read absorbance at 540 nm

Protein content estimated from standard curve.

A standard curve was constructed using BSA of known protein content (Figure 2-14).

Protein estimation by measurement of optical density at 280 nm: was performed by measuring the absorbance at 280 nm of the protein solution in a 1 cm deep quartz cell. It was assumed that an absorbence of one was equivalent to a protein concentration of 1 mg/ml unless the extinction coefficient of the protein involved was known.

(I) ANALYSIS OF PROTEIN COMPOSITION BY SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The principle of this technique has been discussed by Weber and Osborne (1969). Proteins are separated in polyacrylamide gel of a predetermined porosity by migration in an electric field. Addition of the anionic detergent sodium dodecyl sulphate (SDS) imparts a uniform negative charge density on the proteins, so that migration rate is dependent on their molecular weight. The different proteins in a mixture thus separate on the basis of their molecular weight and by running known standards, the molecular weight of a component can be determined and different components can be identified.

Method: The method outlined below was used to make gels 5% with respect to acrylamide. Different concentrations can be made by adjusting the acrylamide concentration. Two glass plates each 16 x 8 cms were rinsed in water and then in ethanol. The plates were then placed on clean tissue paper with the side which was to be in contact with the gel uppermost, and swabbed with an acetone-soaked tissue held in a gloved hand. After a final rinse with ethanol, the plates were allowed to air dry. The plates were then brought facing each other but held apart with plastic spacers of 4 mm thickness. The spacers were attached firmly to the plates using adhesive tape. The plate assembly was then placed in a Pharmacia GSG8 gel-casting apparatus and the spacer comb was placed on top.

The following reagents were used to make polyacrylamide gel:

(a) 0.2 M phosphate buffer pH 7.1 (prepared by making 70 mls 5 M NaOH and 12 ml H_3PO_4 up to one litre with distilled water).

(b) 22.2% acrylamide (22. g + 0.6 g bis in 100 mls H_2O).

(c) 10% SDS.

(d) 10% ammonium persulphate (made up fresh every time).

(e) TEMED (N,N,N',N',-Tetramethylethylenediamine).

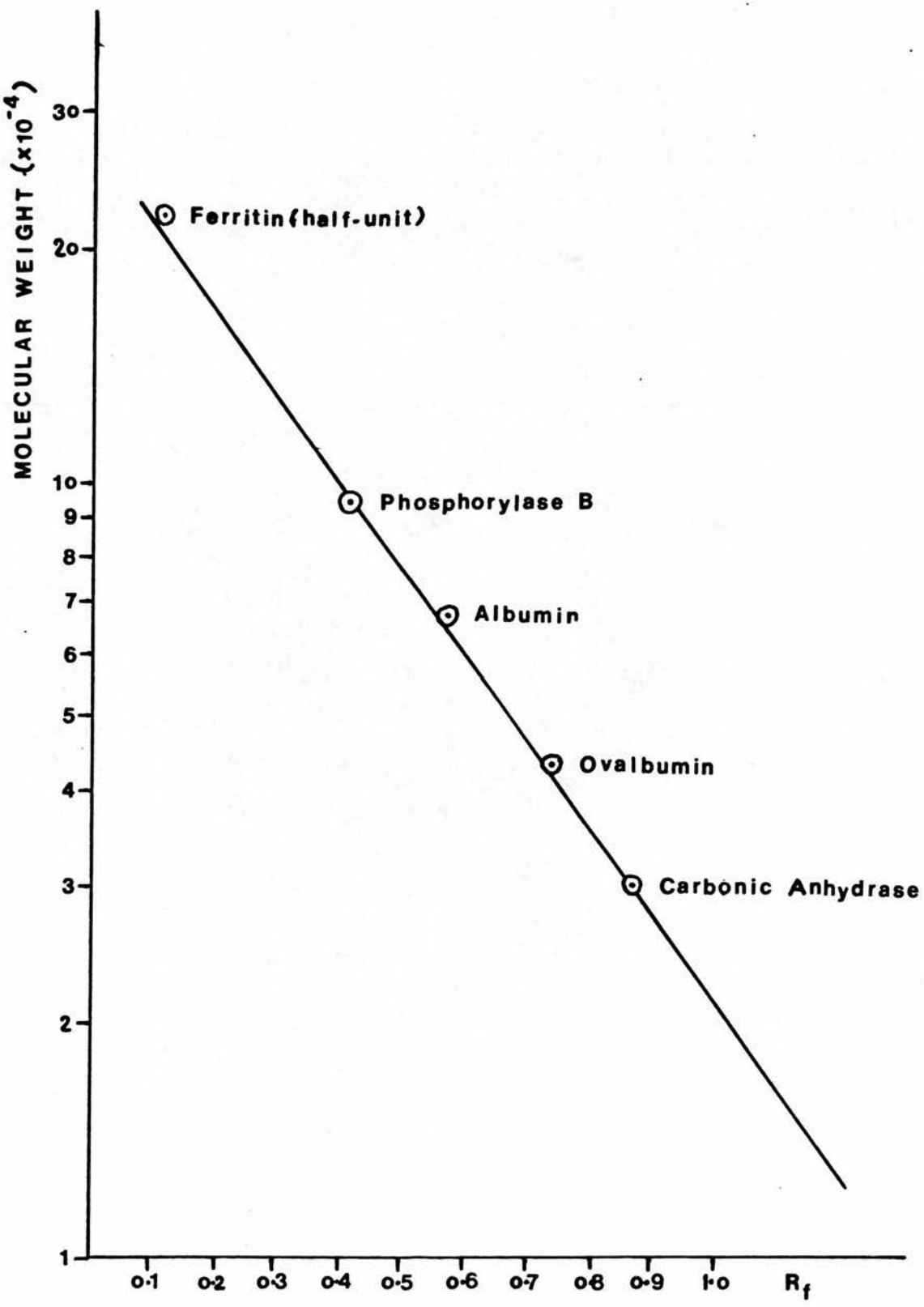
To make 2 gels, 50 ml of (a), 22.5 ml of (b), 10 ml of (c), 1.5 ml of (d) and 0.2 ml of (e) were mixed and made up to 100 ml with distilled water in a measuring cylinder, with gentle mixing to avoid too much air getting into the mixture. The mixture was then poured into the casting apparatus, until the plate assembly was covered. The gels were left for 2 hours at room temperature, excess gel was trimmed away and the gels were subjected to a pre-run for 45 minutes at 30 V, 150 mA in a Pharmacia GE-2/4 apparatus. The tank buffer was 0.1 M phosphate 0.1% SDS pH 7.1. Samples for analysis were mixed with an equal volume of 8 M urea, 0.2 M phosphate 1% SDS 1% bromophenol blue pH 7 and incubated for one hour at room temperature. Mercaptoethanol (1%) was added when proteins were to be reduced. 50 µg of protein in a volume of 80 µl was applied to each sample well on the gel and electrophoresis was carried out for 18 hours at 30 V, 150 mA. The gels were then carefully removed from the glass plate assembly and fixed

FIGURE 2-15

CALIBRATION CURVE FOR MOLECULAR WEIGHT DETERMINATION USING SDS-PAGE

Marker proteins (Pharmacia) were electrophoresed and R_f values were measured

$$R_f = \frac{\text{DISTANCE MIGRATED BY PROTEIN BAND}}{\text{DISTANCE MIGRATED BY DYE FRONT}}$$


MOLECULAR WEIGHT (x10⁻⁴)
30
20
10
9
8
7
6
5
4
3
2
1
Ferritin (half-unit)
Phosphorylase B
Albumin
Ovalbumin
Carbonic Anhydrase
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Rf

for 18 hours in 400 mls of 25% isopropanol, 10% acetic acid. They were then stained for 4 hours in 0.1% Coomassie Blue R, 25% isopropanol, 10% acetic acid and destained with several changes of 14% methanol, 7% acetic acid. If required, gels were scanned densitometrically using a Bio-Rad densitometer. Molecular weight markers (Pharmacia) were incorporated in each run and used to construct a calibration curve (Figure 2-15), from which the molecular weight of different components could be inferred.

(J) STATISTICAL ANALYSIS

Statistical comparisons in this study utilised mainly the student's t-test for paired and unpaired comparisons as appropriate. Data presented in Chapter III regarding the fibrinopeptide A content of blood donations was analysed non-parametrically using the Wilcoxon rank sum test, due to the fact that the results approached zero and were thus not normally distributed.

CHAPTER III

FACTOR VIII IN BLOOD DONATIONS COLLECTED INTO DIFFERENT ANTICOAGULANTS AND USING DIFFERENT TECHNIQUES

INTRODUCTION

The ever increasing demand for factor VIII concentrates has led to many studies into the stability of this protein. Early work, although hampered by a lack of accurate and sensitive assays, quickly established the lability of VIII:C (Penick and Brinkhous 1956, Goldstein et al 1964). This had led to blood banks developing special procedures for processing donations intended for factor VIII concentrate production. However, uncertainty exists as to what factors involved in blood donation and processing influence the stability of factor VIII. The type of anticoagulant has been shown to be an important variable, most studies reporting that Citrate-Phosphate-Dextrose (CPD) improves VIII:C stability compared to Acid-Citrate-Dextrose (ACD) (Schanberge et al 1972, Lane 1981) although some studies claim no difference (Goldstein et al 1964, Slichter et al 1976), and one study claims that ACD is better (Vermeer et al 1976). There is agreement that citrate based anticoagulants are better than oxalate or EDTA for preserving VIII:C (Spaet and Garner 1955, Mustard 1958) and that an optimal citrate concentration exists (Mustard 1958, Weiss 1965). Recent studies have demonstrated marked stability of VIII:C in blood collected in heparin (Rock et al 1979, Smith 1983b) and one group claims higher levels of VIII:C in fresh heparin plasma (Rock et al 1979). Other workers have failed to confirm this (Smit-Sibinga et al 1981).

The level of cellular contamination in the plasma has also been the subject of disagreement; some studies claim an improved stability of VIII:C in cell-free (Nilsson et al 1983) or platelet poor plasma (Mustard 1957), while others claim that cellular contamination has no influence on VIII:C stability (Preston 1967, Pepper et al 1978). High platelet contamination in frozen plasma, however, has been shown to result in low VIII:C levels (Pepper et al 1978) and low yields due to processing difficulties in producing factor VIII concentrate (Smith et al 1977).

Discrepancies also exist regarding optimal conditions for storing blood or plasma prior to the freezing of plasma. The majority of reports indicate retention of 75 to 85% of initial plasma VIII:C levels at temperatures between 4° and 22°C 6 to 8 hours after donation with a subsequent fall to 55 to 75% at 4°C by 18 to 24 hours (Penick and Brinkhous 1956, Rapaport et al 1959, Stibbe et al 1972, Hondow et al 1982a). Losses at room temperature have been reported to be similar (Vermeer et al 1976, Kahn et al 1979) or greater (Pool and Robinson 1959, Rock et al 1980a). Some reports show losses of VIII:C in blood stored at low temperatures (about 2° to 4°C) due to cryoprecipitation of cold insoluble proteins, including the factor VIII complex, and subsequent removal with the cells during centrifugation (Vermeer et al 1976, Rock and Tittley 1979). Other studies have not found this (Hondow et al 1982a,b,

Carlebjörk et al 1983). Although one study claims little loss of VIII:C even at outdate in banked blood (Weaver et al 1967) the many reports confirming lability have led to stringent conditions being formulated for the production of plasma destined for production of factor VIII concentrate. Initial instructions by the American Association of Blood Banks required that such plasma be frozen within 4 hours of collection (American Association of Blood Banks 1970), although this period was subsequently extended to 6 hours (American Association of Blood Banks 1978).

Although most studies have investigated the stability of VIII:C, some work has been published regarding the other activities of the factor VIII complex. VIII:CAg and VIIIR:Ag have been reported to be stable in plasma at room temperature (Rock et al 1983a). At 4°C, VIIIR:Ag in blood and plasma have been reported to be stable in one study (Hondow et al 1982b), but another group reports gradual degradation of VIIIR:Ag over one week at 4°C (Nilsson et al 1983). VIIIR:RCF has been shown to be lost from banked blood (Hondow et al 1982b) and plasma (Rock et al 1983a) after 3 weeks storage at 4°C.

A recent study has emphasised difficulties of processing blood to factor VIII concentrate from plasma with high fibrinopeptide A (FpA) levels due to poor donation procedure (Pflugshaupt and Kurt 1983). Inadequate mixing of blood with anticoagulant has been claimed

to lead to high FpA plasma levels, indicating thrombin formation. This was associated with processing difficulties and poor stability in the factor VIII concentrate derived from such plasma. One other study reports low VIII:C yields from plasma with high FpA levels, induced by deliberate thrombin addition (Törmä and Myllylä 1983). An early study claims 15% differences in VIII:C from mixed and unmixed blood (Perkins et al 1962). Other studies, however, have found no such differences (Preston 1967, Slichter et al 1976).

It is necessary for blood banks to ensure that plasma destined for factor VIII products has a high initial content of factor VIII. Processing to cryo-precipitate indicates that the yield of VIII:C is proportional to the amount in the starting plasma (Kasper et al 1975, Pepper et al 1978). This is not always found when factor VIII concentrate is produced, with equivalent yields being obtained from 4 and 18 hour old plasma (although 4 hour old has a higher initial VIII:C content) (Smith et al 1978,1979). This is probably because the large losses involved in large-scale production mask any differences between the initial content of the various plasma grades. Another manufacturer finds the difference in VIII:C content between 4 and 18 hour old plasma is reflected in higher yields in the final product (Foster et al 1982). In the case of small-scale production by blood banks, processing losses encountered in industrial

fractionation may be minimised by careful attention to processing variables. The final yield may then be expected to reflect more closely the initial VIII:C level.

In this study, the storage lability of factor VIII in CPD and heparin blood donations has been examined, with a view to finding optimal conditions for production of plasma with a high factor VIII content. Addition of calcium back to CPD plasma has been examined as an alternative to heparin collection in maintaining the levels of ionised calcium necessary for optimal VIII:C stability. The amount of citrate in plasma compatible with such stability has also been determined. The effect of different mixing techniques during donation on VIII:C and FpA was also investigated.

METHODS

Blood Collection

CPD donations: Three groups of 10 donations each, were taken into Tuta single blood bags at regular donor sessions. Each group was collected using one of three mixing procedures:

(a) Fully mixed - with the bag inverted so that blood entered through the anticoagulant and with continual gentle manual mixing throughout the donation.

(b) Partly mixed - with the bag inverted and 3 or 4 manual mixes during donation, this being the standard technique in this centre.

(c) Unmixed - with the pack upright and mixing only at the end of the donation.

After donation the bags were mixed and a 10 ml blood sample was obtained and centrifuged for 75,000 g min. at 4°C to give platelet poor plasma. Samples were frozen and stored at -40°C for subsequent VIII:C and FpA assay.

Heparin donations: A group of 6 donations were taken into Fenwal heparin bags (R0601), each containing 2250 units of sodium heparin in 30 ml of phosphate buffered saline.

All donations involved withdrawal of 420 ml of blood from the donor. Donations were not selected for blood group or venesection time although these were noted.

Factor VIII stability in CPD and heparin: Each donation in the CPD partly mixed group was aliquoted into 10 ml tubes; 16 such tubes were collected. The remaining

blood was centrifuged for 75,000 g min. at 4°C and the platelet poor plasma obtained was aliquoted into another 16 10 ml tubes. Tubes of blood and plasma were held at various temperatures and periods of time as outlined in Results. In a further experiment, 20 ml of blood were collected from 3 different donors into CPD. The ratio of blood to anticoagulant was the same as in normal donations (7:1). Plasma aliquots from each donation were incubated for 18 hours and then processed as shown in Results. The heparin collections were treated in the same manner to produce tubes of blood and plasma and held under the conditions outlined in Results. At the end of the stated periods, samples of blood and plasma were centrifuged at the same temperatures as they were incubated and the plasma thus prepared was rapidly frozen in 2 ml aliquots and stored at -40°C for subsequent assay. Samples of CPD plasma were frozen without any prior centrifugation, while heparin plasma samples were centrifuged prior to freezing.

Factor VIII:C stability in plasma with different citrate concentrations: 10 ml aliquots from each of 6 donations of heparin plasma were made to various concentrations of citrate by addition of appropriate amounts of 1 M tri-sodium citrate immediately after collection and separation. The various types of plasma were then held for 18 hours at room temperature and rapidly frozen for subsequent assay.

Factor VIII:C stability in recalcified CPD plasma: 10 ml aliquots from a group of 6 donations of CPD plasma were

made to various concentrations of calcium by addition of different amounts of 1 M calcium chloride containing heparin. In all cases, the amounts of heparin added were such as to result in a concentration of 2 u/ml in the plasma. The various plasmas were held for 18 hours at room temperature and then frozen for subsequent assay.

Cooling rate of blood donations: A bag of outdated CPD whole blood was placed in a 37°C water-bath. The internal temperature of the bag was monitored by a thermocouple (Comark Instruments), placed in the middle of the bag. As soon as the temperature had reached 37°C, the bag was placed in a 4°C cold room and 30 minute recordings of the temperature were taken.

Assays

Factor VIII:C, factor VIIIR:Ag, fibronectin and fibrinopeptide A were measured as described in Chapter II.

Ionised calcium was measured at the Protein Fractionation Centre, Edinburgh, using an ion-specific electrode calibrated with calcium solutions of known concentration. The help of Mrs. Ida Dickson in carrying out these measurements is acknowledged.

Statistical analyses were by the paired t-test or unpaired t-test as appropriate. Logarithmic transformation of data relating to VIII:C did not affect the significance of any of the differences described, despite the reported logarithmic distribution of VIII:C in the plasma from normal individuals (Bangham et al 1971).

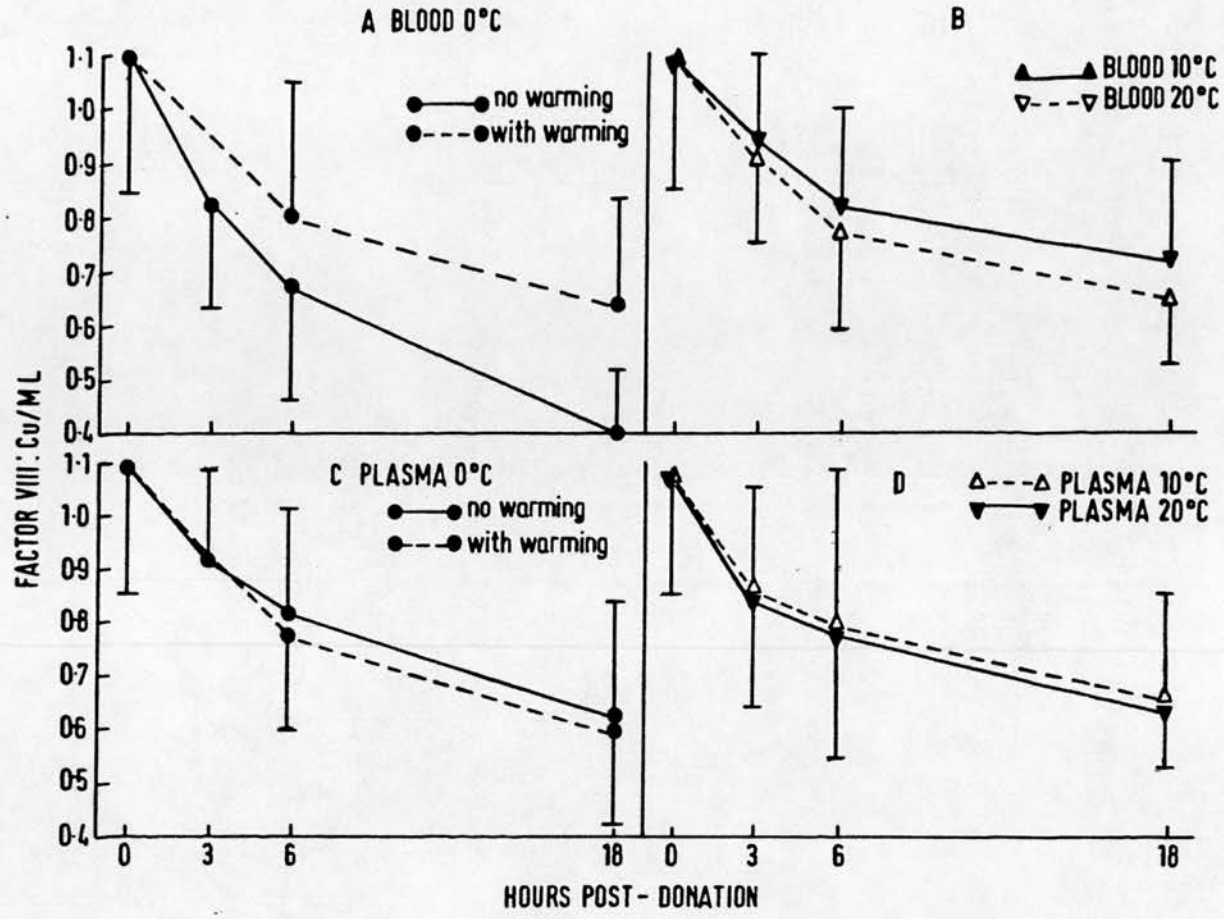
FIGURE 3-1

STABILITY OF FACTOR VIII IN CPD DONATIONS

A,B,C,D show data for VIII:C

E,F,G,H show corresponding values for VIIIR:Ag

Results show the mean and standard deviation for ten individual donations.


A BLOOD 0°C
no warming
with warming
B
BLOOD 10°C
BLOOD 20°C
C PLASMA 0°C
no warming
with warming
D
PLASMA 10°C
PLASMA 20°C
FACTOR VIII: Cu/ML
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0
3
6
18
HOURS POST-DONATION

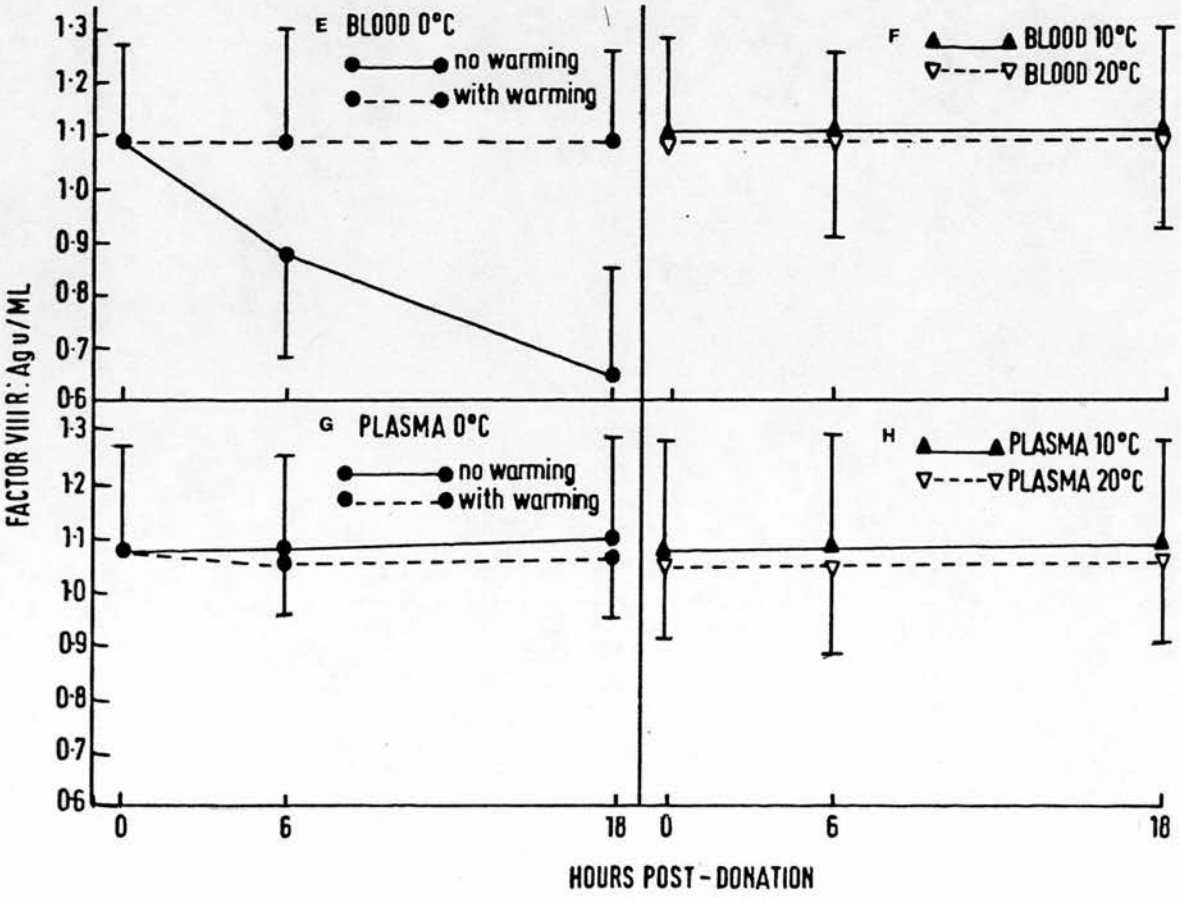

E BLOOD 0°C
no warming
with warming
F
BLOOD 10°C
BLOOD 20°C
G PLASMA 0°C
no warming
with warming
H
PLASMA 10°C
PLASMA 20°C
FACTOR VIII R: Ag u/ML
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0
6
18
HOURS POST-DONATION

TABLE 3-1 STABILITY OF FACTOR VIII AND FIBRONECTIN IN CPD PLASMA AT 0°C

Parameter	Fresh Plasma Levels	Levels after 18 hours at 0°C		
		Centrifuged at 0°C		Centrifuged at 20°C after 30 min at 37°C
		Supernatant	Precipitate	
VIII:C u/ml	0.89	0.28 ± 0.09 (52)*	1.28 ± 0.18 (37)	0.54 ± 0.12
VIIIR:Ag u/ml	0.88	0.51 ± 0.04 (64)	2.12 ± 0.37 (41)	0.80 ± 0.13
Fibronectin antigen u/ml	0.83	0.4 (53)	3.3 ± 1.05 (68)	0.76 ± 0.14

Aliquots of plasma (3.2 ml.) were held in melting ice and processed as described. Samples held and centrifuged at 0°C showed a small precipitate which was dissolved in 0.5 ml of 15 mM citrate, 150 mM NaCl, pH 6.9.

* Amount found as a percentage of the warmed sample.

RESULTS

(A) FACTOR VIII STABILITY IN CPD DONATIONS

Stability of factor VIII was investigated at 3 temperatures: 0°C (achieved by holding tubes in ice/water); 10°C (using a controlled temperature water bath, Grant Instruments) and room temperature (measured at 20°C). Figure 3-1 shows the results. Factor VIII:C appears equally stable in plasma at all 3 temperatures studied, and similar results were obtained at 10°C and 20°C in blood. In blood at 0°C VIII:C appeared less stable. This loss, however, was shown to be due to cryoprecipitation of VIII:C into the cell layer at this temperature as warming samples at 37°C for 30 minutes prior to plasma separation led to VIII:C recoveries very similar to those observed at higher temperatures (Figure 3-1A). Although this effect was not observed in plasma at 0°C (Figure 3-1C,E), these samples were not centrifuged prior to plasma freezing and storage. However, a further experiment on a smaller group of donors confirmed that cryoprecipitation of factor VIII occurs in plasma held at 0°C (Table 3-1). Measurement of fibronectin antigen showed precipitation of this protein. Fibrinogen was not precipitated to the same extent.

Corresponding assays for VIIIR:Ag (Figure 3-1 E-H) showed great stability in both blood and plasma over 24 hours at all temperatures studied, with the same cryoprecipitation occurring in blood at 0°C as occurred for

FIGURE 3-2

COOLING OF BLOOD BAG AT 37°C PLACED IN 4°C AIR
The temperature probe was secured to a thin wooden stick and placed in the middle of the bag through an opening in the transfusion port. After allowing the temperature to rise to 37°C in a water-bath, the bag was placed in a 4°C cold-room and regular temperature readings were taken.

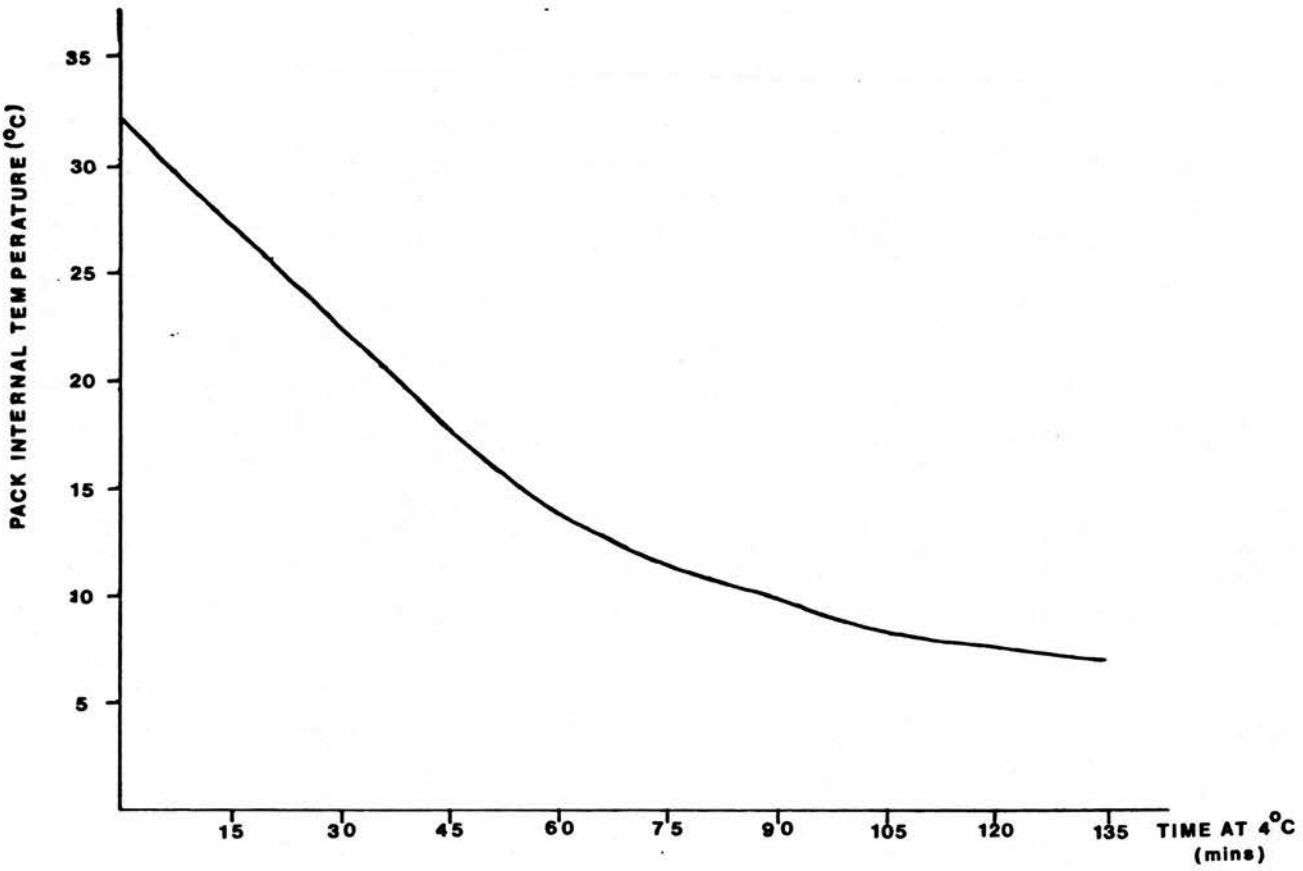

PACK INTERNAL TEMPERATURE (°C)
35
30
25
20
15
10
5
15
30
45
60
75
90
105
120
135
TIME AT 4°C
(mins)

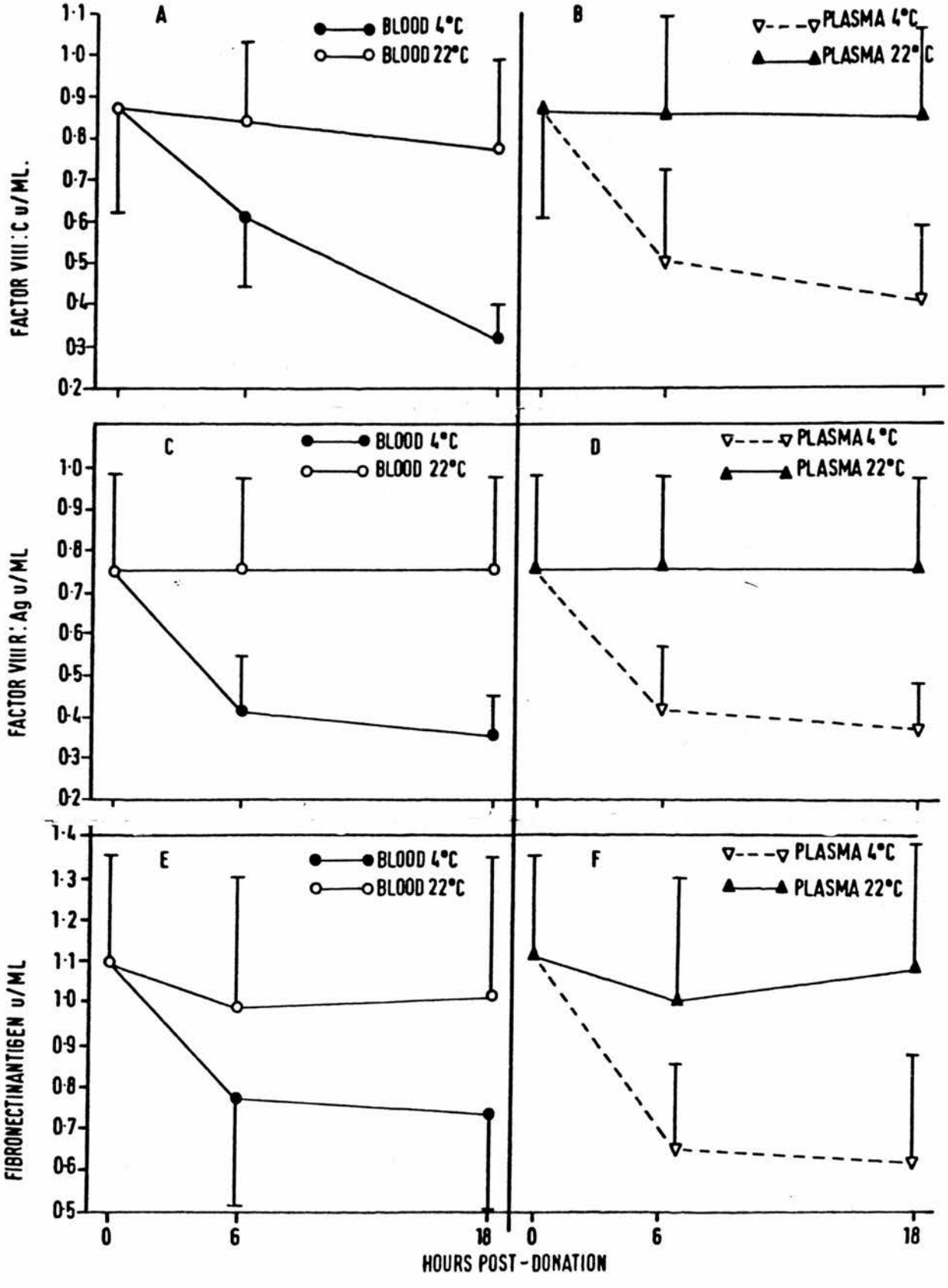
FIGURE 3-3

STABILITY OF FACTOR VIII AND FIBRONECTIN ANTIGEN IN HEPARIN DONATIONS

A & B show data for VIII:C

C,D & E,F show corresponding data for VIIIR:Ag and fibronectin antigen (1 u of fibronectin antigen was taken as being the amount in 1 ml of normal pooled plasma)

Results show the mean and standard deviation from six individual donations.


A
BLOOD 4°C
BLOOD 22°C
B
PLASMA 4°C
PLASMA 22°C
C
BLOOD 4°C
BLOOD 22°C
D
PLASMA 4°C
PLASMA 22°C
E
BLOOD 4°C
BLOOD 22°C
F
PLASMA 4°C
PLASMA 22°C
FACTOR VIII:C u/ML.
FACTOR VIII R:Ag u/ML
FIBRONECTINANTIGEN u/ML
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0
6
18
HOURS POST-DONATION

VIII:C. Warming to 37°C led to full recovery of VIIIR:Ag.

The cooling rate of a blood donation at 37°C placed in 4°C refrigeration is shown in Figure 3-2. It took over 2 hours for the internal temperature to fall to 6°C.

(B) FACTOR VIII STABILITY IN HEPARIN DONATIONS

Stability of factor VIII was studied at two temperatures: 4°C (blood bank cold room) and 22°C (room temperature). Figure 3-3 shows the results.

Factor VIII:C in heparin blood showed no significant instability over 18 hours at room temperature ($t = 1.5$, $p > 0.1$ compared to fresh plasma VIII:C) in contrast with VIII:C in CPD blood, which showed marked instability over the same period ($t = 6$, $p < 0.01$ compared to fresh plasma VIII:C). This was confirmed for VIII:C in heparin plasma. The starting VIII:C levels (0.9 ± 0.14 u/ml) were not significantly different from a group of CPD donations with the same blood group distribution.

Striking losses of VIII:C were observed at low temperatures in blood. Although a normal blood bank refrigeration temperature of 4°C and not 0°C was used, apparent cryoprecipitation of VIII:C was observed as was seen for CPD blood. In this experiment warming at 37°C was not performed. However, cryoprecipitation was indicated as being the mechanism for VIII:C loss as parallel losses were observed in VIIIR:Ag (Figure 3-3C) at this temperature, with VIIIR:Ag being stable at room temperature. Plasma samples in this series of

TABLE 3-2 IONISED CALCIUM IN PLASMA COLLECTED INTO DIFFERENT ANTICOAGULANTS

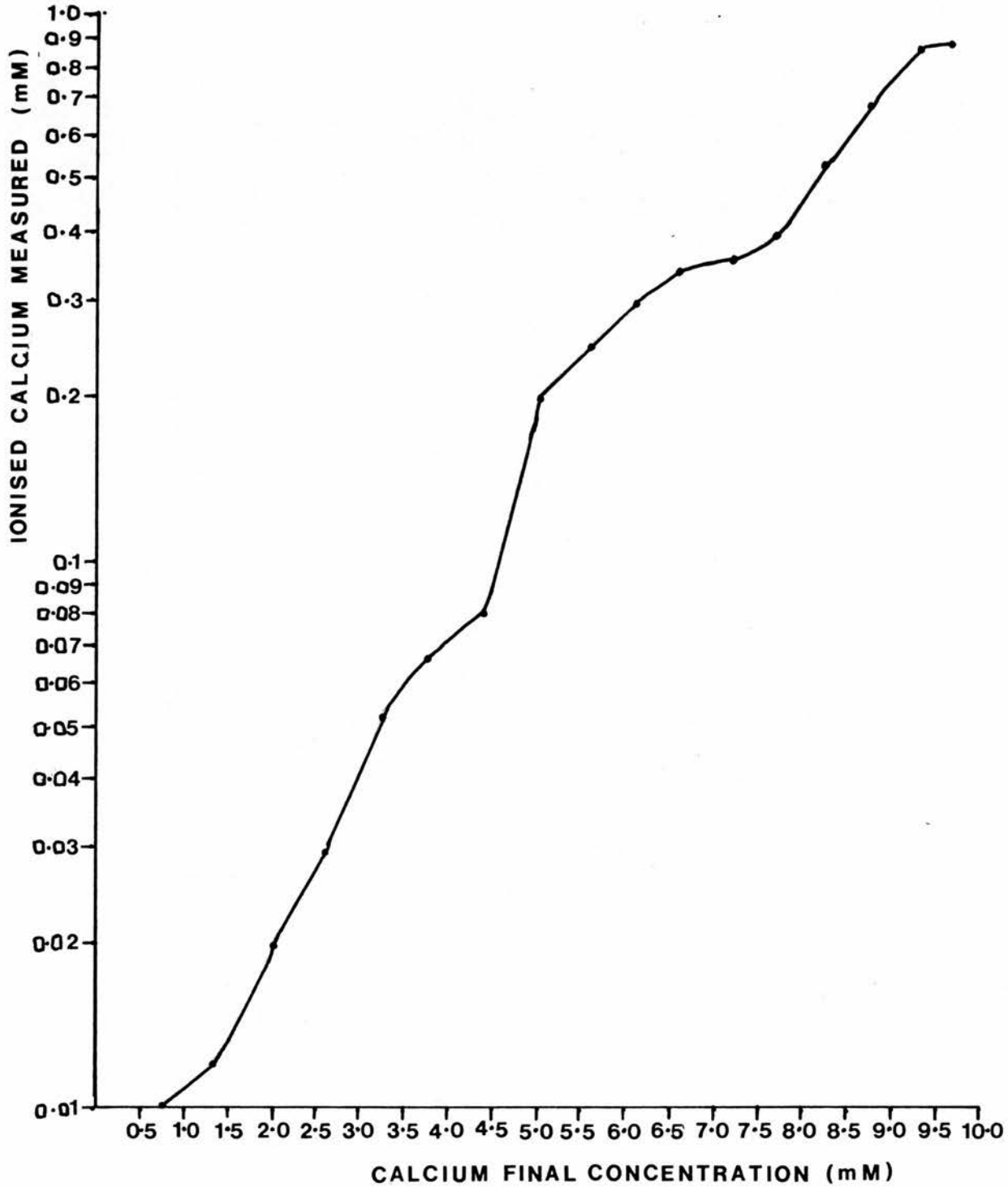
Anticoagulant	Calcium Ion-Specific Electrode Reading (mV)	Ionised Calcium mM
Heparin (3 u/ml)+	- 90	0.56
CPD + Heparin (3 u/ml)	- 50	< 0.01

+ Final heparin concentration in plasma

FIGURE 3-4

TITRATION OF CPD/HEPARIN PLASMA TO IONISED CALCIUM LEVELS FOUND IN HEPARIN PLASMA

55 mM $CaCl_2$ was added to a 4 ml plasma sample.


IONISED CALCIUM MEASURED (mM)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·09
0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
0·5 1·0 1·5 2·0 2·5 3·0 3·5 4·0 4·5 5·0 5·5 6·0 6·5 7·0 7·5 8·0 8·5 9·0 9·5 10·0
CALCIUM FINAL CONCENTRATION (mM)

experiments were centrifuged prior to sampling for assay, unlike corresponding samples in the CPD experiments. Figures 3-3B and 3-3D show loss by cryoprecipitation of VIII:C and VIIIR:Ag in heparin plasma at 4°C, with stability of both activities at room temperature.

Measurement of fibronectin antigen levels in both blood and plasma showed cryoprecipitation of this protein in the cold (Figures 3-3E and 3-3F) with stability being observed at room temperature.

(C) IONISED CALCIUM IN PLASMA WITH DIFFERENT ANTICOAGULANTS

Two samples of blood were collected from the same individual into equal volumes of CPD/heparin and heparin anticoagulant (final plasma heparin (about) 3 u/ml). The ratio of blood to anticoagulant was the same as used in normal blood donation i.e. (about) 7:1. Plasma was separated from both these samples. The ionised calcium levels in the paired samples measured with an ion-specific electrode and shown in Table 3-2.

A stock solution of calcium chloride (55 mM) was then used to bring the ionised calcium level of the CPD/heparin plasma sample as recorded by the electrode, to the level of the heparin sample. The resulting titration curve is shown in Figure 3-4. About 0.7 ml of stock solution were needed to bring the ionised calcium level of a 4 ml CPD/heparin sample to that of the heparin sample. It can be seen that calcium had to be added back to a level of about 8 mM to overcome the

TABLE 3-3 EFFECT OF RECALCIFICATION OF CPD BLOOD AND PLASMA ON VIII:C STABILITY

Sample	Hours Post-Donation	Factor VIII:C u/ml*
Blood	6	0.83 ± 0.17
Blood	Calcium/heparin+ added 3 hrs, separated 5 hrs	1.03 ± 0.27†
Blood	18	0.73 ± 0.17
Blood	Calcium/heparin added 18 hrs, separated 20 hrs	0.65 ± 0.14§
Plasma	6	0.84 ± 0.25
Plasma	Calcium/heparin added 3 hrs, frozen 5 hrs	1.1 ± 0.4 †
Plasma	18	0.69 ± 0.12
Plasma	Calcium/heparin added 18 hrs, frozen 20 hrs	0.77 ± 0.25§

* Mean and standard deviation for 10 individual donations

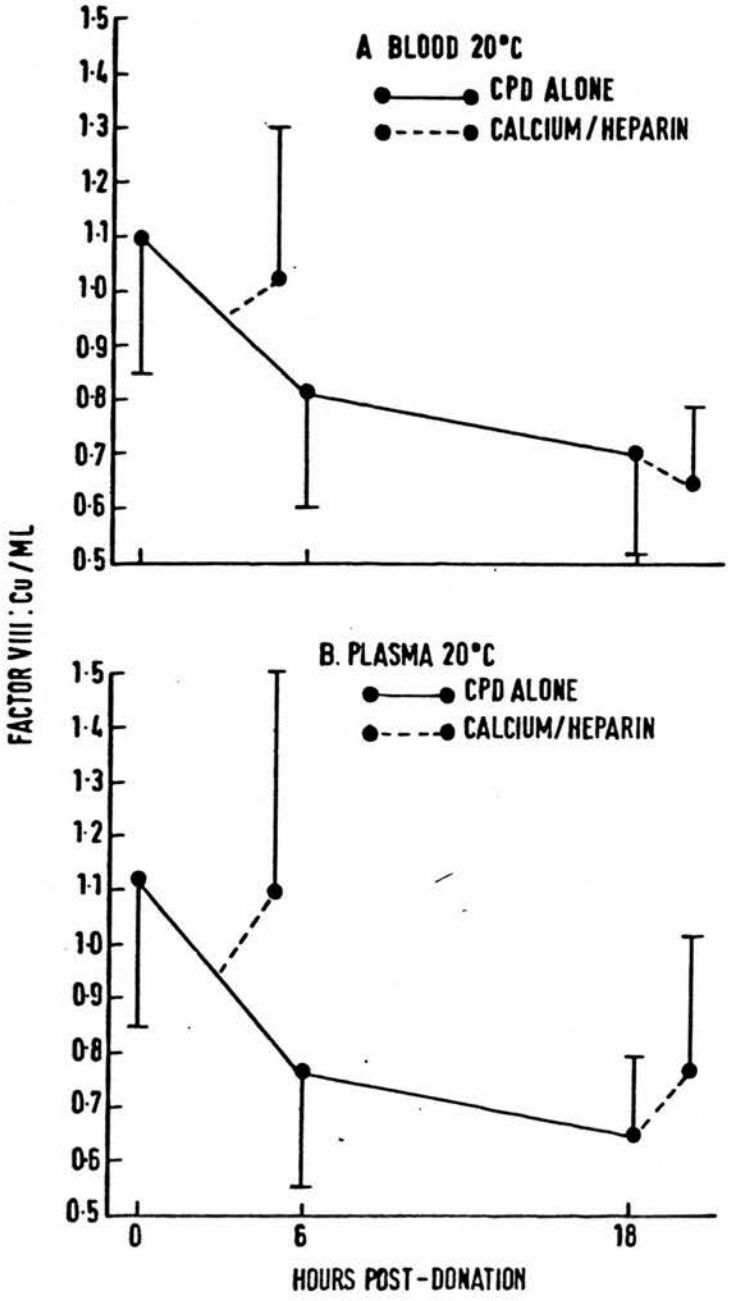
\+ In each case, the calcium/heparin was added in an amount sufficient to result in a plasma concentration of 10 mM calcium, 5 u/ml heparin

† Significantly higher than control, $p < 0.01$ for blood, $p < 0.05$ for plasma

§ Difference from control not significant

FIGURE 3-5

EFFECT OF RECALCIFICATION OF CPD DONATIONS
Results show the effect of addition of a calcium/ heparin solution to give final concentrations of (about) 10 mM and 5 u/ml in the plasma.
Results show the mean and standard deviation of ten individual donations.


A BLOOD 20°C
CPD ALONE
CALCIUM/HEPARIN
B. PLASMA 20°C
CPD ALONE
CALCIUM/HEPARIN
FACTOR VIII : Cu / ML
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0
6
18
HOURS POST-DONATION

effect of the CPD.

(D) RECALCIFICATION OF CPD DONATIONS

On the basis of the calcium level measurements just outlined, a $CaCl_2$/sodium heparin solution was added to samples of CPD blood and plasma, held at room temperature, 3 and 18 hours post donation. The amounts added were such as to result in a final concentration of 10 mM calcium and 5 u/ml heparin in the plasma. Plasma from such samples was separated and frozen for assay 5 and 20 hours post donation respectively. Subsequent VIII:C assay gave the results shown in Table 3-3. It can be seen that calcium/heparin addition 3 hours post donation led to recovery of VIII:C activity, such that blood which had been thus treated had higher VIII:C levels 5 hours post donation than blood which had not been recalcified. This difference was significant ($t = 3.48$, $p < 0.01$ - paired t-test comparing recalcified sample with 6 hour post donation sample in CPD alone). Likewise, plasma recalcified after 3 hours showed higher VIII:C levels after 5 hours than plasma in CPD alone ($t = 3.04$, $p < 0.05$, comparison as for blood). Figure 3-5 demonstrates this effect.

Addition to blood or plasma of calcium/heparin at 18 hours resulted in VIII:C levels at 20 hours that did not differ significantly from those in samples held for 18 hours in CPD alone (Table 3-3).

(E) EFFECT OF RECALCIFICATION WITH DIFFERENT AMOUNTS OF CALCIUM ON VIII:C STABILITY

Plasma from 6 CPD donations were made to

TABLE 3-4 EFFECT OF RECALCIFICATION WITH DIFFERENT AMOUNTS OF CALCIUM ON VIII:C STABILITY IN CPD PLASMA

Calcium/(Heparin) Added to Final Concentration mM (u/ml)	VIII:C After Overnight Incubation at 22°C u/ml*	Ionised Calcium After Overnight Incubation at 22°C mM
0 (0)	(starting levels) 1.07 ± 0.09	
0 (0)	0.67 ± 0.17	< 0.01
10 (3)	0.86 ± 0.18+	0.07
15 (3)	0.78 ± 0.13†	0.33
20 (3)	0.89 ± 0.16†	0.44
25 (3)	0.82 ± 0.15†	0.96
30§ (3)	0.78 ± 0.09†	0.78

* Mean and standard deviation of 6 individual donations

\+ Significantly higher than control, $p < 0.01$

† Difference from 10 mM value not significant

§ Precipitate formed after overnight incubation

TABLE 3-5 DEPENDENCE OF VIII:C STABILITY IN HEPARIN PLASMA ON CITRATE CONCENTRATION

Citrate Added Final Concentration mM	VIII:C After Overnight Incubation at 22°C u/ml*	Ionised Calcium After Overnight Incubation at 22°C mM
	(starting levels) 1.07 ± 0.06	
0	0.85 ± 0.2	0.21
5	0.91 ± 0.11	0.03
10	0.83 ± 0.1	< 0.01
15	0.66 ± 0.06	< 0.01
20	0.58 ± 0.04+	< 0.01
0 (+ 10 mM calcium)	1.03 ± 0.19†	2

All donations taken into standard heparin anticoagulant, with a final concentration of about 8 u/ml in the plasma

* Mean and standard deviation of 6 individual donations

\+ Significantly lower than control, $p < 0.02$

† Significantly higher than control, $p < 0.05$

different calcium concentrations 5 hours post donation and left overnight at room temperature. Table 3-4 shows the effect of such addition on VIII:C stability. CPD plasma showed the usual pattern, with 63% of the starting VIII:C being left after overnight incubation. This instability was corrected to a considerable extent by calcium/heparin addition to 10 mM total calcium. Addition to higher calcium levels did not improve stability over that observed with 10 mM calcium and at higher calcium levels, an insignificant drop in stability was observed. At 30 mM calcium, a precipitate was observed after overnight incubation. It is possible that this represented precipitation of calcium phosphate formed by reaction with phosphate ions derived from the CPD anticoagulant.

(F) DEPENDENCE OF VIII:C STABILITY ON CITRATE CONCENTRATION

Plasma collected into heparin was held overnight, together with aliquots of the same plasma that had been made to different concentrations of tri-sodium-citrate. Table 3-5 shows the results. VIII:C levels remained high in heparin plasma after overnight incubation. This was maintained at citrate concentrations of 5 and 10 mM. At 15 mM citrate, however, a loss of VIII:C was observed, although this only became significant at 20 mM citrate ($t = 3.52$, $p < 0.02$). The difference between 15 and 20 mM citrate was also significant ($t = 2.95$, $p < 0.05$). Adding calcium to

TABLE 3-6 EFFECT OF MIXING PROCEDURE ON PLASMA LEVELS OF VIII:C AND FpA

Mixing Procedure*	VIII:C (u/ml) Mean and Standard Deviation (range)	FpA ng/ml+			
		Median	Range	% Donation with < 7 ng/ml	% Donation with < 30 ng/ml
Fully Mixed	0.97 ± 0.21 (0.74 - 1.37)	13.9	5.6 - 50	10	80
Partly Mixed	0.82 ± 0.13 (0.62 - 0.98)	15.5	3.5 - 25.8	20	70
Unmixed	0.87 ± 0.31 (0.41 - 1.5)	7.9	2.9 - 26.8	40	100

* 10 donations in each group were studied

\+ Because of the wide range in values, statistical significance was assessed by the Wilcoxon test

No significant difference in VIII:C or FpA was observed between the 3 procedures.

TABLE 3-7 FACTOR VIII:C IN FRESH CPD PLASMA*

Mixing Procedure	Group O	Group A + B + AB	All Groups
Unmixed	0.74 ± 0.23 (0.41 - 1.12,7)	1.18 ± 0.29 (0.94 - 1.5,3)	0.87 ± 0.31 (0.41 - 1.5,10)
Partly Mixed	0.80 ± 0.13 (0.62 - 0.97,5)	0.83 ± 0.14 (0.63 - 0.98,5)	0.82 ± 0.13 (0.62 - 0.98,10)
Mixed	0.84 ± 0.18 (0.74 - 1.1,4)	1.06 ± 0.19 (0.77 - 1.37,6)	0.97 ± 0.21 (0.74 - 1.37,10)

* Results show mean ± standard deviation (range, number of donations)

heparin plasma improved VIII:C stability ($t = 2.66$, $p < 0.05$) over that of plasma to which additional calcium had not been added.

(G) INFLUENCE OF MIXING PROCEDURE DURING DONATION ON PLASMA QUALITY

Factor VIII:C and FpA assays were performed on frozen plasma samples derived from the donations collected using different mixing techniques. The results are shown in Table 3-6. Although a wide range in individual FpA levels was found, this was not related to mixing techniques; indeed no mixing gave the lowest levels. Factor VIII:C was also unaffected by mixing technique, the slightly higher VIII:C in the fully mixed group levels being accounted for by a higher incidence of blood groups A, B and AB in this group (Table 3-7).

DISCUSSION

This study confirms the lability of VIII:C during storage under blood bank conditions. Results for blood drawn into standard CPD anticoagulant indicate that temperature of storage is not as crucial a factor as has been previously thought; indeed storage at low temperatures can be detrimental because of the cryoprecipitation described in this study and in others (Vermeer et al 1976, Rock and Tittley 1979). However, it must be noted that the data in such studies have been obtained from experiments with small volumes of blood - a blood donation stored in standard donation bags takes much longer to cool down (Figure 3-2) especially if large numbers of bags are packed together (Rock and Tittley 1979). Thus, low temperature storage of blood under standard blood bank conditions might not result in loss of factor VIII by cryoprecipitation. Indeed, data for VIII:C levels in donations stored at 18 hours at 4°C prior to plasma separation indicates VIII:C levels of about 0.6 u/ml (Prowse 1984) in contrast to the value of 0.4 u/ml found in this study. However, the finding that low temperature storage is not essential for VIII:C stability might remove some of the logistical difficulties in procuring plasma suitable for factor VIII fractionation. Other studies have indicated that a prolonged room temperature hold is not detrimental for other blood components (Avoy et al 1978, Snyder et al 1983).

No difference was observed in the stability of VIII:C between CPD blood and plasma. Thus, any VIII:C loss observed is not the result of cellular contamination of liquid plasma as has been claimed in some studies (Mustard 1957, Nilsson et al 1983). It has been shown that adding a wide range of proteolytic inhibitors has no effect on the lability of VIII:C stored under blood bank conditions (Stibbe et al 1972, Rock et al 1983b) although one study claims that some stabilisation was obtained by adding diisopropyl-flouro-phosphate (D.F.P.) (Rock et al 1983b). It is thus unlikely that VIII:C loss is through degradation induced by cellular proteases.

This study confirms that the decay of VIII:C over the period studied (18 hours) is biphasic, with about 4.4% of the initial VIII:C being lost each hour up to 6 hours post donation, and about 1.1% being lost each hour for the next 12 hours. Thus, rapid separation and freezing within 6 hours of donation can produce substantial improvements in plasma VIII:C content which would be lost if further delay occurs. This implies that blood donations collected in mid and late afternoon would require processing by staff working on an overtime or shift basis, with resultant increased costs. Any factors which would help stabilise VIII:C would thus allow blood banks to extend the time period between donation and separation. This would increase substantially the amount of plasma suitable for factor VIII fractionation without excessive additional costs.

Given the above as the current situation, improvements in plasma quality are suggested by the findings in this study and others (Rock et al 1979, Krachmalnicoff and Thomas 1983, Mikaelsson et al 1983a) which demonstrate the stability of VIII:C in blood collected in heparin. It has been known since the work of Weiss (1965) that VIII:C is stabilised by ionised calcium. Collection of blood in heparin - a non-chelating anticoagulant - permits maintenance of ionised calcium and stabilises VIII:C in both blood and plasma (Figure 3-3) at room temperature. Losses of VIII:C, VIIIR:Ag and fibronectin were observed in heparinised blood and plasma stored at 4°C. These losses are probably due to cryoprecipitation as was observed in CPD blood and plasma at 0°C. Although the effect of warming to 37°C was not assessed, parallel losses in antigen and activity point to a physical removal of molecules. It is possible that the heparin induced precipitation of fibronectin and factor VIII in chilled plasma is also contributing to the effect observed (Amrani et al 1982) although in the present study VIII:C was also precipitated. Amrani et al reported that VIII:C was not precipitated from chilled heparinised plasma but they used different amounts of heparin.

Several options are possible for exploiting the improved stability of VIII:C in physiological calcium levels. Collection of blood in heparin anticoagulant would require that CPD be added back to the cells after

plasma separation, as otherwise the short shelf-life (3 days) (Mollison et al 1942) of such red cell concentrates would decrease their usefulness. This procedure produces red cell concentrates with normal in vivo and in vitro characteristics (de Jonge et al 1983); however, in these studies separation and CPD replenishment was effected shortly after donation. The effect, on red cell concentrates, of prolonged storage of heparin blood followed by CPD replenishment has not been assessed. Plasma collection by plasmapheresis would avoid this problem and allow collection of heparin plasma. However, plasmapheresis is expensive and cannot substitute for whole blood collection to meet red cell concentrate needs. Addition of calcium/heparin mixtures to normal CPD blood units after donation has been proposed (Rock 1982). The present study shows that addition of calcium to a plasma concentration of 10 mM 3 hours after donation gave recovery of VIII:C in both blood and plasma (Figure 3-5). Addition after 18 hours resulted in no significant effect on VIII:C (Table 3-3). This data indicates that recalcification must be made shortly after donation. Other studies (Rock 1982, Rock et al 1983b) indicate that recalcification must be made within 4 hours of donation for maximal recovery of VIII:C. This can be effected by adding sufficient calcium and heparin to the standard anticoagulant solution in the bag so as to result in physiological levels of plasma ionised calcium. Such an approach, however, would require that additional citrate

be added to the red cells after plasma separation. A preferable option would be to add calcium/heparin to plasma separated within 6 hours of donation and allow recovery of VIII:C (2-4 hours) (Figure 3-5) (Krachmalnicoff and Thomas 1983, Rock et al 1983b) prior to plasma freezing.

The data in Table 3-5 suggests that citrate concentrations of up to 10 mM in the plasma do not affect VIII:C stability. It has been shown by Mishler et al (1978) that using half-strength CPD anticoagulant (final plasma citrate concentration about 14 mM) does not affect the storage characteristics of banked blood. Plasma collected by automated plasmapheresis with low citrate anticoagulant has been shown to give greater VIII:C concentrate yields than plasma collected manually in normal anticoagulants (Robinson et al 1983). In whole blood collection, however, use of low citrate levels might result in coagulation of red cell concentrates. The 10 mM plasma citrate concentration found in this study to be compatible with VIII:C stability might require supplementation with heparin to allow production of stable red cell concentrates.

Measurement of ionised calcium in heparin plasma supplemented with citrate (Table 3-5) gave values lower than expected, considering the established normal levels of plasma ionised calcium (about 1 mM) (Penny 1983). It is known that the concentration of free ionised calcium is markedly dependent on pH (Thode et al 1983)

with levels dropping sharply with increasing pH. The lack of a buffering anticoagulant in heparin and citrated heparin plasma samples leads to marked increases in pH as carbon dioxide is lost from the plasma (Krachmalnicoff and Thomas 1983). This may explain the low calcium levels which were measured.

Table 3-4 indicates that addition of 10 mM calcium to CPD donations is sufficient to improve VIII:C stability, further addition giving no additional benefit. The residual ionised calcium levels measured by the electrode were rather low compared to the amounts added. This is probably the result of calcium ion sequestration by other ions including phosphate in the CPD and by binding to plasma protein. A precipitate was seen to form after overnight incubation in the 30 mM calcium sample and this might represent insoluble inorganic calcium salts. Addition of further calcium to heparin plasma did increase VIII:C stability (Table 3-5) a finding noted in one other study (Mikaelsson et al 1983a). This suggests that further calcium addition in CPD plasma is ineffective in increasing VIII:C stability due to the sequestration outlined above.

When all factors are considered, it is probable that the best way to exploit the increased stability of VIII:C in physiological calcium levels is to use this effect to bring about recovery of VIII:C levels in plasma separated up to 6 hours after donation. The data in this and other studies indicates that recalcification during

this period would lead to recovery of VIII:C activity which would be preserved by subsequently freezing the plasma. This procedure would require that calcium/ heparin be added to the plasma bag. Such an additive system is at present not on the market but can doubtless be manufactured if a demand for it exists. The extra cost would be justifiable if a significant improvement in plasma VIII:C content is achieved. However, use of heparin as an anticoagulant has been observed to be unsuitable for production of intermediate purity factor VIII concentrate (Smith qu. Penny 1983) and other purification methods (Chapter V). Thus, the plasma VIII:C content might not be the only factor to be considered.

The results of the study on different bag mixing techniques indicate that the three methods of mixing had no effect on FpA or VIII:C levels. The work of Pflugshaupt and Kurt (1983) claims that inadequate mixing results in thrombin formation and high FpA levels. Further work in this laboratory (Prowse et al 1984a) suggests that donation time and the stripping of the donor lines are parameters that can affect plasma FpA. Plasma quality may be further improved by pumping anticoagulant into the donation line itself, rather than relying on mixing in the bag. A device which ensures proportional admixture of blood and anticoagulant in the donation line has been reported to give plasma and platelets of better quality than are obtained by routine methods (Unger et al 1983).

In summary, this Chapter concludes:

1) Mixing technique during donation using the current plastic bag system is not a crucial parameter for good quality plasma and the use of specially purchased machines for this purpose is not justified. Adequate stripping of donor lines and short venesection times lead to plasma with a good VIII:C content and low FpA levels, which is suitable for fractionation.

2) Plasma separation and freezing within 0 to 4 hours of donation yields plasma with a high VIII:C content; further delays lead to losses. However, plasma in standard donations 18 hours after donation still had about 0.65 u/ml in this study and is still acceptable for fractionation. Storage temperature is not crucial; as far as VIII:C goes, room temperature (about 20°C) is satisfactory. Small scale experiments indicate that factor VIII may be lost by cryoprecipitation in blood or plasma given prolonged storage and sufficient cooling. However, in standard donations stored at 18 hours at 4°C this effect is not seen.

3) Use of anticoagulants which result in plasma ionised calcium levels of 1 mM lead to enhanced VIII:C stability and permit extension of the period between donation and plasma separation/freezing. This would require the use of special plastic bag systems and would lead to the presence of heparin in the plasma, which might affect fractionation.

CHAPTER IV

EFFECT OF PLASMA FREEZING RATE AND STORAGE CONDITIONS ON CRYOPRECIPITATE QUALITY

INTRODUCTION

Although being gradually superseded by lyophilised concentrates, blood bank cryoprecipitate (cryo) still constitutes an important source of therapeutic factor VIII in developed nations such as Britain (Rizza and Spooner 1983) and the United States (Aledort 1982). In underdeveloped countries, cryoprecipitate and fresh frozen plasma, when available, often constitute the sole form of replacement therapy, unless expensive foreign concentrates are purchased.

Thus, any factors influencing the quality of cryoprecipitate have an important bearing on the logistics of haemophilia care. Many studies have investigated the optimal conditions for cryoprecipitate preparation (Kasper et al 1975, Slichter et al 1976, Vermeer et al 1976, Regional Transfusion Directors' Committee 1978). An early report claimed that fast-thawing of the plasma in an 8°C waterbath resulted in much better VIII:C yields than the original overnight thaw in air (Brown et al 1967). This was denied by one study (Bloom et al 1969) which claimed that slow-thawing was better. Subsequent investigations, however, tended to support the benefits of 'fast' versus 'slow' thaw (Vermeer et al 1976, Prowse and McGill 1979, Wensley and Snape 1980) although some find no difference between the two types of thawing (Kasper et al 1975, Rock and Tittley 1977). In 1978, Mason introduced a modified method of fast plasma thawing,

in which the thawed supernatant was continuously siphoned over leaving the frozen cryoprecipitate in the original plastic pack; this was claimed to improve markedly the factor VIII yield. Studies comparing different thawing techniques confirmed these results (Prowse and McGill 1979, Kang 1980).

Although much data has been published regarding thawing methods, indicating the superiority of fast thawing, less certainty exists regarding the mode of freezing plasma. Early studies (Pool and Robinson 1959, Britten and Grove-Ramussen 1966) suggest an inevitable loss of around 15% of the VIII:C when plasma is frozen. Freezing of small volumes of plasma has been reported to reduce this loss (Britten and Grove-Ramussen 1966, Kasper et al 1975) suggesting that fast freezing is necessary, but other reports find no difference between large and small volumes (Preston 1967). Placement of fresh plasma packs in a -30°C or -20°C freezer results in complete freezing taking over 6 hours, with resultant losses in the plasma VIII:C (Fiets and Feitsman 1982) and decreased cryoprecipitate yields compared to faster freezing (Slichter et al 1976, Rock and Tittley 1979). On the question, "How fast is fast?", Kasper et al (1975) report no difference in cryo VIII:C yields between plasma frozen in 30 minutes in -70°C ethanol dry ice and plasma frozen in one hour between dry ice sheets. Smith (1983a) reports no difference between the VIII:C content of 5 litre pools frozen in a Grant plate freezer (90-120 minutes) and

single packs frozen in a liquid nitrogen controlled rate freezer (10-20 minutes) suggesting that the volume of plasma frozen is not critical per se, as long as freezing to about -30°C is achieved in about one hour. Different freezing rates have been reported to affect the protein composition of cryoprecipitate while not influencing factor VIII yields (Vermeer et al 1976); slower freezing resulting in a higher amount of fibrinogen in the cryoprecipitate (Carlebjork and Blombäck 1983).

Installation of liquid nitrogen controlled rate freezers or airblast freezers would involve greater capital and maintenance costs than simple chest freezers or use of cold ethanol. Considerations of product quality and safety are also important, freezing in dry ice/ethanol, for example, although comparatively cheap, has been reported to result in passage of ethanol into the plasma resulting in fibrinogen deposition in cryoprecipitate being much increased (Lane 1981).

Controversy also exists regarding optimal conditions for storing frozen plasma. Some early studies suggest that plasma VIII:C is rather labile at -20°C (Penick and Brinkhous 1956, Pool and Robinson 1959) while other studies report no loss at -20°C for periods of up to 12 months (Preston 1967, Koerner and Stampe 1982). Newman et al (1971) state that storage of frozen plasma for periods longer than 3 to 6 months affects proteins other than factor VIII and can result in processing difficulties during concentrate production, due to high

fibrinogen content.

Measurements based on the theory of eutectics (Mackenzie 1982 qu. Foster 1983a) show that phase changes occur in plasma at temperatures of -27°C and -40°C (and possibly as low as -80°C). Storage at temperatures below these points might be expected to have advantages. Practical considerations, however, play a role in what kind of storage temperatures are used in practice, the relative costs of refrigeration to -50°C, -40°C and -30°C being 4.5, 1.6 and 1.2 fold that of -20°C storage (Watt 1982).

In this chapter, the effect of changing the rate of freezing plasma on the quality of thaw-siphon cryoprecipitate has been investigated. The effect of storage of plasma at different temperatures for varying periods of time has also been determined, in order to find optimal conditions for frozen plasma storage. In this regard, plasma was also deliberately subjected to large temperature fluctuations during storage.

METHODS

Plasma used in this study was CPD plasma prepared from normal donations within 3 hours of blood donation. Plasma pools of 6 donations were used in each experiment, thus avoiding the variations in factor VIII content found in different individuals. The plasma was pooled in a 2 litre bag and aliquots of 200 ml were distributed into 300 ml Fenwal R2011 transfer packs. The plasma was then frozen and stored as follows:

'Slow Freezing': Packs of plasma were placed in thin aluminium cassettes and placed in a -40°C cabinet freezer. The cassettes were placed vertically and the packs had the outlet ports down.

'Fast Freezing': Packs were placed in cassettes as in slow freezing but were frozen in a -70°C ethanol bath which had been cooled using liquid nitrogen.

Freezing Rates: For both these modes of freezing, plasma temperature was monitored by a thermocouple (Comark Instruments) placed in the middle of a pack which was connected to a pen-recorder (Vitatron).

Storage Studies: Plasma pools prepared as above were aliquoted into 6 separate packs and the plasma was frozen in -70°C ethanol. Three packs from each pool were then placed in a -20°C chest freezer, while the other 3 were placed in a -40°C cabinet freezer. Six pools of plasma were prepared and stored in this way. Daily checks ensured that the freezer temperatures were keeping to the reasonable limits (± 4°C) of the temperatures stated.

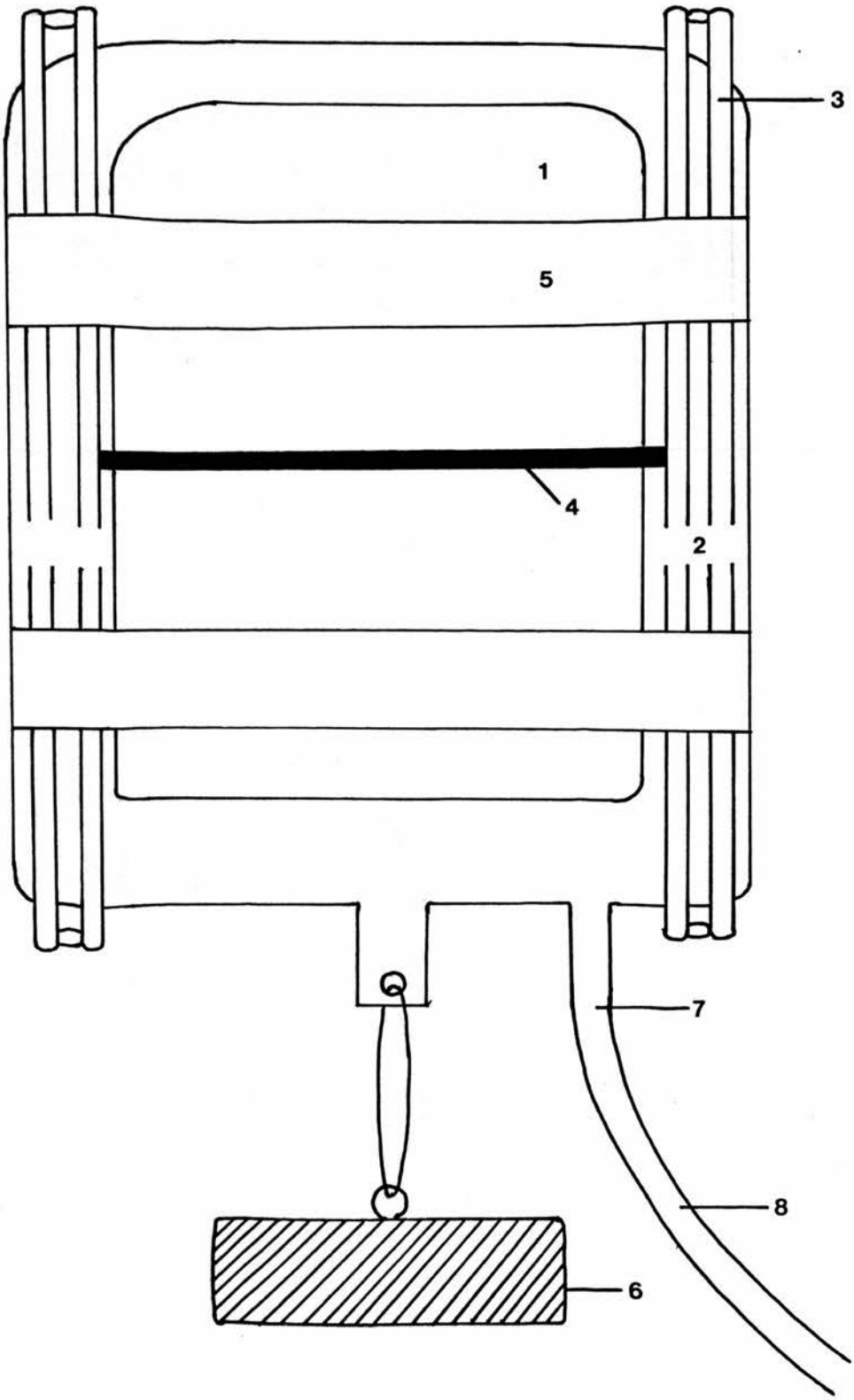
Temperature Insult Studies: Six donation pools were again prepared and plasma was aliquoted into three 300 ml packs. These packs were all frozen in -70°C ethanol and placed in -40°C storage. One pack was stored for one week. The other 2 were removed after 2 days and placed in a 4°C cold room for 4 hours. They were then replaced in -40°C storage. One of these packs was subjected to the same procedure a second time. One week after starting the experiment the packs were all processed to cryoprecipitate. This process was done for 6 plasma pools; a separate pack with a temperature probe monitored the fluctuations in the packs subjected to temperature insult. The remaining 3 packs from each pool were frozen in a -40°C freezer and used for storage studies as outlined above.

Cryoprecipitate Preparation: This was according to Mason et al (1981) with some modifications. The plasma packs were taken and 2 elastic bands, each 15 mm wide, were placed one-third and two-thirds of the way down the pack. Two metallic rods were placed through the slits along each of the 2 lateral edges of the pack and clipped together. The rods were joined to each other by a piece of string, and together with the elastic bands provided tension on the pack during thawing. The outlet tube of the pack was joined to an empty 300 ml pack, and the plasma pack was immersed in a 4°C water bath (Grant Instruments) with the outlet ports at the bottom (i.e. inverted thawing). The pack was weighed down with a

FIGURE 4-1

PLASTIC PACK WITH FROZEN PLASMA SET UP FOR THAWING

1. Frozen plasma block
2. Pack margins with slits
3. Hinged metal rods passed through pack margins
4. String joining metal rods
5. Elastic bands
6. Lead weights
7. Outlet port of pack
8. Siphon tube connecting pack to empty satellite pack


3
1
5
4
2
7
8
6

lead weight. Siphoning of the thawed supernatant was initiated after about 10 minutes by raising the pack momentarily out of the water bath. Thawing was continued until the residual mass of cryoprecipitate was enmeshed in a lump of lightly-pigmented ice, usually when 20-30 ml were still left in the pack. The cryoprecipitate pack was then sealed off and the cryo dissolved in a water bath at 37°C for about 5 minutes. The cryoprecipitate and cryosupernatant plasma volumes were then measured, the 2 components were sampled and the samples were frozen and stored in 2 ml aliquots for subsequent assay. Figure 4-1 shows the details of the plasma pack set up for the thawing process. No attempt was made to remove residual factor VIII e.g. by saline washing.

Assays:

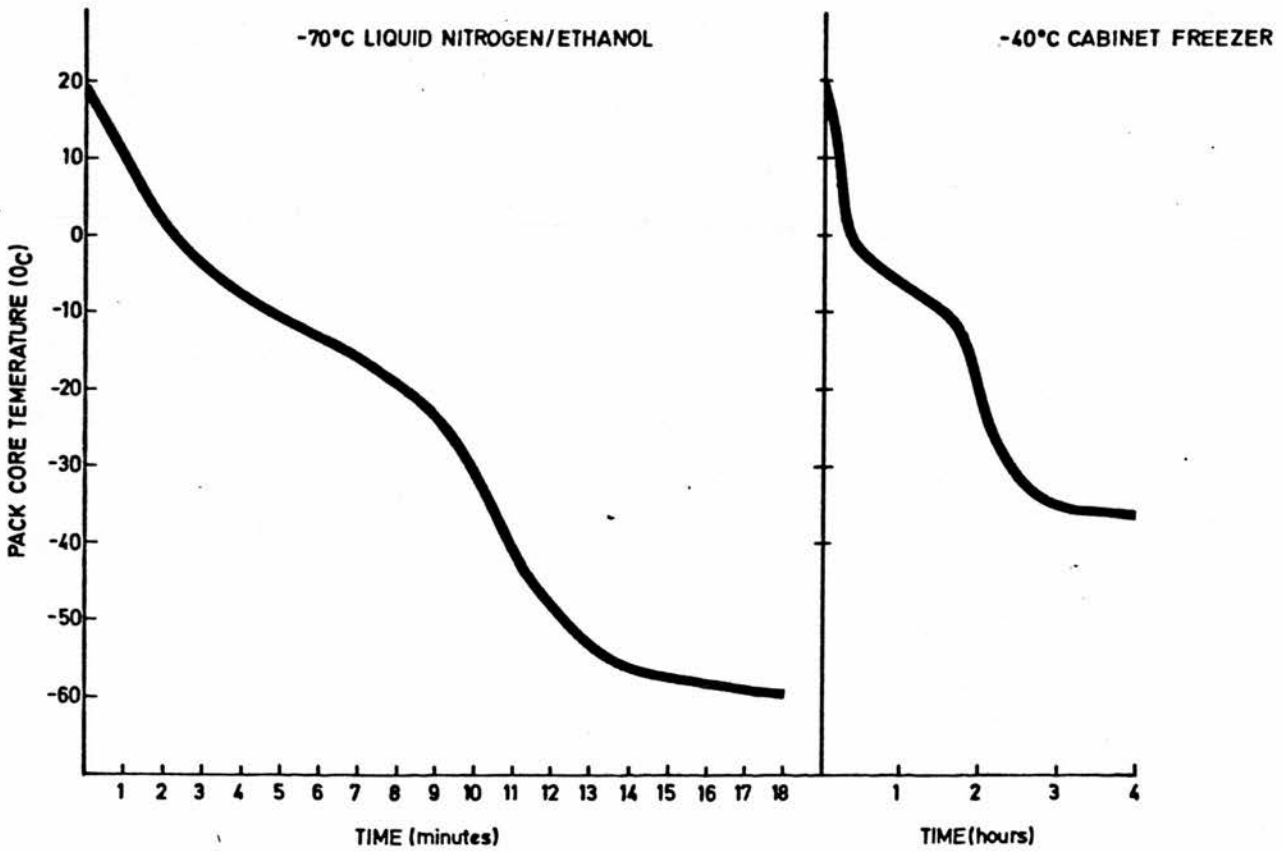
Factor VIII:C, Factor VIII:CAg, Factor VIIIR:Ag, Fibrinogen and Total Protein were assayed as described in Chapter II. Factor VIIIR:Ag was assayed by electro-immunoassay, except for samples of cryosupernatant plasma, the VIIIR:Ag in which gives indistinct precipitin lines (Over et al 1978). For these samples an immunoradio-metric assay (IRMA, Ch.II) was used. Fibrinogen was assayed by electroimmunoassay.

FIGURE 4-2

TEMPERATURE PROFILES OF PLASMA FROZEN IN DIFFERENT MEDIA

Results show temperature changes recorded by probe placed in middle of pack. Probe was connected to a pen-recorder which traced the changes in temperature shown.

TEMPERATURE RECORDED IN A PLASMA PACK FROZEN IN DIFFERENT MEDIA



RESULTS

(A) EFFECT OF PLASMA FREEZING RATE ON CRYOPRECIPITATE QUALITY

Figure 4-2 shows the temperature profiles of plasma packs frozen in a -70°C alcohol bath and a -40°C deep freeze. Plasma frozen in -70°C alcohol took only about 10 minutes to reach -30°C at the core of the pack, while plasma frozen in the -40°C deep freeze took nearly 2½ hours to reach this temperature. From these recordings, it can be estimated that for fast freezing (-30°C in 10 min), 23% of the time was needed for the plasma temperature to reach 0°C, and 77% to drop further to -30°C, while the corresponding figures for fast freezing (-30°C in 2½ hours) are 10% and 90%.

It was noticed that plasma that had been frozen slowly had an even translucent appearance, while plasma that had been frozen fast showed variations in pigment density over the frozen surface. After overnight storage at -40°C, plasma frozen under both these conditions was processed to thaw-siphon cryo as described in Methods. It was immediately noticed that plasma frozen slowly produced a steady flux of particulate material in the siphon lines during thawing. Plasma frozen fast, however, produced a clear supernatant during thawing, with no particles, unless thawing was actually allowed to proceed until the residual cryo also siphoned over.

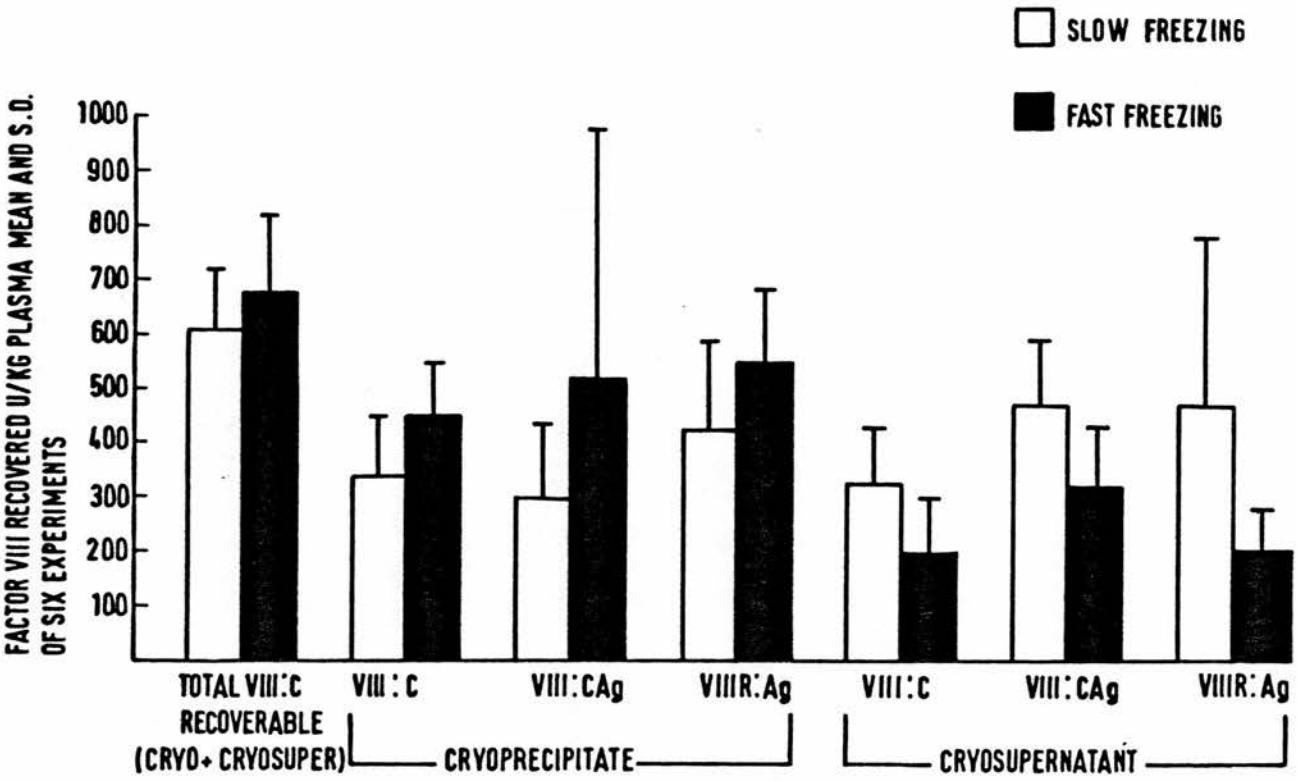


FIGURE 4-3 DISTRIBUTION OF FACTOR VIII RELATED ACTIVITIES IN PLASMA FRACTIONS DERIVED FROM PLASMA FROZEN AT DIFFERENT RATES

Figure 4-3 shows the distribution of factor VIII related activities in cryoprecipitate and cryosupernatant prepared from the 2 types of plasma. Slow freezing results in a lower amount of VIII:C being recovered (expressed as the sum of the VIII:C recovered in cryo and cryosupernatant) upon thawing the plasma; the difference however is not significant (paired t-test, $t = 1.16$, $p > 0.1$). Furthermore, the VIII:C in slowly frozen plasma seems to be less cryoprecipitable, with only 47% of the residual VIII:C being recovered in the cryoprecipitate. These two effects lead to a comparatively poor yield of VIII:C when plasma is frozen slowly. Fast-frozen plasma, however, gave much better results - 425 u/kg of plasma was recovered in the cryo, compared with 318 u/kg for slowly frozen plasma, a difference that was significant ($t = 3.19$, $p < 0.05$). The poor cryoprecipitability of factor VIII in slowly frozen plasma was confirmed by assays of other factor VIII related activities in cryoprecipitate and cryosupernatant - Figure 4-3.

Fibrinogen was measured in cryoprecipitates produced from both types of plasma. Plasma frozen by fast-freezing gave higher yields of fibrinogen in cryo than plasma produced by slow-freezing - 824 mg/kg plasma compared with 522 mg/kg plasma ($t = 4.6$, $p = 0.01$). In general, the flow of particulate material coming over during thawing of slow-frozen plasma seems to represent cryoprecipitate that for some reason is not retained

TABLE 4-1A EFFECT OF DIFFERENT STORAGE CONDITIONS ON PLASMA FROZEN IN -70°C ETHANOL. RESULTS SHOW MEAN ± S.D. FOR SIX DIFFERENT EXPERIMENTS

Temperature of Storage	Period of Storage	Total VIII:C Recovered (Cryo + Super) u/kg Plasma	VIII:C Cryo Yield u/kg Plasma	Fibrinogen Cryo Yield mg/kg Plasma
-20°C	16 hours	623 ± 108	426 ± 80	605 ± 172
-20°C	3 months	698 ± 123	500 ± 118	609 ± 187
-20°C	6 months	690 ± 82	416 ± 67	538 ± 115
-40°C	16 hours	698 ± 125	493 ± 116	607 ± 292
-40°C	3 months	718 ± 102	522 ± 77	577 ± 51
-40°C	6 months	730 ± 101	449 ± 47	542 ± 47

TABLE 4-1B EFFECT OF STORAGE PERIOD ON PLASMA FROZEN IN -40°C DEEP FREEZE. RESULTS SHOW MEAN ± S.D. FOR SIX DIFFERENT EXPERIMENTS

Period of Storage	Total VIII:C Recovered (Cryo + Super) u/kg Plasma	VIII:C Cryo Yield u/kg Plasma	Fibrinogen Cryo Yield mg/kg Plasma
16 hours	610 ± 101	318 ± 111	522 ± 270
3 months	611 ± 183	306 ± 62	502 ± 79

during the siphoning process, leading to lower levels of cryoprecipitated proteins in the cryo derived from such plasma. The total protein yield in cryo was 4.3% and 5.9% for slow- and fast-frozen plasma respectively, a difference which was not significant. In this regard it must be noted that most of the cryo-protein comes from the supernatant plasma left after siphoning is terminated. The amount of this protein is thus expected to be relatively unaffected by changes affecting the proteins (such as factor VIII and fibrinogen) specifically concentrated by cryoprecipitation.

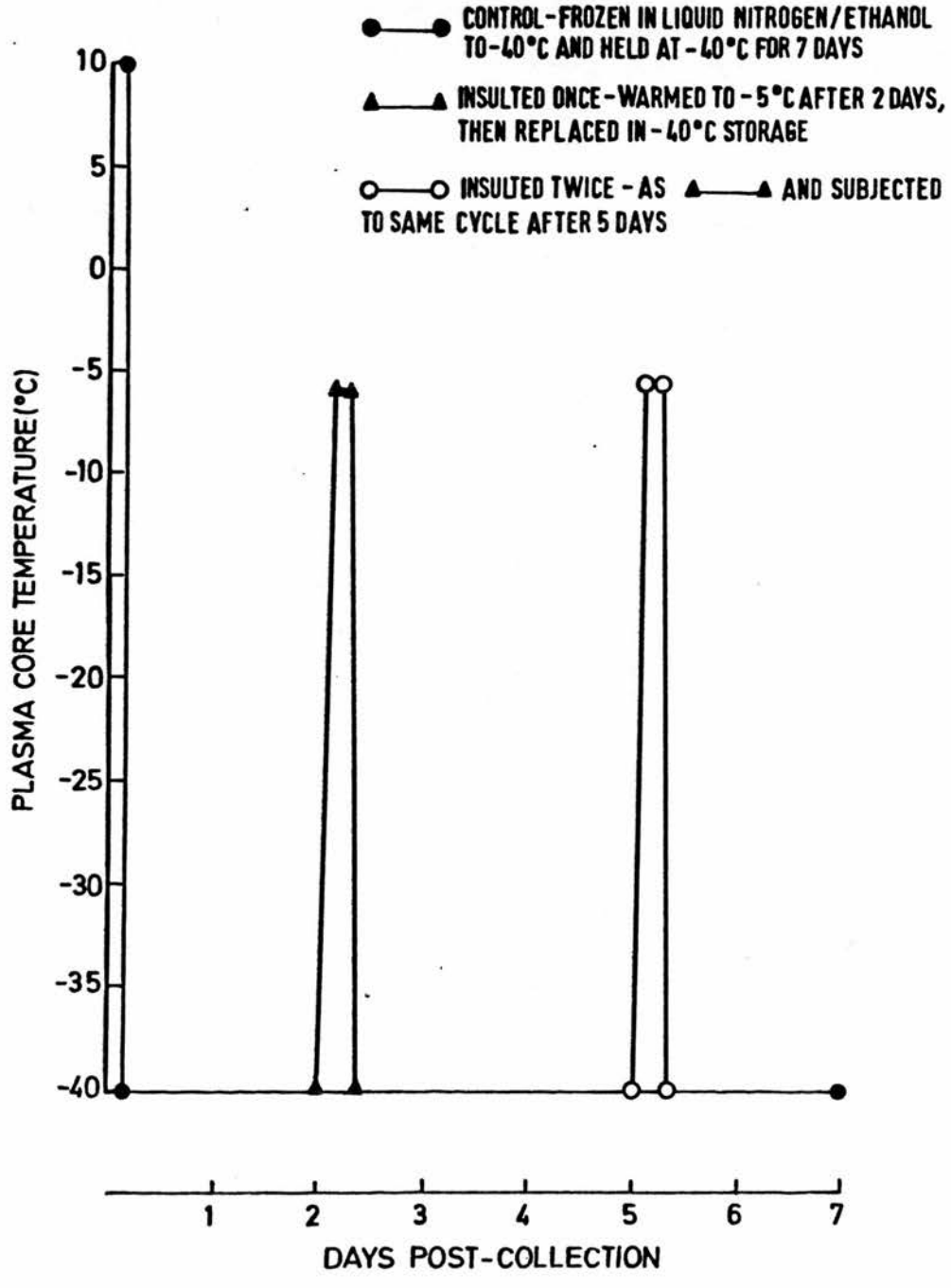
(B) EFFECT OF PLASMA STORAGE CONDITIONS ON CRYOPRECIPITATE QUALITY

The effect of different storage conditions on plasma pools frozen in -70°C ethanol is shown in Table 4-1A. Table 4-1B shows similar data for pools frozen in a -40°C deep freeze.

Initial freezing rate can be seen to be a much more important determinant for factor VIII yields than storage conditions. As long as constant storage temperature is maintained, there is no significant difference in cryo factor VIII yields between plasma stored at -20°C and -40°C. Cryo factor VIII yields did not change during storage periods of up to 6 months for plasma stored at both temperatures, the slight drop in yield after 6 months storage being insignificant. Fibrinogen content in cryoprecipitate likewise was unaffected at both temperatures throughout the period of

FIGURE 4-4

TEMPERATURE RECORDED IN A PLASMA PACK SUBJECTED TO TEMPERATURE INSULT



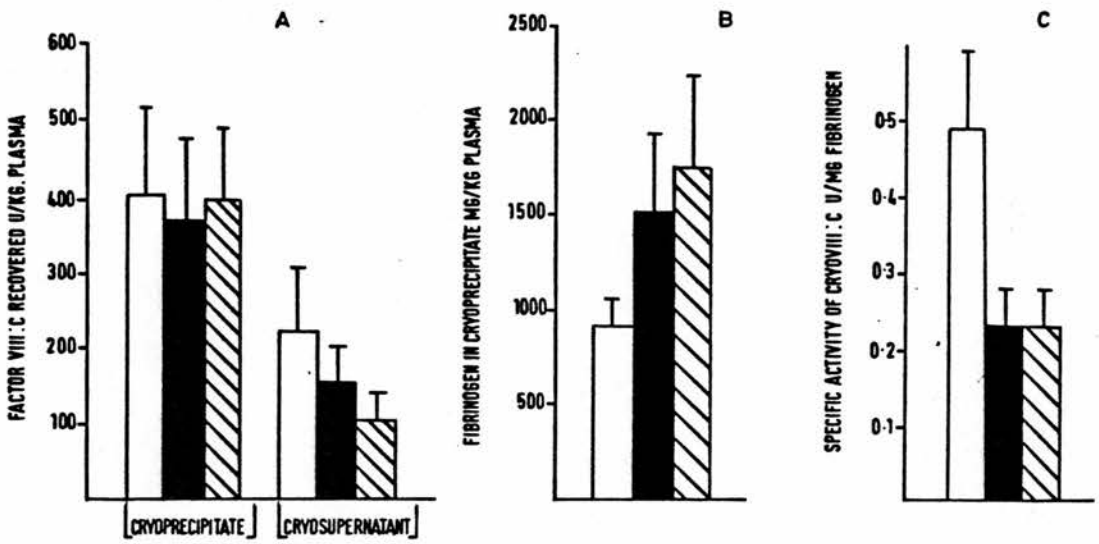


FIGURE 4–5 EFFECT OF TEMPERATURE INSULT (WARMING AND REFREEZING) ON CRYOPRECIPITATE

(A) YIELD OF VIII:C IN PLASMA FRACTIONS

(B) YIELD OF FIBRINOGEN IN CRYOPRECIPITATE

(C) SPECIFIC ACTIVITY OF CRYOPRECIPITATE VIII:C

storage. The same findings are apparent from the data for slowly frozen pools of plasma.

(C) EFFECT OF TEMPERATURE FLUCTUATIONS DURING PLASMA STORAGE ON CRYOPRECIPITATE QUALITY

Figure 4-4 shows the temperature fluctuations recorded in the core of a plasma pack subjected to the temperature insult described in Methods. The effect of these fluctuations on cryoprecipitate production is shown in Figure 4-5.

Although warming and refreezing frozen plasma resulted in a 20% drop in the total amount of factor VIII:C recoverable from such plasma, this was not reflected by a proportionate drop in cryoprecipitate factor VIII:C yields. Rather, the amount of factor VIII:C in cryosupernatant declined sharply upon temperature insult. The fibrinogen content of cryoprecipitate was affected by temperature changes, insulted plasma yielding twice as much fibrinogen in cryoprecipitate (Figure 4-5B) a difference that was highly significant ($t = 4.31$, $p < 0.01$). A second cycle of temperature insult produced no further significant change. The specific activity of cryoprecipitate factor VIII:C (expressed as u/mg of fibrinogen) was thus halved upon temperature insult of the stored plasma.

DISCUSSION

In the thaw siphon technique, continuous removal of thawed supernatant plasma maintains the cryoprecipitate in the frozen state throughout the process, thus ensuring that the temperature is kept close to 0°C. This results in minimal loss of cryoprecipitate factor VIII:C by dissolution and/or enzymatic degradation. On the large scale production of bulk cryoprecipitate for processing to factor VIII concentrate, a similar result is achieved by plasma crushing and continuous thawing with rigid temperature control (Foster et al 1982).

It was the intention of this study to investigate the effects of two variables on cryoprecipitate quality, (i) the rate of plasma freezing and (ii) the conditions under which frozen plasma is stored. It was felt important to use the high yielding thaw-siphon technique as a thawing method, as a method involving high losses during thawing might have masked any differences in yield due to the other variables under investigation. At the same time, it was the intention of the study to investigate the effect of the two variables on the purity of cryoprecipitate, so a modification of the original technique ensuring a higher factor VIII specific activity was used. Compared to the original technique, the modified method produces a lower factor VIII:C yield - about 500 u/kg plasma compared to about 600 u/kg plasma - but gives a higher specific activity - 0.17 u/mg protein versus 0.08 u/mg. The drop in yield was felt acceptable

in order to obtain a higher purity. The specific activity obtained in this study is lower than that reported by Mason et al - 0.17 u/mg compared to 0.5 u/mg. This is probably because of less efficient tensioning during thawing with subsequent retention of excess plasma protein with the cryoprecipitate. This is confirmed by measuring the ratio of cryoprecipitate fibrinogen to total protein using the present method - 0.16 compared to 0.7 in the method described by Mason et al.

Previous studies using the thaw-siphon technique all used plasma frozen by fast-freezing methods. The importance of fast-freezing is shown by the results of this study. Following slow-freezing, a continuous flux of particulate matter in the siphon lines is accompanied by a marked decrease of all the factor VIII related activities in the residual cryoprecipitate, with a corresponding increase in the cryosupernatant (Figure 4-3). The loss of cryoprecipitated material is also reflected by lower levels of fibrinogen in the cryoprecipitate. Thus, although the total recoverable VIII:C content was not different using the two freezing methods, the loss of cryoprecipitate led to factor VIII recoveries comparable or inferior to yields obtained by less optimal thawing techniques (see Chapter V). Attempts to assay the contents of the observed particles were unsuccessful, as these dissolved by the end of the thawing period even when the supernatant was collected in a flask placed in melting ice.

The different results obtained with slow and fast-freezing suggest a different distribution of cryoprecipitate in the plasma pack obtained by the two methods. In fast-frozen plasma, cryoprecipitate appears to be concentrated in the final 20 mls or so of siphoning material. Slow-freezing seems to result in cryoprecipitate formation throughout the whole plasma block, as evinced by the appearance of particles in the siphon lines as soon as thawing commences, leading to loss of cryoprecipitated proteins such as factor VIII and fibrinogen in the supernatant. Mason et al (1981) have proposed that fast-freezing involves a primary precipitation of cryoglobulins (including fibrinogen and factor VIII) followed by precipitation of other proteins as the concentration of electrolyte in the liquid phase increases. Upon thawing, the various proteins then elute in the reverse order to that in which they separated during freezing. It is possible that with slow-freezing precipitation of cryoglobulins occurs throughout the freezing process, leading to cryoprecipitate formation through the whole plasma block. Elution of factor VIII and other cryoglobulins would then occur throughout siphoning, resulting in the losses observed.

The results of this study show that as long as steady storage conditions are maintained, plasma can be stored for long periods without affecting the quality of cryoprecipitate. The work of Mackenzie cited above suggests that complete freezing in plasma is achieved at

below -80°C. Although it has been suggested that storage below the temperature of complete freezing is to be preferred (Watt 1976), a temperature of -20°C was found to be adequate. This finding has important implications, as the cost of -20°C refrigeration is considerably lower that that of -40°C. Storage period also did not have a crucial effect, the slight drop in VIII:C yield after 6 months being insignificant. There was no change in the total fibrinogen and total protein in cryoprecipitate after 6 months storage, and no difference between the two temperatures studied.

Storage involving deliberate temperature insult resulted in a marked drop in the total factor VIII:C recoverable from plasma after processing to cryoprecipitate and cryosupernatant. This drop, however, was not reflected in the amount of VIII:C recovered in cryoprecipitate, which remained relatively constant, but was due to a sharp drop in VIII:C recovered in the cryosupernatant. The increased lability of cryosupernatant VIII:C compared to cryoprecipitate VIII:C has been documented (Over et al 1978) and it seems that the deleterious effects of temperature insult result in loss of this form of VIII:C.

The marked increase in cryoprecipitate fibrinogen that results from temperature insult may be one of the reasons for the variation in cryoprecipitate mass that has been noted during bulk plasma fractionation (Hershgold et al 1966, Newman et al 1971). Foster (1983a)

has reported similar increases in cryo-fibrinogen in plasma that has been stored at constant temperature for increasing periods. The present results indicate that temperature fluctuations are more likely to be the reason, no increase in fibrinogen being apparent when a constant storage temperature was maintained. Fluctuations may occur during storage and/or transportation of bulk frozen plasma, although warming of large plasma blocks is bound to be less rapid than for the single donations used in this study. It is uncertain why the cryoprecipitated fibrinogen should increase after temperature insult. Increased aggregation of fibrinogen molecules leading to greater insolubility could be one explanation (Newman et al 1971). Increased cryoprecipitate fibrinogen is responsible for difficulties in extracting and further processing factor VIII concentrates and results in poor filterability and solubility. Maintenance of steady temperatures during frozen plasma storage is therefore important.

In summary, this study concludes:

1) Cryoprecipitation using the thaw-siphon technique requires plasma that is frozen fast for good factor VIII yields. The results of this and other studies suggest that attainment of -30°C in the core of the pack in about one hour should be sufficient. Ethanol cooled to -70°C with dry ice can achieve this very efficiently. Placing the packs in a plastic bag should decrease any risk of ethanol leaking into

the pack, although this was never encountered in the course of this work.

2) Storage of frozen plasma at -20°C until processing is perfectly adequate in terms of product quality and is much cheaper than refrigeration at lower temperatures.

3) Maintenance of refrigeration equipment is very important to avoid temperature fluctuations leading to the effects outlined in Results. This is especially important if -20°C storage is used, as minor breakdown of such equipment is more likely to result in rapid temperature rises. Daily or continuous monitoring of temperature is recommended.

CHAPTER V

APPROACHES TO PRODUCING A FACTOR VIII CONCENTRATE FROM HUMAN PLASMA IN BLOOD BANKS

INTRODUCTION

At present, about 98% of the world's production of factor VIII concentrates starts off with the production of cryoprecipitate (De Vreker 1980). Cryoprecipitate yield and quality depend on many variables (Chapter I) and yields of 20 to 85% of the plasma VIII:C have been reported (Masure 1969). By modifying the thawing technique and using optimal freezing methods, yields of about 500 u/kg of plasma can be obtained on a blood bank scale (Chapter IV). Such thaw-siphon cryoprecipitate, however, cannot substitute, in terms of convenience and ease of storage and administration, for lyophilised concentrate, which is produced at much lower yields.

Although cryoprecipitation is widely utilised, the mechanism of the process is poorly understood. Polson (1972) has proposed that the well known salting-out of proteins is involved. In this theory, the increasing salt concentration in the intercrystalline spaces of frozen plasma causes precipitation of the most insoluble proteins, including factor VIII and fibrinogen. An alternative explanation proposed a steric exclusion mechanism (Owen and Wagner 1972), in which exclusion of high molecular weight proteins from volumes of solvent by increasing concentrations of plasma protein leads to their precipitation. A better understanding of the mechanism of cryoprecipitation might allow improvements in product quality and yield.

The ease and simplicity of cryoprecipitation have led to few investigations into alternatives for concentrating factor VIII from plasma. The early observations of Polson et al (1964) suggested that polymer precipitation might provide a method of plasma protein fractionation, but in the case of factor VIII, polymers such as polyethylene glycol (Newman et al 1971) have only been used in the later stages of purification. Use of such polymers, which act through a steric exclusion mechanism (Polson and Ruiz-Bravo 1972), might enable the high molecular weight difference between factor VIII and other plasma proteins to be exploited. So far, this difference has mainly been used, in gel filtration, for separations on an analytical scale (Chapter II, Ratnoff et al 1969).

It is the purpose of this Chapter to develop a simple and reliable process for the concentration of factor VIII from plasma using methods applicable in blood banks. The method sought after needed to give a high yield of factor VIII with the product characteristics of current lyophilised concentrates. In addition, the method had to allow utilisation of other blood components after concentration of factor VIII.

In this regard, some attempts were made to improve cryoprecipitate yield by adding heparin (Rock et al 1980b) or P.E.G. (Johnson et al 1979) to plasma which was then thawed by different methods. Studies were made on the mechanism of cryoprecipitation, in order to

delineate conditions for better VIII:C yields. As an alternative to cryoprecipitation, polymer precipitation of unfrozen plasma was examined. Conditions derived for factor VIII purification on the small scale were then utilised on medium scale plasma volumes, thereby modelling factor VIII concentrate production under blood bank conditions.

METHODS

Cryoprecipitate production: Plasma for these experiments consisted of pools prepared as in Chapter III or as described in Results. All plasma was frozen by fast-freezing as in Chapter IV.

Thawing: Two basic thawing methods were used:

1. Fast-thawing. 200 ml packs of frozen plasma were placed in a water bath at 4°C. The plasma was allowed to thaw until visual inspection showed that only a few ice crystals were left. This took about 100 minutes from the start of thawing. The cryo-precipitate was then sedimented either by (a) centrifugation of the bags in a Mistral 6L centrifuge for 36,000 g min. at 0°C. All but 20 ml of supernatant plasma was then expressed out of the pack using a Fenwal plasma expressor and 3 ml of 15 mM citrate, 150 mM NaCl, pH 6.9 (citrate-saline) were added to the cryoprecipitate (cryo) bag. The cryo was then solubilised in a 37°C water bath, its volume was measured and small aliquots (2 ml) sampled, fast frozen and stored at -40°C until assayed; or (b) transfer of the thawed plasma to polycarbonate bottles and centrifugation in a Sorvall RC-2B centrifuge for 80,000 g min. at 0°C. The supernatant was then decanted and the cryoprecipitate was dissolved in 10 ml of citrate-saline.
2. Thaw-siphoning and centrifugation. This was performed according to Kang (1980). Packs of frozen

plasma were placed in a 4°C water bath. The outlet tubing was attached to an empty 200 ml bag and clamped. After about 10 minutes in the water bath, the clamp was removed and 2 elastic bands were placed around the pack as described in Chapter IV. The pack was replaced in the water bath and siphoning was initiated using a pair of Fenwal (R4415) tube-strippers to force air out of the siphon line. Siphoning was allowed to continue until about 60 ml of frozen material remained in the bag. This point was reached about 60 minutes from the start of thawing. The siphon line was the clamped and the residual material was allowed to thaw in the bag until a few ice crystals remained. The thawed plasma was then transferred to polycarbonate bottles and centrifuged for 80,000 g min. in a Sorvall RC-2B centrifuge. The supernatant was decanted completely and combined with the supernatant obtained from siphoning. The cryoprecipitate was dissolved in 10 ml of citrate-saline.

Small scale cryoprecipitation of plasmas of different composition:

1. Preparation of plasma of different ionic strengths.

   Low ionic strength: 10 ml plasma aliquots were dialysed against 5 litres of distilled water for 4 hours at room temperature. Control samples were held at room temperature for the same period, and then adjusted to the same volume with isotonic saline.

High ionic strength: 10 ml plasma aliquots were made to 2% NaCl by addition of solid crystals of NaCl and stirring until dissolution was observed.

2. Preparation of plasma of low albumin content. Albumin poor plasma was prepared by gel filtration of 20 mls of plasma on a column (70 x 1.5 cms) of Sepharose CL-4B (Pharmacia). The column was eluted with citrate-saline pH 6.9. 6 ml fractions were collected and assayed for VIII:C, VIIIR:Ag, fibrinogen and fibronectin as described in Chapter II. Total protein was assayed by measuring the absorbance of fractions at 280 nm. Fractions containing the bulk of VIIIR:Ag, fibrinogen and fibronectin were pooled and concentrated to 20 ml by ultrafiltration on an Amicon PM-10 membrane. A control sample was allowed to stand at room temperature for the length of the procedure, which took about 22 hours from when the plasma was collected.

3. Preparation of fibrinogen-depleted plasma. 10 ml plasma aliquots were clotted with 0.5 mls of Reptilase reagent (Pentapharm) at 37°C for 1 hour. The resultant clots were removed with a thin wooden stick, the clot being squeezed to express any entrapped fluid. Controls were incubated for the same period with 0.5 ml of saline.

4. Preparation of fibronectin-depleted plasma. 10 ml plasma aliquots were batch adsorbed with gelatin-agarose (3 ml settled gel; prepared as in

Chapter II) for 3 hours at room temperature. Controls were adsorbed with 3 ml of Sepharose 4B. The plasma was separated after centrifugation for 20,000 g min.

The small aliquots of plasma prepared as described above were frozen in a -40°C cabinet freezer. Cryoprecipitate was prepared by thawing in a 4°C water bath, centrifugation for 20,000 g min. at 4°C and solution of the cryo in 1 ml of citrate-saline.

Salt precipitation of plasma (Polson and Ruiz Bravo 1972):

Aliquots of plasma were made 20% (w/w) NaCl by addition of solid NaCl at room temperature with constant stirring. After 15 minutes at room temperature, the mixtures were centrifuged for 13,000 g min. and the precipitate was dissolved in 0.1 of the plasma volume of citrate-saline.

Polymer induced precipitation of plasma:

Polymer stock solutions were made by dissolving 40% w/w of polymer in isotonic saline. The following polymers were used: polyethylene glycol (PEG) Mn = 6000 (Union Carbide); polyvinylpyrrolidone Mn = 10,000 and Mn = 40,000 (PVP 10, PVP 40) (Sigma); Dextran T 70, Dextran T 150 and Ficoll 70 (Pharmacia Fine Chemicals); hydroxyethyl starch Mn = 40,000, Mn = 350,000 and Mn = 450,000 (HES 40, HES 350, HES 450) were generously supplied by the Army Blood Supply Depot, Aldershot. A 40% stock solution of human albumin was made by concentrating a 20% solution (Immuno) by ultrafiltration on an Amicon

PM-10 membrane.

Polymer induced precipitation: Dilutions of stock polymer solutions were added to plasma until the desired final concentration was reached. The mixtures were then held under the conditions described in Results, after which the precipitates formed were sedimented by centrifugation for 13,000 g min. Experiments were performed on small scale (20 ml volumes of plasma) and the conditions derived were used to process medium scale volumes (200-1500 ml).

Plasma concentration and cold precipitation: Plasma was concentrated by ultrafiltration on an Amicon PM-10 membrane or by dialysis against 50% PEG 6000 in 20 mM Tris, 150 mM NaCl, pH 7.2 as described by Owen and Wagner (1972). The concentrated plasma was then held at 0°C (melting ice bath) for 30 minutes, centrifuged for 13,000 g min. at 4°C and the resultant precipitate dissolved in 0.1 of the original volume of buffer as specified in Results.

TABLE 5-1 EFFECT OF HEPARIN ON FAST-THAW CRYOPRECIPITATE

Conditions	n	VIII:C Yield u/kg plasma	VIIIR:Ag Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity VIII:C u/mg protein
No heparin added	3	364 ± 53.5	529 ± 55	678 ± 73	0.13 ± 0.03
Heparin added to plasma, 1 u/ml	3	347 ± 74	519 ± 58	678 ± 89	0.12 ± 0.03
Heparin added to plasma, 5 u/ml	1	435	680	690	0.11
Heparin added to blood bag pre-donation, 2 u/ml in plasma*	2	475	608	730	0.18

Cryoprecipitate was produced by fast-thawing and centrifugation of plasma bags for 36,000 g min. as described in Methods.

Results show mean and standard deviation of experiments using plasma pools of 6 donations.

* Heparin was introduced in the blood bag with CPD anticoagulant before donation, so as to result in a concentration of about 2 u/ml in the plasma

TABLE 5-2 EFFECT OF HEPARIN ON THAW-SIPHON/CENTRIFUGE CRYOPRECIPITATE

Conditions	n	VIII:C Yield u/kg plasma	VIIIR:Ag Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity VIII:C u/mg protein
No heparin added	3	637 ± 150	690 ± 165	505 ± 84	0.74 ± 0.19
1 u/ml heparin added 1 hour post donation	3	625 ± 126	699 ± 142	512 ± 123	0.70 ± 0.1
1 u/ml heparin added 3 hours post donation	3	637 ± 163	672 ± 128	537 ± 83	0.71 ± 0.14

Results show mean and standard deviation of experiments using plasma pools of 6 donations

TABLE 5-3 EFFECT OF ANTICOAGULANT ON CHARACTERISTICS OF FAST-THAW CRYOPRECIPITATE

Anti-coagulant	n	VIII:C Yield u/kg plasma	VIIIR:Ag Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity VIII:C u/mg protein
CPD	6	386 ± 120	580 ± 135	680 ± 136	0.12 ± 0.04
Heparin*	6	533 ± 156	641 ± 150	735 ± 187	0.09 ± 0.02

Results show mean and standard deviation of experiments using single, unpooled donations

* Heparin in the blood bag was at an amount which gave a concentration of 5 u/ml in the blood

TABLE 5-4 EFFECT OF PEG 6000 ON CHARACTERISTICS OF FAST-THAW CRYOPRECIPITATE

Conditions	n	VIII:C Yield u/kg plasma	VIIIR:Ag Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity VIII:C u/mg protein
No PEG added	5	591 ± 153	796 ± 284	490 ± 111	0.6 ± 0.15
PEG added to 1% w/w in plasma	6	563 ± 123	667 ± 172	906 ± 210*	0.32 ± 0.04*

Cryoprecipitate was produced by fast-thawing and transfer of thawed plasma to bottles for centrifugation for 80,000 g min. as described in Methods

Results show mean and standard deviation of experiments using single, unpooled donations

* Significantly different from non-PEG assisted cryoprecipitation $p < 0.01$

RESULTS

(A) EFFECT OF HEPARIN ON CRYOPRECIPITATE FACTOR VIII YIELDS

Table 5-1 shows that addition of heparin to CPD plasma had no effect on the factor VIII yield in fast-thaw cryoprecipitate. Increasing the amount of heparin added did not improve yield and neither did adding heparin to the anticoagulant in the blood bag instead of addition to the plasma after separation.

Using the thaw-siphon/centrifugation technique, VIII:C yields were significantly increased compared to yields using the fast-thaw method ($p < 0.05$) (Table 5-2). Addition of heparin to the plasma did not affect yields. Delaying addition of heparin until 3 hours after the initial donation also did not affect yields.

Collection of blood in heparin anticoagulant resulted in increased yields of cryo VIII:C compared to collection in CPD (Table 5-3) but the difference was not significant.

(B) EFFECT OF POLYETHYLENE GLYCOL (P.E.G.) ON CRYOPRECIPITATE FACTOR VIII YIELDS

Table 5-4 shows the characteristics of cryoprecipitate prepared by the fast-thaw technique with hard centrifugation and complete drainage of the supernatant. Addition of PEG 6000 to plasma to a final concentration of 1% w/w did not increase VIII:C yields, but the amount of fibrinogen in the cryoprecipitate was

TABLE 5-5 SALT PRECIPITATION OF PLASMA

Number of Experiments	Plasma Volume	VIII:C Yield u/kg plasma	VIIIR:Ag Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity Protein VIII:C u/mg
3	253 ± 75	378 ± 85	812 ± 23	2350 ± 424	0.14 ± 0.02

Results show characteristics of precipitates obtained by plasma precipitation with 20% NaCl

Results are the mean and standard deviation of experiments using plasma pools from 6 different donors

TABLE 5-6 CHARACTERISTICS OF PLASMA OF DIFFERENT COMPOSITION

Variable Investigated	VIII:C u/ml	VIIIR:Ag u/ml	Fibrinogen mg/ml	Fibronectin mg/ml[+]	Total Protein mg/ml	Conductivity mMho
A) Ionic Strength:						
Control*	0.96 ± 0.18	1.0 ± 0.25	2.3 ± 0.8	0.36 ± 0.10	56 ± 9	7.65 ± 0.2
Low	1.1 ± 0.22	1.1 ± 0.28	2.3 ± 0.6	0.38 ± 0.15	54 ± 7	1.27 ± 0.05
B) Protein Content:						
Control	0.73	1.04	2.85	0.24	47	7.5
Low	< 0.05	0.72	1.57	0.16	14	7.7
C) Fibrinogen Content:						
Control	1.23 ± 0.3	1.24 ± 0.2	3.1 ± 0.15	0.49 ± 0.21	61 ± 10	7.7 ± 0.1
Low	1.23 ± 0.14	1.1 ± 0.21	1.4 ± 0.4×10^{-2}	0.33 ± 0.11	59 ± 6	7.6 ± 0.1
D) Fibronectin Content:						
Control	0.98 ± 0.14	1.07 ± 0.14	2.31 ± 0.35	0.25 ± 0.03	63 ± 12	7.6 ± 0.3
Low	1.1 ± 0.2	1.21 ± 0.14	3.24 ± 1.29	4.6 ± 0.5×10^{-3}	63 ± 17	7.5 ± 0.2

Plasma and controls were prepared as described in Methods. All results show the mean and standard deviation of 6 different plasma except (B) where 2 separate experiments were performed, each from a plasma pool of 6 donations.

* High ionic strength plasma had the same characteristics but had a conductivity of 24.8 mMho

\+ Assuming mormal pooled plasma = 0.33 mg/ml (Mosseson and Amrani 1980).

TABLE 5-7 EFFECT OF PLASMA COMPOSITION ON CRYOPRECIPITATE

Plasma Type	VIII:C Yield in Cryo % plasma	VIIIR:Ag Yield in Cryo % plasma	Fibrinogen Yield in Cryo % plasma	Fibronectin Yield in Cryo % plasma
Physiologic Ionic Strength	47 ± 4	64 ± 12	34 ± 17	56 ± 22
Low Ionic Strength	60 ± 13	51 ± 6	42 ± 14	34 ± 12
High Ionic Strength	5 ± 2	32 ± 19	19 ± 7	89 ± 47
Physiologic Protein Content	53	84	50	94
Low Protein Content	*	10	2	2
Physiologic Fibrinogen Level	47 ± 8	73 ± 24	55 ± 28	68 ± 26
Low Fibrinogen	11 ± 6	11 ± 6	92 ± 33	13 ± 26
Physiologic Fibronectin Level	42 ± 4	48 ± 20	32 ± 6	88 ± 23
Low Fibronectin	3 ± 1	5 ± 3	2 ± 1	25

Cryoprecipitate was prepared by thawing in a 4°C water bath, centrifugation and solution of the precipitates in 1 ml of 15 mM citrate, 150 mM NaCl, pH 6.9

* Plasma VIII:C level was initially very low and yields could not be measured

significantly increased ($p < 0.01$). The specific activity of cryo VIII:C was also significantly lowered ($p < 0.01$) when PEG was added to the plasma.

(C) PLASMA PRECIPITATION WITH NaCl

Table 5-5 summarises the characteristics of the precipitate obtained by adding NaCl to a final plasma concentration of 20% w/w. Although most of the VIIIR:Ag precipitated, the VIII:C yield was comparatively poor. Most of the plasma fibrinogen also precipitated, leading to a low specific activity of VIII:C in the precipitate. It was also noticed that the precipitates showed great instability, with clotting occurring after one cycle of freeze-thawing.

(D) CRYOPRECIPITATION OF PLASMAS OF DIFFERENT COMPOSITION

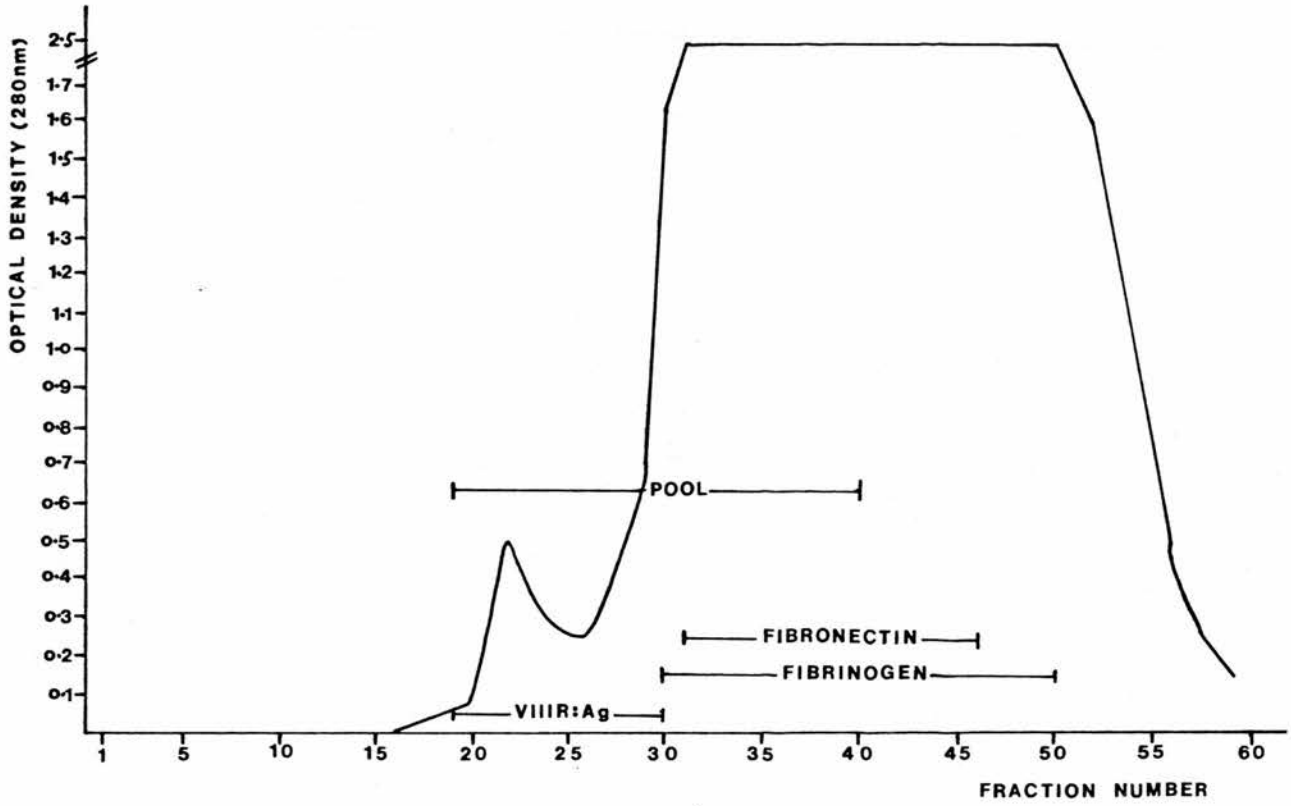
Table 5-6 lists the characteristics of 'plasma' of different composition prepared as described in Methods. Table 5-7 shows the effect of these differences on the characteristics of cryoprecipitate.

1. Plasma of different ionic strengths:

Decreasing the ionic strength of plasma by dialysis increased the yield of VIII:C in cryo but the difference compared to an untreated control was not significant. Increasing the plasma ionic strength by salt addition however resulted in significantly decreased yields of cryo VIII:C ($p < 0.001$) and fibrinogen ($p < 0.05$). Cryo VIIIR:Ag was also

FIGURE 5-1

GEL FILTRATION OF 20 mls POOLED CPD PLASMA ON SEPHAROSE CL-4B COLUMN (70 x 1.5 cm)
Elution was with 15 mM citrate, 150 mM NaCl, pH 6.9 at 35 ml /hour. 6 ml fractions were collected. The positions of elution of VIIIR:Ag, fibrinogen and fibronectin are shown. Fractions containing the bulk of these proteins were pooled as shown and concentrated as described in Methods.


OPTICAL DENSITY (280nm)
2·5
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
POOL
FIBRONECTIN
FIBRINOGEN
VIIIR:Ag
1
5
10
15
20
25
30
35
40
45
50
55
60
FRACTION NUMBER

decreased, although not to the same extent as VIII:C, while fibronectin yield was unaffected. It was noted that some degree of clotting had occurred in the cryo from high ionic strength plasma after removal of the cryosupernatant.

2. Plasma of low protein content:

Gel filtration of plasma on Sepharose CL-4B gave the elution pattern shown in Figure 5-1. The relatively large sample:column volume ratio did not permit sharp separation of the main proteins according to their molecular weights. Nevertheless, by pooling the fractions shown and concentrating them to the initial volume, a low protein plasma with the characteristics shown in Table 5-6 was obtained. Although this material was somewhat depleted in fibrinogen and fibronectin, substantial amounts of these cryoprecipitable proteins were present, as was VIIIR:Ag. The production method led to loss of most of the VIII:C however. Such protein-poor plasma gave a very small cryoprecipitate, with low levels of all the normally cryoprecipitable proteins.

3. Fibrinogen depleted plasma:

Clotting with Reptilase allowed fibrinogen removal without affecting VIII:C (Funk et al 1971) (Table 5-6). Cryoprecipitation gave low levels of the other cryo-proteins, factor VIII and fibronectin (Table 5-7) in the precipitate.

FIGURE 5-2

SMALL SCALE PRECIPITATION OF CPD PLASMA WITH HYDROPHILIC POLYMERS

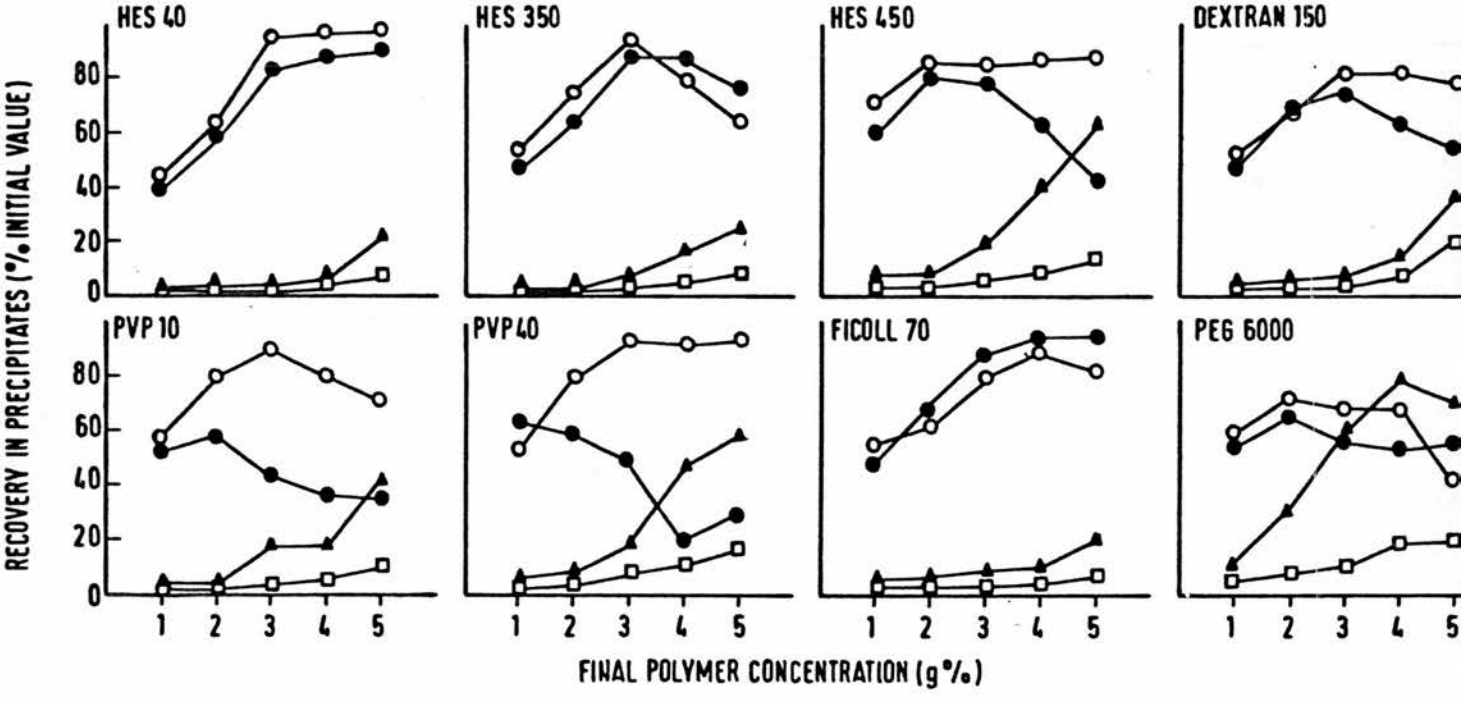
20 ml aliquots of plasma were made to the appropriate polymer concentration and held for 2 hours in melting ice. The precipitates were then recovered by centrifugation for 13,000 g min. at 4°C, dissolved in 2 ml of 60 mM NaCl, 130 mM glycine, 70 mM dextrose, 20 mM citrate, pH 6.8 ('glycine-dextrose' buffer) and stored at -40°C

O —— O VIII:C

● —— ● VIIIR:Ag

▲ —— ▲ Fibrinogen

□ —— □ Total protein


HES 40
HES 350
HES 450
DEXTRAN 150
PVP 10
PVP 40
FICOLL 70
PEG 6000
RECOVERY IN PRECIPITATES (% INITIAL VALUE)
0
20
40
60
80
1
2
3
4
5
FINAL POLYMER CONCENTRATION (g %)

4. Fibronectin depleted plasma:

Adsorbtion of plasma with gelatin-agarose resulted in specific depletion of fibronectin (Table 5-6). Subsequent cryoprecipitation led to low yields of all the cryo-proteins in the precipitate (Table 5-7).

(E) POLYMER INDUCED COLD PRECIPITATION OF PLASMA

1. Small scale precipitation:

Preliminary observations indicated that certain polymers formed precipitates when added to plasma at low temperatures. Figure 5-2 shows dose response results for the various polymers when tested on 20 ml amounts of plasma under the conditions described. All polymers tested produced selective precipitation of VIII:C and VIIIR:Ag, but some resulted in a better purification than others. In particular, the various hydroxyethyl starches and Ficoll 70 gave quantitative recoveries of VIII:C and VIIIR:Ag at concentrations which produced minimal precipitation of fibrinogen and total protein. At higher concentrations, increased fibrinogen precipitation was observed and, in the case of HES 350 and HES 450, the precipitates were somewhat insoluble. At concentrations of between 2 and 4%, maximal recovery and purification of factor VIII was obtained.

Other polymers were found to be less selective. Except at the lowest concentrations tested, PEG precipitated factor VIII and fibrinogen

FIGURE 5-3

SMALL SCALE PRECIPITATION OF CPD PLASMA WITH HUMAN ALBUMIN

Conditions for precipitation and recovery of precipitates were as in Figure 5-2.

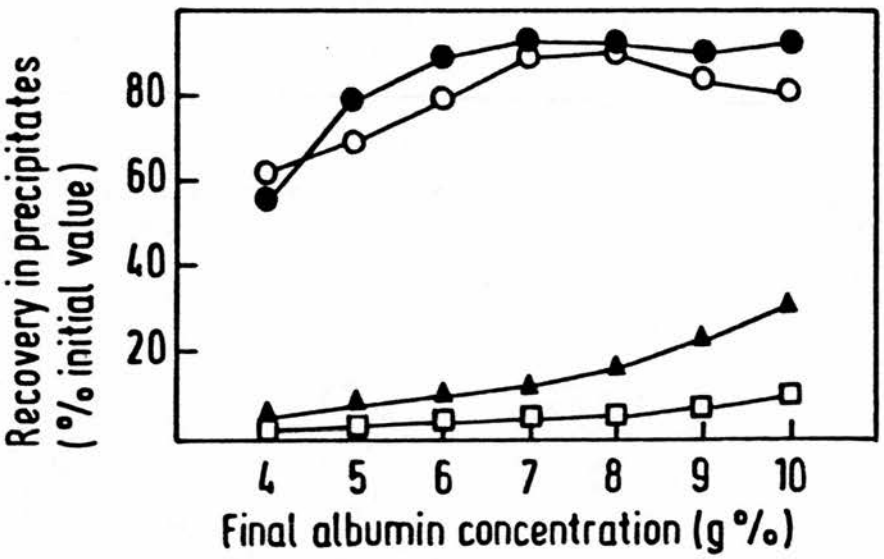

Recovery in precipitates
(% initial value)
80
60
40
20
4
5
6
7
8
9
10
Final albumin concentration (g %)

TABLE 5-8 SMALL SCALE (20 ml) POLYMER PRECIPITATION OF FRESH PLASMA. CONDITIONS FOR OPTIMAL FACTOR VIII PURIFICATION

Polymer	Final Concentration (% w/w)	n	VIII:C u (% yield)	VIIIR:Ag u (% yield)	Fibrinogen mg (% yield)	Total Protein mg (% yield)	Specific Activity u/mg
HES 40	4	6	14.2 (89)*	19.4 (108)	5.4 (9)	24 (2)	0.59
HES 350	3	6	14.9 (93)	17.1 (95)	5.4 (9)	12 (1)	1.24
HES 450	2	3	13.6 (85)	15.5 (86)	5.4 (9)	48 (4)	0.28
Dextran T-70	2	4	12.6 (79)	13 (72)	4.2 (7)	36 (3)	0.35
Dextran T-150	3	4	11.7 (73)	14.4 (80)	4.2 (7)	36 (3)	0.33
PVP 10	3	5	6.7 (42)	15.8 (88)	10.8 (18)	48 (4)	0.14
PVP 40	2	4	9.8 (61)	14.4 (80)	4.8 (8)	24 (2)	0.41
Ficoll 70	4	5	14.6 (91)	15.5 (86)	4.8 (8)	48 (4)	0.30
PEG 6000	2	3	9.8 (61)	12.8 (71)	18.6 (31)	96 (8)	0.10
Percoll	3.5	3	4.3 (27)	9 (50)	0.7 (1.1)	12 (1)	0.36
Human Albumin	6	7	14.2 (89)	15.8 (88)	4.8 (8)	24 (2)	0.59

Results presented as the mean amounts of the respective activities recovered in the precipitates, with the respective yields as % of starting plasma in brackets

* To allow clarity standard deviations have not been shown above but were typically ± 15 percent from the mean values shown

TABLE 5-9 MEDIUM SCALE POLYMER PRECIPITATION OF FRESH PLASMA

Process	n	VIII:C u/kg	VIII:CAg u/kg	VIIIR:Ag u/kg	Fibrinogen mg/kg	Fibronectin mg/kg	Total Protein mg/kg	Specific Activity u/mg
A) Two Hour Incubation:								
4% HES 40	8	576 ± 146	576 ± 108	990 ± 250	448 ± 84	168 ± 92	744 ± 186	0.93 ± 0.21
4% Ficoll 70	6	568 ± 138	522 ± 171	750 ± 230	420 ± 112	N.D.	868 ± 310	0.84 ± 0.23
B) Overnight Incubation:								
4% HES 40	2	525	N.D.	960	616	N.D.	2604 ± 806	0.24 ± 0.06
4% Ficoll 70	6	421 ± 163	540 ± 207	980 ± 170	840 ± 336	N.D.	2170 ± 1054	0.21 ± 0.15

Results show yields over plasma values immediately post-donation. Precipitates were harvested after the appropriate incubation period at 0°C by centrifugation for 80,000 g min. at 0°C and were then dissolved in 0.03 plasma volumes of 0.06 M NaCl, 0.13 M glycine, 0.07 M dextrose, 0.02 M citrate pH 6.8

N.D. not determined

together and formed very insoluble precipitates. Precipitates obtained by dextran addition were sparingly soluble in the buffer used. Dextran solutions tended to be very viscous and polymer was retained in the precipitate. Since no attempt was made to wash these precipitates, the insolubility could be due to retention of dextran. Precipitates obtained with PVP were unstable as some clotting was observed upon freezing and thawing samples.

Precipitation of factor VIII from plasma by albumin addition is shown in Figure 5-3. At final concentrations of between 5 and 6% added albumin, virtually complete precipitation of factor VIII was obtained with little fibrinogen or other proteins being precipitated.

Table 5-8 summarises the optimal conditions for factor VIII precipitation on a small scale (20 ml).

2. Medium scale precipitation:

The optimal conditions derived for small scale precipitation were applied to plasma precipitation on a medium scale (between 400 and 1800 ml). The effect of an overnight incubation at 0°C as well as the standard 2 hour one was also investigated. The results are summarised in Table 5-9. Precipitations on a medium scale for 2 hours resulted in concentrates containing the bulk of the factor VIII with little contaminating proteins. Overnight

TABLE 5-10 EFFECT OF ANTICOAGULANT ON POLYMER PRECIPITATION OF PLASMA

Anticoagulant	VIII:C Yield u/kg plasma	Fibrinogen Yield mg/kg plasma	Specific Activity VIII:C u/mg protein
CPD	590 ± 85	525 ± 180	0.82 ± 0.±8
Heparin*	(630 ± 120)+	(1525 ± 295)	(0.32 ± 0.16)

Six donations of each anticoagulant were processed. Precipitation with 4% Ficoll was carried out with plasma in polythene bottles at 0°C for two hours. The bottles were then centrifuged for 80,000 g min. and dissolved in 0.05 plasma volumes of glycine-dextrose buffer. Results show mean and standard deviation.

* Blood was collected in heparin to a final concentration of 5 u/ml

\+ Analyses were only possible on plasma and cold supernatant samples, as the precipitates were insoluble. Results show the estimated precipitate values described as the difference between the plasma and the supernatant. These data are thus only approximate.

incubation, however, resulted in decreased yields of VIII:C and increased precipitation of other proteins.

3. Medium scale precipitation of plasma collected in different anticoagulants:

Six donations of CPD plasma were made 4% with respect to Ficoll 70, and incubated for 2 hours at 0°C. The resulting precipitates had the characteristics shown in Table 5-10. Six donations of heparin plasma were similarly processed, but the precipitates obtained were observed to be much denser than those from CPD plasma. These precipitates proved very difficult to dissolve even at 37°C, and reprecipitation occurred at room temperature. Table 5-10 shows that in heparin plasma, considerably more fibrinogen was precipitated than from CPD plasma.

(F) USE OF HES 40 TO PRODUCE A BLOOD BANK FACTOR VIII CONCENTRATE

Table 5-11 shows the characteristics of a concentrate prepared from a plasma pool of 1800 mls by cold precipitation with 4% HES 40. The resulting precipitate was processed according to the method of Newman et al (1971) for bulk cryoprecipitate, i.e. by extraction into 20 mM Tris-HCl pH 7 at 20°C, addition of 20 mM citrate, pH adjustment to pH 7 and freeze-drying. Adsorbtion with alumina was not performed in this experiment. Upon reconstituting the freeze-dried concentrate, only 60% of the VIII:C that was aliquoted into the vial was recovered.

TABLE 5-11 PREPARATION OF FREEZE-DRIED FACTOR VIII CONCENTRATE USING HES 40

Sample	VIII:C u/ml	VIII:C % yield	VIII:CAg u/ml	Fibrinogen mg/ml	Protein mg/ml	Specific Activity VIII:C u/mg
Plasma	0.92	100	0.98	2.44	56	0.016
Supernatant	0.3	34.4	0.39	2.12	54	0.006
Dissolved precipitate	18.5	60.3	15.6	8.8	20.2	0.92
Dried precipitate reconstituted in dispensed volume	10.9	36.2	14.9	8.4	21.7	0.50

A 6 donation pool of 1800 mls of CPD plasma was cold precipitated with 4% HES 40 for 2 hours. The precipitate was recovered by centrifugation for 45,000 g min. at 4°C, extracted into 20 mM Tris pH 7, made 20 mM citrate and freeze-dried over 3 days. The dried powder was reconstituted in distilled water to the original dispensed volume.

TABLE 5-12 PRODUCTION OF FACTOR VIII CONCENTRATES USING 4% HES 40 PRECIPITATION

Component	VIII:C u/ml	pH
1) Dissolved precipitate	10.5 ± 3.8	6.9 ± 0.1
2) Dried precipitate dissolved in dispensed volume	10.3 ± 4.5	7.2 ± 0.06
3) Dissolved precipitate adsorbed with $Al(OH)_3$	9.8 ± 2.4	6.8 ± 0.08
4) Dried adsorbed precipitate dissolved in dispensed volume	10.2 ± 3.5	7.3 ± 0.14
5) (1) after 24 hours at 22°C	8.2 ± 4.0	6.9 ± 0.17
6) (2) after 24 hours at 22°C	5.7 ± 2.6	7.4 ± 0.12
7) (3) after 24 hours at 22°C	8.3 ± 1.9	7.0 ± 0.03
8) (4) after 24 hours at 22°C	5.9 ± 3.6	7.5 ± 0.19

Six donations of CPD plasma were each precipitated with 4% HES 40. The precipitates were each dissolved in 16 ml. of glycine-dextrose buffer pH 6.8. One portion (7 ml) of each dissolved precipitate was freeze-dried, the other (7 ml) portion was adsorbed with alumina, the pH was adjusted to about 6.9 and it was then freeze-dried.

TABLE 5-13 CHARACTERISTICS OF FREEZE-DRIED HES 40 PRECIPITATE OF PLASMA

Freeze-dried material from 12 individual donations was reconstituted to 10 u FVIII:C/ml and had the following characteristics:

Factor VIII:C	At Reconstitution	9.6 ± 2.7 u/ml
	After 6 hours	9.2 ± 1.9 u/ml
	After 24 hours	6.5 ± 2.4 u/ml
	Recovery from Plasma	594 u/kg
	Specific Activity	0.67 ± 0.2 u/mg
Factor VIII:CAg		8 ± 2.8 u/ml
Factor VIIIR:Ag		14 ± 4.6 u/ml
Factor VIIIR:RCF		14.3 ± 5.2 u/ml
Fibrinogen		8.7 ± 3.2 mg/ml
Fibronectin		3.2 ± 1.1 mg/ml
Total Protein		14.4 ± 1.5 mg/ml
pH		6.8 ± 0.03
Conductivity		5 ± 0.2 mMho

(Material from 4% HES 40 precipitation was dissolved in 0.06 M sodium chloride, 0.13 M glycine 0.07 M dextrose, 0.02 M citrate pH 6.5, and freeze-dried)

It was felt that the Tris buffer used for extracting the HES precipitate might be unsuitable for freeze-drying. The HES precipitate had a much higher specific activity than the bulk cryoprecipitate which is usually extracted into Tris buffer. A further experiment was performed in which 6 individual CPD plasma donations were each precipitated with 4% HES 40 at 0°C. The resulting precipitates were dissolved in a glycine-dextrose buffer (Milligan et al 1981) at 37°C, and freeze-dried. Table 5-12 shows the characteristics of these precipitates. Stability over freeze-drying was much improved using the glycine-dextrose buffer. However, stability of the reconstituted material over 24 hours was rather poor, with only 55% of the VIII:C immediately after reconsititution remaining after 24 hours. Adsorbtion of the initial dissolved precipitate with alumina did not improve stability. The pH of the reconstituted product was above 7 (Table 5-12) and rose further during incubation. It was felt that a lower pH might improve VIII:C stability. In a further experiment, the pH of the dissolved precipitates was adjusted to 6.6 prior to freeze-drying. This resulted in a pH of about 6.8 upon reconstitution. Table 5-13 lists the characteristics of concentrates from 12 individual donations processed in this manner. As can be seen, pH adjustment increased the 24 hour post-reconstitution stability to 68%, a difference which was significant ($p < 0.05$). Figure 5-4 shows the two-dimensional

FIGURE 5-4

TWO-DIMENSIONAL IMMUNOELECTROPHORESIS OF VIIIR:Ag IN FRACTIONS PRODUCED BY 4% HES-40 PRECIPITATION OF PLASMA

Electrophoresis was at 200 V for 6 hours and 120 V for 18 hours in the first and second dimensions respectively. The anode was to the right in the first dimension and at the top in the second dimension. Antiserum concentration in the second dimension was 0.2%.

The arc produced by the supernatant fraction was traced over with a pen in order to improve clarity.


PRECIPITATE
SUPERNATANT
PLASMA

TABLE 5-14 PURIFICATION OF FACTOR VIII BY CONCENTRATION OF PLASMA PROTEIN AND SUBSEQUENT COOLING

Method of Concentration	N	VIII:C u/kg	VIIIR:Ag u/kg	Fibrinogen mg/kg	Fibronectin mg/kg	Specific Activity u/mg
Dialysis vs 50% PEG	1*	414	738	216	274	0.85
Ultrafiltration on PM-10 Membrane	4+	772 ± 148	801 ± 162	116	N.D.	1.53 ± 0.41

* Plasma volume was 1200 ml.

\+ Plasma volumes averaged 320 ml

Plasma was concentrated to about 55% of the initial volume. Concentrated plasma was held at 0°C for 30 min. and centrifuged for 30,000 g min. at 0°C. Precipitates were dissolved in 0.03 plasma volumes of glycine-dextrose buffer

immunoelectrophoretic pattern of the VIIIR:Ag in the 4% HES supernatant and precipitate. It can be seen that slower migrating forms of VIIIR:Ag are concentrated in the precipitate, with fast-migrating forms being left in the supernatant.

(G) PLASMA CONCENTRATION AND COLD PRECIPITATION

Two methods were used to concentrate plasma protein by water removal:dialysis against concentrated PEG and ultrafiltration on a polycarbonate membrane. The concentrated plasma was then incubated at 0°C and the resultant precipitates were dissolved in glycine-dextrose buffer. Table 5-14 shows the results. The one experiment utilising dialysis gave a rather low yield in VIII:C, although most of the VIIIR:Ag was precipitated. Concentration using ultrafiltration gave a very good purification of VIII:C at high yield, but using the equipment available took over 6 hours to effect a two-fold concentration of the plasma.

DISCUSSION

Most studies reporting on the routine production of cryoprecipitate agree that a yield of 40-60% of the plasma VIII:C is the best that can be obtained (Kasper et al 1975, Slichter et al 1976). Any factors that increase VIII:C yields in cryoprecipitate without affecting the production of other blood components are bound to have a profound effect on the logistics of haemophilia care. The recent association of development of the AIDS syndrome with use of commercial concentrates has resulted in increased use of cryoprecipitate in the United States (Sandler and Katz 1984). Thus, it is likely that efforts to improve cryoprecipitate quality will continue.

This study does not support the claim that addition of heparin to CPD plasma increases VIII:C cryoprecipitate yields (Rock et al 1980b, Hanratty 1983). Addition of heparin might be assumed to inhibit any thrombin present in the plasma, and thus prevent the long known degradation of VIII:C by thrombin (Rapaport et al 1963). Indeed, the amount of heparin used (1 u/ml) has been shown to inhibit totally the effect of exogenously added thrombin on VIII:C in plasma (Sussman and Weiss 1978). However, thrombin formation in fresh plasma used in this study is minimal, as evinced by low FpA levels (Chapter III) and heparin did not affect the yield of VIII:C in cryoprecipitate. Collecting blood directly into heparin/CPD anticoagulant also did not

affect VIII:C yields (Table 5-1), thus negating the possibility that thrombin formation had occurred during plasma production and heparin had been added too late to prevent VIII:C degradation. It has also been observed that antithrombin III is not concentrated in cryoprecipitate (Cosgriff et al 1983) making it unlikely that heparin can prevent VIII:C degradation even if thrombin generation occurred during cryoprecipitate production. It is thus difficult to explain the studies cited which claim benefit in adding heparin. It has been suggested that rather than working through a protective effect on VIII:C from the action of thrombin, heparin acts by increasing the physical cryoprecipitability of factor VIII and other proteins (Rock 1983b). However, the results of the present study show that provided cryo separation is efficient, VIIIR:Ag yields approach 800 u/kg of plasma without heparin addition (Table 5-4) suggesting that VIII:C loss under these conditions is by inactivation. Rock (1983b) claims that heparin increases the amount of fibrinogen and total protein in the cryo, but the data from the present study and others (Smit-Sibinga 1983) do not confirm this. It should be noted that Rock reports a yield of 9% in cryo-fibrinogen derived from CPD plasma, a value that must be considered abnormally low. Yields of 20 to 30% as found in the present study are the normal range for cryo-fibrinogen. The study reported by one of the groups (Hanratty 1982) uses the thaw-siphon/ centrifugation technique to produce cryoprecipitate. As

shown in this study, use of this technique leads to increased VIII:C yields without heparin addition. It has been claimed that addition of heparin to CPD plasma must be made immediatley after donation if good results are to be obtained. The data in this study, however, find no effect of heparin addition upon donation, or one hour and 3 hours after (Tables 5-1 and 5-2).

One factor resulting in low VIII:C yields is loss by redissolving into the cryosupernatant. This is shown by decreased yields in VIII:C and VIIIR:Ag using fast-thawing compared to thaw-siphon/centrifugation (Tables 5-1 and 5-2). Using the latter technique, supernatant plasma is removed continuously throughout most of the thawing period, thus minimising loss of cryoprecipitate through redissolving. In the fast-thawing method, cryoprecipitate remains suspended in supernatant plasma throughout thawing, allowing re-dissolving. The conditions for the final separation of cryoprecipitate are however also of importance. Thus, when centrifuging bags of thawed plasma after fast-thawing, it was observed that the cryoprecipitate often failed to sediment fully, with fragments of precipitate remaining floating in the supernatant. This is probably due to an insufficiently hard centrifugation using the Mistral machine, and to cryoprecipitate fragments being trapped in creases in the plasma bag. Substantially improved yields were obtained by transferring fast-thawed plasma to polycarbonate bottles and using harder

centrifugation. Using this latter technique, drainage of the supernatant could be effected much more efficiently than in bags, so that the cryo obtained had a much higher specific activity (Tables 5-3 and 5-4).

Collection of blood in heparin led to increased VIII:C yields in cryo, although compared to a group of CPD donations the difference was not significant (Table 5-3). In this experiment, sedimentation was achieved by spinning the bags of thawed plasma, with the problems noted above. This might explain why the yields are lower than reported by other studies (Rock et al 1979, Smit-Sibinga et al 1981).

Transferring of thawed plasma from plastic bags to bottles for centrifugation would involve abandoning the closed plastic bag system. At the same time, adoption of bottles for the whole process of plasma processing to cryo cannot be recommended, as freezing and thawing plasma in bottles would greatly prolong freezing and thawing times, with possible resultant loss in yield. Use of the plastic bag has the advantage that the plasma is frozen and thawed as a flat slab, and the thin bag ensures quick freezing and thawing. It might be possible to adopt the approach described of transferring thawed plasma to bottles but checks for sterility would be essential. Hanratty (1982,1983) has shown that use of such an open system can maintain sterility, using a sterile room and laminar flow cabinets.

Addition of PEG to 1% did not result in

improved VIII:C yields, in contrast to the findings of Johnson et al (1979). Fibrinogen in the cryoprecipitate, however, was greatly increased and specific activity of VIII:C was reduced. PEG addition led to the formation of heavier cryoprecipitates, which might sediment better during low speed centrifugation and lead to higher yields. Using adequate centrifugation, however, no benefit was obtained.

The findings shown in Table 5-7 do not support the suggestion of Polson (1972) that cryoprecipitation involves a form of salting-out of proteins. Decreasing the salt content of plasma had no effect on cryoprecipitate composition, while increasing the ionic strength depressed cryoprecipitation, possibly by dissolution of cryo-proteins into plasma, after thawing at this salt concentration (Smith et al 1979). Decreasing the protein content of plasma greatly decreased cryoprecipitation. Owen and Wagner (1972) have suggested that a steric exclusion of high molecular weight proteins by increasing concentrations of low molecular proteins during freezing is responsible for the precipitation of cryo-proteins such as factor VIII, fibrinogen and fibronectin. This is also supported by the precipitation of factor VIII upon addition of albumin to plasma (Figure 5-3), and by precipitation of cryo-proteins upon increasing the plasma protein concentration (Table 5-14). The findings in this study indicate that both of the other major cryo-proteins (fibrinogen and fibronectin)

are necessary for efficient factor VIII cryoprecipitation to occur, a finding noted in one other report (Mazurier et al 1983). The cold precipitation of plasma factor VIII and fibronectin (Chapter III) also points to a cold induced association of these proteins.

Alternatives to cryoprecipitation as a means of concentrating plasma factor VIII were investigated. Addition of NaCl to plasma, as described by Polson and Ruiz-Bravo (1972) was not an effective method, as low yields of VIII:C associated with unstable precipitates were obtained. This method is also unsuitable because it would preclude use of the supernatant plasma as a volume expander or for further fractionation, due to the high salt content.

Use of hydrophilic polymers led to more promising results (Figure 5-2). Precipitation of chilled citrated plasma with albumin, HES or Ficoll 70 was particularly effective and allowed a single-step purification of factor VIII at a specific activity of 0.5 to 1.0 units/mg protein in greater than 80% yield, in 2 hours. Casillas and Simonetti (1982) have reported that the use of PVP also results in good factor VIII purification. In the present study, however, precipitates obtained with PVP tended to clot, possibly due to the presence of impurities in the polymer (Zuber and Morgenthaler 1982). The other polymers tested, while resulting in selective precipitation of factor VIII, gave inferior results to those described above. Previous

studies by Alexander et al (1975,1978) with low concentrations of polymers added to chilled plasma indicated that prolonged incubation (48 hours) resulted in precipitation of fibrinogen and factor VIII with dextran. Although no data was given, HES was stated to be a less efficient precipitant, while albumin was found to be ineffective. In the present study, both HES and albumin were found to be effective agents for precipitating factor VIII selectively. The experimental conditions used by Alexander et al differed from those used in this study and this could explain these discrepancies.

It seems likely that the mechanism of precipitation by the polymers described in this study is a molecular exclusion process similar to that described for dextran (Laurent 1963) and PEG (Juckes 1971). In this regard, it may be significant that the discrimination between factor VIII and fibrinogen/total protein was worst with a linear flexible polymer - PEG - while compact polymers such as Ficoll 70 and albumin gave much better discrimination. HES has a branched structure leading to a more compact shape, and this polymer was also found to be a selective precipitant of factor VIII. Another factor influencing discrimination between factor VIII and fibrinogen is the incubation period at 0°C; 2 hours gives significantly better discrimination than 20 hours. It appears that factor VIII is rapidly and completely precipitated within 2 hours, whereas fibrinogen

is slowly (and incompletely) precipitated over 20 hours. Fibronectin is also precipitated by the polymers (Table 5-13). As shown in this study (Chapter III) incomplete precipitation of factor VIII and fibronectin occurs in chilled plasma without polymer addition. It appears that polymer addition results in equilibrium between precipitate and supernatant phases being reached sooner. Cold precipitation of fibrinogen without polymer addition is very low, but 20 hour incubation in the presence of polymer results in substantial precipitation of this protein. Figure 5-4 shows that precipitation with HES preferentially precipitates the slower-migrating forms of VIIIR:Ag from plasma. Zimmerman et al (1975b) have shown that these forms represent the higher molecular weight multimers of VIIIR:Ag. This supports the hypothesis that polymer precipitation works through a steric exclusion mechanism with higher molecular weight proteins being precipitated first as the polymer concentration is increased.

HES 40 was used to further develop polymer induced cold precipitation of plasma factor VIII into a method for producing a blood bank factor VIII concentrate. This polymer is already used in haematological practice for several purposes, including the preparation of leucocyte-poor blood and the freezing of red cell concentrates (Mishler 1982). Use of a glycine-dextran buffer to dissolve the precipitates allowed lyophilisation with retention of the VIII:C activity (Table 5-12).

Loss of activity was observed with use of the Tris citrate solution used to dry conventional intermediate purity concentrate (which is of lower purity than the precipitates produced by HES precipitation). It is known that higher-purity materials are less stable to freeze-drying than materials of lower-purity (Bidwell 1955). The reconstituted products showed rather poor VIII:C stability over 24 hours, which was not improved by alumina adsorbtion (Table 5-12). Stability was improved by adjusting the pH of the solubilised precipitate to 6.6 prior to lyophilisation. As has been shown by Liu et al (1980), carbon dioxide loss during drying results in a higher pH in the reconstituted product, leading to lowered VIII:C stability. In fact, adjustment of pH to 6.6 prior to drying resulted in a pH of 6.8 in the reconstituted precipitate and the stability over 24 hours was improved from 55% to 68%. Foster et al (1983b) have shown that citrate addition, while being necessary for adequate solubility and filterability, destabilises VIII:C during processing; improvement of VIII:C stability was attained by addition of ionised calcium to levels existing prior to citration. The buffer used to dry the starch precipitates contained 20 mM citrate and further stabilisation may well be attained by calcium addition. In this study, however, this was not evaluated.

Table 5-13 shows that concentrates belonging to the 'high-purity' grade (Smith and Bidwell 1979) can

be produced from CPD plasma donations at yields of about 600 u/kg. HES precipitation represents an attractive alternative to cryoprecipitation as factor VIII is obtained at a high yield and purity without the need for freezing plasma. The concentrate can easily be obtained using the multiple plastic bag system, as plasma can be separated into a secondary or tertiary bag containing enough concentrated HES to result in about 4% concentration in the plasma. Cold precipitation and centrifugation would allow harvesting of the precipitate. It is suggested, however, that such single donation 'wet' products suffer from several disadvantages as does cryoprecipitate, notably lack of standardisation and the need for frozen storage. High speed centrifugation would also be necessary for harvesting the precipitates, and the use of plastic bags might lead to the problems described for cryo. A standardised dried product can easily be produced, provided that special precautions are taken to ensure sterility. Cold precipitates produced from single donations could be stored frozen until a sizable number have been collected. These could then be solubilised and pooled in freeze-drying buffer, ampouled and lyophilised to give standard units of dried concentrate. Sterile filtration of the solutions would be necessary but should not present a problem in view of the low fibrinogen content. A sterile freeze-dryer would also be necessary. However, the fact that the final product can be subjected to

standard pharmaceutical quality control and can be labelled with the factor VIII content may justify the capital expenditure involved in purchasing the required equipment.

The presence of 4% HES in the supernatant plasma might be seen as a disadvantage. However, such plasma can be safely used as a blood volume expander as HES itself has been used for this purpose (Mishler 1982). Although it is probable that 4% HES will interfere with the Cohn fractionation process to produce gamma-globulin and albumin, this process is unlikely to be used by blood banks with a small scale component programme. New ion-exchange chromatographic techniques (Curling 1980, Suomela 1980) are likely to be much more suitable for production on a blood-bank scale. The presence of an uncharged polymer like HES should still allow such procedures.

An attractive alternative to additive-induced cold precipitation is shown by the data in Table 5-14. Concentration of plasma followed by cold precipitation led to concentrates of high-purity and at good yields. This study did not pursue this approach further, as the equipment available did not allow fast concentration of the plasma. Use of more efficient systems, e.g. hollow fibres might be expected to effect concentration much more quickly. It is possible that the high initial capital costs might be justified if this method can be adapted as a process to produce factor VIII concentrate.

In summary, this Chapter concludes:

1) Cryoprecipitate yields by good technique are unaffected by addition of heparin or PEG to plasma, but are improved if loss of factor VIII by dissolution and/or inactivation are minimised. This can be achieved by thaw-siphoning, but attention to the final separation of cryo from supernatant plasma can improve yields in fast-thawing.

2) Cryoprecipitation cannot be ascribed to a salting-out mechanism, and salt-induced plasma precipitation is an unsatisfactory method for factor VIII concentration. Cryoprecipitation appears to involve a precipitation of the cryo-proteins - factor VIII, fibrinogen and fibronectin - induced by low temperatures and by high concentrations of other plasma proteins produced during freezing.

3) Polymer induced precipitation of chilled unfrozen plasma is an attractive alternative to cryoprecipitation and can be adapted to produce a standardised dried factor VIII concentrate in a blood bank. Simple concentration and cold precipitation of plasma without any additives is also an attractive possibility, but would require high initial capital costs (Chapter VIII).

CHAPTER VI

PRODUCTION OF FACTOR VIII CONCENTRATES OF HIGHER PURITY

INTRODUCTION

More than 80% of infusions which haemophiliacs receive are for the so-called 'spontaneous' bleeds not associated with any obvious trauma, (Biggs 1978). The level of VIII:C desired in a patient's blood to treat such bleeds is 15 to 20% of normal. It is estimated that a dosage of 10 u/kg body weight should have the desired effect. This would imply infusion of about 700 units of VIII:C for a 70 kg man. Correspondingly, less would be needed for boys, for whom, however, the frequency of such bleeds is often higher (Rizza 1976a). Factor VIII concentrates of intermediate purity can usually be dissolved at about 15 to 20 units per ml. This potency is enough to ensure that for the majority of bleeds treatment can be administered by syringe infusion, by the patient himself if on home therapy, in volumes which have no appreciable effect on the blood volume.

For dealing with major trauma and for surgery, levels of 100 to 150% of normal are desirable (Rizza 1976b). The volumes of concentrate would be correspondingly larger if material of intermediate purity were used, necessitating lengthy infusions by drip. This can present problems if high levels are required immediately. Massive doses are also sometimes used to overcome VIII:C inhibitors (Rizza 1981). Thus, a minority of clinical situations exist in which high-purity concentrates,

which can be dissolved in concentrations of 30 to 50 units per ml, are needed.

The tendency among commercial manufacturers appears to be to produce concentrates of ever increasing purity. Such products are often attractive to patients due to their high solubility and conveniently small size, allowing easy storage. Indeed inducements such as the ability of a haemophiliac to carry 'his concentrate in his pocket' are sometimes used in marketing these products. However, present methods for the industrial production of high-purity concentrates result in yields which are far smaller than those obtained for intermediate purity concentrates. Published data shows that yields of less than 200 u/kg of plasma are obtained (Smith and Bidwell 1979). High-purity concentrates are produced by further purification of intermediate purity materials. Quantitatively, the most important impurity is fibrinogen. Most high-purity production methods consist of two steps - the first to selectively remove fibrinogen and the second to concentrate factor VIII (Chapter I). The first step usually involves heavy losses of factor VIII, which can be as high as 50%. Such losses in yield are generally of lesser importance for commercial manufacturers who obtain their starting plasma from paid donors and can 'pass on' any additional costs in its procurement, in the interest of obtaining a purer, more attractive product. For state owned concerns who rely on a limited voluntary system of blood donation, losses

of this kind are unacceptable.

In addition to the reasons described above, another reason exists for developing an efficient method of fibrinogen removal. Sterilisation of factor VIII concentrates by heat or chemical treatment has been extensively investigated recently (Heimburger et al 1981, Prince et al 1983). Fibrinogen is rapidly denatured by high temperatures and is precipitated by a variety of chemical agents such as ether, which has been proposed as a viral inactivating agent (Prince et al 1984). Thus, fibrinogen reduction is a necessary preliminary to current methods designed to reduce the viral infectivity of factor VIII concentrates.

In this Chapter, methods of further purification of intermediate purity concentrates have been investigated. The main scope has been the removal of as much fibrinogen as possible, while retaining factor VIII yields, in order to produce a solution that might be sterilised by heating (Chapter VII).

METHODS

Source materials for these studies consisted of cryoprecipitates prepared as described below. In some experiments, conditions derived for purifying cryoprecipitate were used to process other materials as outlined in Results.

Cryoprecipitate ('cryo'): Two types of cryo were prepared:-

1. Small-scale cryo was prepared from 200 ml of fresh plasma derived from 6 donation pools or single donations. The cryo was prepared by thaw-siphoning or fast-thawing, as described in Chapters IV and V, and was dissolved for processing in its own supernatant plasma or in 15 mM citrate, 150 mM NaCl pH 6.9 (citrate saline) as specified in Results.
2. Tris extract of bulk cryo was prepared as described by Newman et al (1971). 250 ml donations of fresh plasma were frozen in dry ice/ethanol as described in Chapter IV and stored at -40°C. For processing, 2 to 3 kilograms of this plasma were allowed to soften in a 4°C cold room for 5 hours. The bags of plasma were then placed in a 4°C water bath and allowed to thaw. When thawing was complete, the plastic bags were opened and the contents were transferred to polycarbonate bottles and centrifuged as described in Chapter V. After complete decantation of the cryosupernatant plasma, the cryo was extracted at 22°C with 20 mM Tris-HCl pH 7. The

TABLE 6-1 PRODUCTION OF TRIS-EXTRACTED CRYOPRECIPITATE

Component	Yield in Component % of Starting Plasma					Specific Activity VIII:C u/mg Protein
	VIII:C	VIIIR:Ag	Fibrinogen	Fibronectin	Total Protein	
Plasma	100	100	100	100	100	0.014 ± 0.002
Cryosupernatant	15 ± 7	12 ± 3	62 ± 18	19 ± 7.5		
Tris Extract of Cryo	56 ± 12	72 ± 18	30 ± 5.8	72 ± 27	2.1 ± 1.2	0.44 ± 0.23
Residue from Extraction	< 1	6.8 ± 2.5	3.5 ± 0.7	4.6 ± 1.9	0.002	

Results show mean ± standard deviation of 4 individual batches

volume of extracting buffer was 30 ml for each kilogram of starting plasma, and extraction was performed by stirring the cryo-buffer mixture with a magnetic stirrer for 10 minutes. The mixture was then centrifuged for 40,000 g min. at 22°C and the factor VIII-rich supernatant was decanted and stored at -40°C. The residue from extraction was solubilised at 37°C in citrate-saline and stored at -40°C. Table 6-1 summarises the various stages of the production of this material.

Purification Methods

Cold insoluble globulin precipitation: Small-scale cryo was solubilised at 37°C in the residual supernatant plasma or in buffer as described in Table 6-2 of Results. The cryoprecipitate solution was then placed in 40 ml polycarbonate tubes and held for 2 hours in a melting ice bath. The pH of the solution was 7.1 ± 0.2 in all instances. After 2 hours the tubes were centrifuged for 60,000 g min. at 0°C in a Sorvall RC2B centrifuge. The supernatant was removed and sampled. The precipitate was dissolved at 37°C in 10 mls of citrate-saline. The solubilised precipitate and samples from each step of the process were stored at -40°C prior to assay.

Polymer induced precipitation: Stock solutions of various polymers were prepared as described in Chapter V. Precipitation of small-scale cryo and extracted cryo was performed under various conditions as specified in Results.

Glycine induced precipitation: This was performed according to Blombäck and Thorell (1982). Small-scale cryo prepared by fast-thawing and complete drainage of the supernatant was used as starting material. The cryo was dissolved in 20 ml of 55 mM citrate pH 6.8. 40 ml of a buffer containing 3 M glycine, 125 mM NaCl and 25 mM imidazole pH 6.8 were then added to the cryo solution. The addition was carried out in a 30°C water bath with continuous mixing. The mixtures were allowed to equilibrate for 15 minutes, after which the resultant precipitate was harvested by centrifugation for 60,000 g min. at 22°C, and dissolved in 20 ml of citrate-saline at 37°C. The supernatant was then adjusted to pH 7.5 with 0.1 M NaOH and was cooled to 0°C in a melting ice bath. Sufficient 40% w/w PEG 6000 was added to produce a final concentration of 6.5% w/w PEG. The mixture was left at 0°C for 30 minutes, after which it was centrifuged for 60,000 g min. at 0°C. The supernatant was removed and the precipitate was dissolved in 5 ml of glycine-dextrose buffer (Chapter V). Samples of every stage of the procedure were stored at -40°C for subsequent assay.

Extraction in the presence of glycine: Cryo was extracted into buffer as described above for preparation of Tris extract with the exception that instead of Tris, glycine-imidazole pH 6.8 (2 M glycine, 83 mM NaCl, 17 mM imidazole) was used. The extraction was performed at 30°C on a stirrer/hot plate with careful temperature monitoring. The extraction was performed for 15 minutes

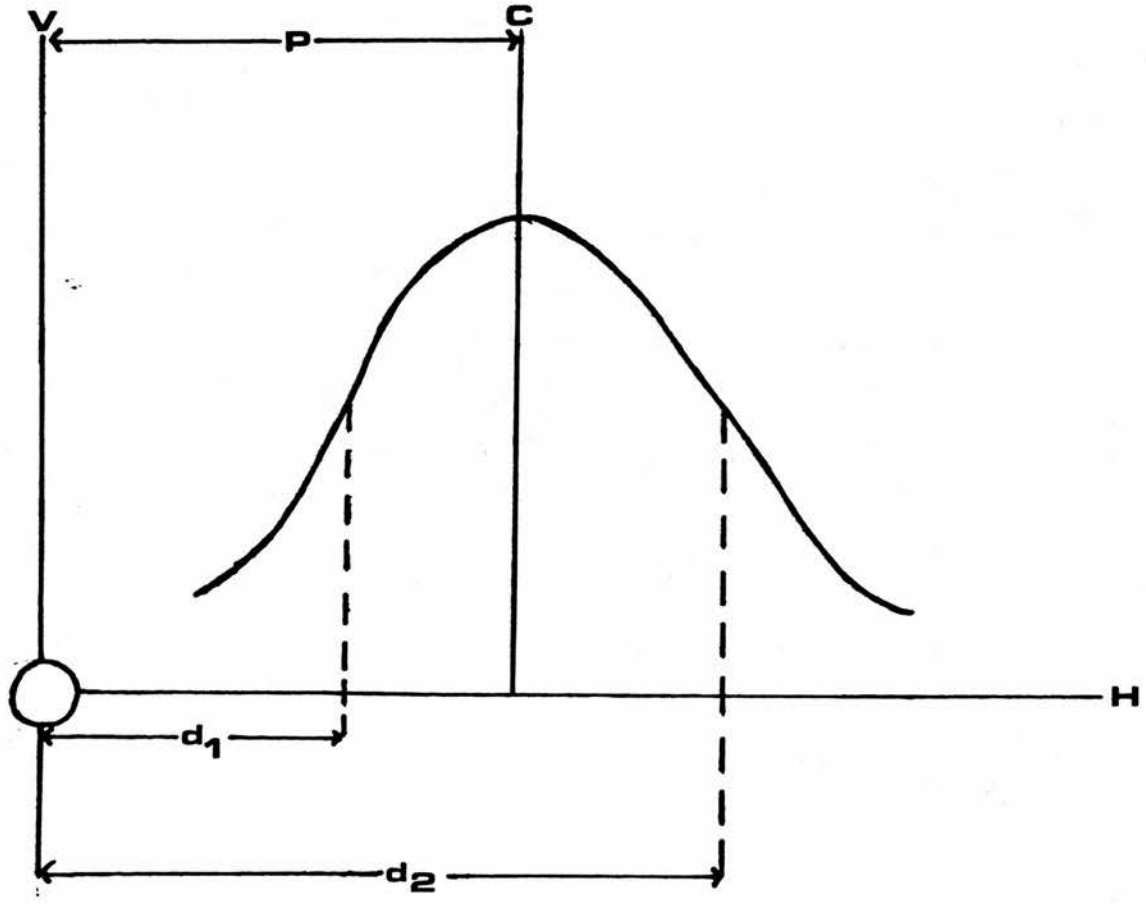
and the mixtures were then centrifuged for 60,000 g min. at 22°C. The extract was removed and the residue was dissolved at 37°C in citrate-saline. Samples of all the fractions were stored at -40°C for assay.

Zinc-induced precipitation: (Foster et al 1983a). Tris extract of cryo was brought to 1 mM zinc by addition of a 5 mM zinc acetate solution. The addition was made slowly, with continuous stirring, at room temperature (Foster et al 1983a). The mixture was allowed to equilibrate for 15 minutes. The gelatinous precipitate which formed was removed with a thin wooden stick; in some cases small pieces of precipitate were removed by centrifugation for 60,000 g min. at 22°C. The precipitate proved to be poorly soluble. Some solubilisation was achieved by warming the precipitate to 40°C in a volume of citrate-saline equal to 2 to 3 times the volume of the initial extract. Insoluble material was then removed by centrifugation. Samples of all the fractions were stored at -40°C for subsequent assay.

Extraction in the presence of zinc: Cryo was extracted into Tris buffer as described above, but 1 mM zinc acetate was included in the extraction buffer. Extraction was carried out for 15 minutes, after which extract and residue were separated by centrifugation. The residue was partly solubilised as described above. Samples of extract and residue were stored at -40°C until assayed.

FIGURE 6-1

DIAGRAMATIC REPRESENTATION OF A PRECIPITIN ARC IN A TWO-DIMENSIONAL IMMUNOELECTROPHORESIS, TO ILLUSTRATE THE MIGRATION DISTANCES MEASURED


V
P
C
H
d_1
d_2

Analysis of factor VIIIR:Ag in two dimensional immuno-electrophoresis (2DIEP): Details of this technique are found in Chapter II. 2DIEP was performed on samples of various concentrates, the samples being analysed together on one plate so that conditions were uniform for all the samples. The precipitin arcs obtained were then analysed as described by Ekert and Chavin (1977). The arcs were divided into 3 electrophoretically different regions, as shown in Figure 6-1. Tracings of the individual arcs were made and migration distances were measured and interpreted as follows:

1. Perpendicular and horizontal lines V and H were drawn through the centre of the sample well.
2. A perpendicular line C was drawn between the centre of the peak and line H, and the distance p was recorded.
3. The length of the ascending and descending arms of the precipitin arc were measured from the perpendicular C to the end of the visible precipitin line. A vertical line was drawn through the middle of the ascending and descending arms to intersect with line H; d_1 and d_2 were then measured and recorded in mm.

An increase in distance d_1 is interpreted to be the result of a decrease in the amount of antigen with slower electrophoretic mobility. An increase in d_2 is the result of a larger proportion of antigen with faster mobility. An increase in p reflects an increase in the

mobility of the median fraction of antigen. Decreases in these measurements are taken to be the result of the opposite changes in the antigen population.

All other assays and analytical procedures are described in Chapter II.

TABLE 6-2 PURIFICATION OF CRYOPRECIPITATE USING COLD INSOLUBLE GLOBULIN PRECIPITATION

Yield of Protein*		Cryo Produced by Fast-Thaw, Dissolved in 10 mls of Residual Plasma, and Held for 2 Hours at 0°C			Cryo Produced by Thaw-Siphon/ Centrifugation with Complete Drainage of Plasma, Dissolved in 10 mls of Citrate-Saline, and Held for 2 hours at 0°C	
		Anticoagulant (n)			Anticoagulant (n)	
		CPD (6)	CPD/Heparin+(3)	Heparin†(6)	CPD (3)	CPD/Heparin+(3)
VIII:C Yields:	1	42.5 ± 17	45 ± 12.5	58 ± 17.5	71 ± 7.5	76 ± 12
	2	74.2 ± 11.8	69 ± 23.7	73.8 ± 25	22.8 ± 5.6	23.7 ± 5.9
VIIIR:Ag Yield:	1	63 ± 12.3	65 ± 17	64 ± 15	82 ± 10.5	79 ± 13
	2	65.5 ± 17	74.6 ± 18	71.2 ± 15.8	19.7 ± 8.6	25 ± 7.5
Fibrinogen Yield:	1	32.5 ± 7.8	38 ± 12.5	41 ± 15	28 ± 6.8	31 ± 4
	2	57.3 ± 12	48.9 ± 12	41.6 ± 6	10.6 ± 4.8	12.3 ± 10
Fibronectin Yield:	1	71 ± 16.8	78 ± 22.4	76 ± 25	84.5 ± 18.5	91 ± 23
	2	95.6 ± 38	92.6 ± 32	95.1 ± 17	54.6 ± 18	78.7 ± 23.5
Specific Activity Cryo		0.12 ± 0.04	0.12 ± 0.03	0.09 ± 0.02	0.74 ± 0.19	0.7 ± 0.1
Specific Activity Final Precipitate		0.32 ± 0.11	0.31 ± 0.12	0.35 ± 0.14	0.51 ± 0.12	0.38 ± 0.22

Results show mean ± standard deviation. Cryoprecipitates were produced from 200 ml of plasma, using either of the two methods described in detail in Chapter V. Final precipitates obtained by cold precipitation were dissolved in 10 ml of citrate-saline.

* Two stage yields are shown: 1 refers to the yield in cryoprecipitate as a % of starting plasma
2 refers to the yield in the final precipitate as a % of cryoprecipitate

\+ Plasma heparin concentration = 1 u/ml

† Plasma heparin concentration = 10 u/ml

FIGURE 6-2

POLYMER PRECIPITATION OF THAW-SIPHON CRYOPRECIPITATE
Incubations at 0°C and 10°C were carried out for 2 hours in melting ice and a thermostated water bath respectively. Precipitates were dissolved at 37°C in 0.5 volumes of glycine-dextrose buffer (Chapter V)

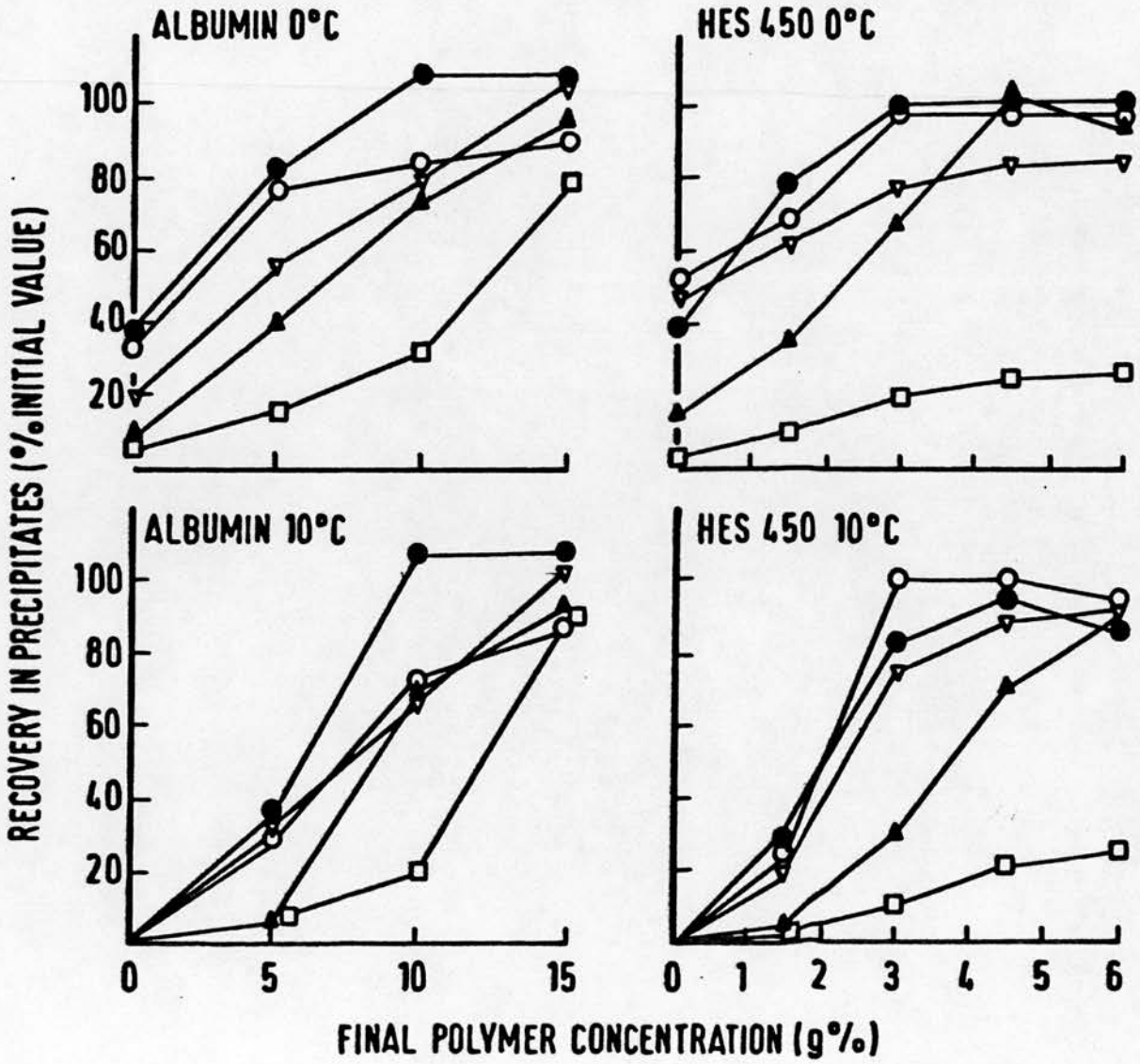
● ——— ● VIII:C

▽ ——— ▽ VIII:CAg

O ——— O VIIIR:Ag

▲ ——— ▲ Fibrinogen

□ ——— □ Total Protein


ALBUMIN 0°C
HES 450 0°C
ALBUMIN 10°C
HES 450 10°C
RECOVERY IN PRECIPITATES (% INITIAL VALUE)
FINAL POLYMER CONCENTRATION (g%)
0
20
40
60
80
100
0
5
10
15
0
1
2
3
4
5
6

RESULTS

(A) COLD PRECIPITATION OF SOLUBILISED CRYOPRECIPITATE

Table 6-2 shows that a significant purification of cryo solubilised in residual plasma can be achieved by a second precipitation at 0°C. The cold insoluble fraction that formed contained most of the factor VIII and fibronectin of the initial cryoprecipitate. Fibrinogen was also precipitated to a lesser extent. As described in Chapter V, the 'fast-thaw' cryo gave lower factor VIII yields than cryo produced by the thaw-siphon/centrifugation method, which is also purer because of complete drainage of the supernatant plasma. This type of cryo was therefore subjected to cold precipitation, in an attempt to improve the yield and purity of the final fraction. However, using this method, precipitation of cryo proteins was significantly decreased ($p < 0.02$) compared to fast-thaw solubilised in supernatant plasma.

Incorporation of heparin into the system, either by processing heparin plasma or adding heparin to CPD plasma, did not affect factor VIII yields. Heparin addition, however, resulted in a significant increase in the amount of fibronectin precipitated when cryo dissolved in buffer was held at 0°C ($p < 0.05$).

(B) POLYMER INDUCED PRECIPITATION OF CRYOPRECIPITATE

Figure 6-2 shows the results of polymer assisted precipitation of cryoprecipitate at low temperatures. It

FIGURE 6-3

PRECIPITATION OF CRYO EXTRACT AT DIFFERENT IONIC STRENGTH

Cryo extract was prepared from FFP and precipitated at 0°C with albumin. Ionic strength was increased by making the 20 mM Tris extract 0.2 M with respect to NaCl

● ——— ● VIII:C

O ——— O VIIIR:Ag

▲ ——— ▲ Fibrinogen

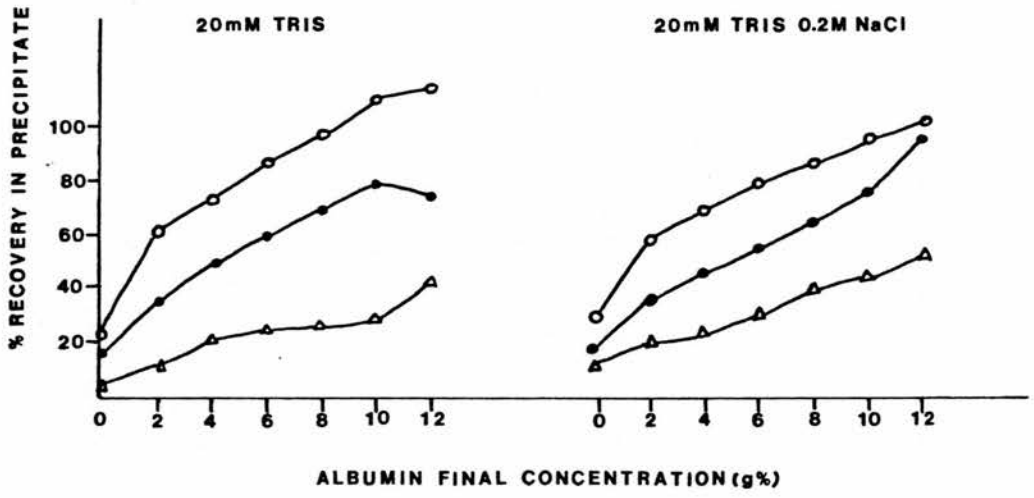

20mM TRIS
20mM TRIS 0.2M NaCl
% RECOVERY IN PRECIPITATE
100
80
60
40
20
0
2
4
6
8
10
12
ALBUMIN FINAL CONCENTRATION (g%)

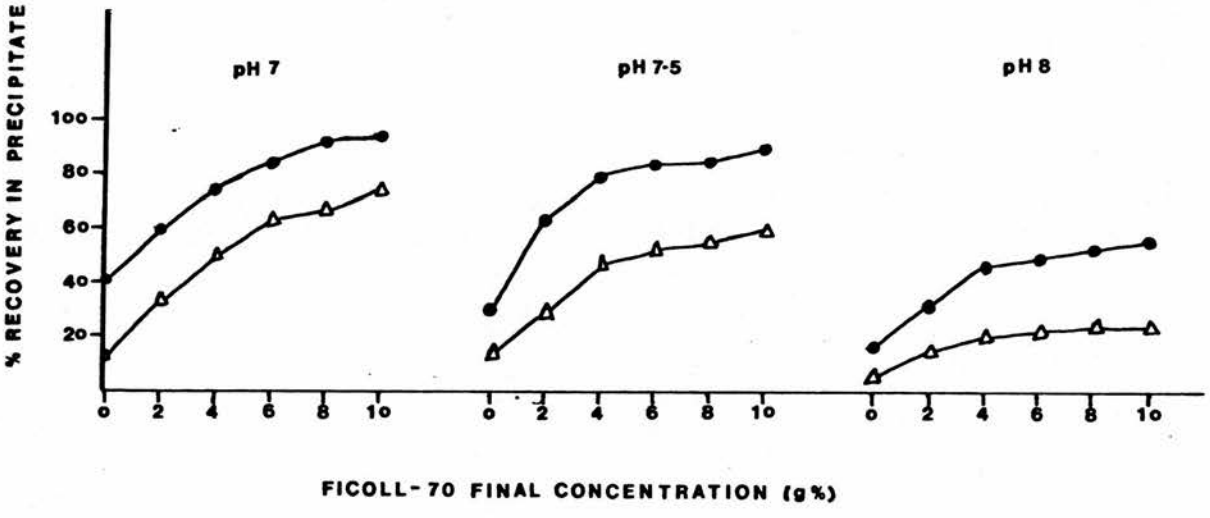
FIGURE 6-4

PRECIPITATION OF CRYOEXTRACT AT DIFFERENT pH VALUES

Cryo extract was prepared from FFP and precipitated at 0°C with Ficoll-70. pH was varied by addition of 0.1 N NaOH

● ——— ● VIII:C

▲ ——— ▲ Fibrinogen


% RECOVERY IN PRECIPITATE
pH 7
pH 7·5
pH 8
100
80
60
40
20
0
2
4
6
8
10
FICOLL-70 FINAL CONCENTRATION (g%)

can be seen that, in contrast to plasma (Chapter V) cryoprecipitate fibrinogen tended to precipitate with factor VIII. Precipitation of reconstituted lyophilised concentrate (Protein Fractionation Centre, Edinburgh) produced similar results.

Attempts were made to suppress fibrinogen precipitation at low temperatures. Increasing ionic strength has been shown to increase fibrinogen solubility at pH values between 6 and 7 (Leavis and Rothstein 1974, Smith et al 1979). However, increasing the salt content of extracted cryo by 0.2 M (Smith et al 1979) did not improve discrimination between factor VIII and fibrinogen in albumin-induced precipitation (Figure 6-3). Increasing the pH of the mixture in order to work as far from the isoelectric point of fibrinogen as possible did not improve the separation of factor VIII from fibrinogen in Ficoll-induced precipitation (Figure 6-4).

Fractional precipitation with PEG at room temperature according to the method of Newman et al (1971) (Chapter I) led to high losses of factor VIII in the first precipitate (4.5% w/w PEG) designed to remove fibrinogen, although a two-fold purification of VIII:C in the second precipitate (11% w/w PEG) was achieved.

(C) PURIFICATION USING GLYCINE

Table 6-3 shows that addition of glycine to a concentration of 2 M at 30°C precipitates most of the fibrinogen in cryoprecipitate while leaving most of the factor VIII in the supernatant. Addition of PEG to the

TABLE 6-3 PURIFICATION OF CRYOPRECIPITATE USING GLYCINE

Stage of Purification	Yield in Fraction, % of Starting Cryo				Specific Activity VIII:C u/mg Protein
	VIII:C	VIIIR:Ag	Fibrinogen	Fibronectin	
Solubilised Cryo	100	100	100	100	0.6 ± 0.2
2 M Glycine Precipitate	24 ± 6	21 ± 4	76 ± 7	32 ± 12	
2 M Glycine Supernatant	89 ± 24	76 ± 13	18 ± 5	60 ± 15	1.1 ± 0.3

Cryos were prepared by thawing 200 ml frozen plasma donations in a 4°C water bath, transferring the thawed contents to polycarbonate bottles and harvesting the precipitate by centrifugation. Results show the mean and standard deviation of experiments with 5 separate donations.

TABLE 6-4 CONCENTRATION OF GLYCINE-PURIFIED FACTOR VIII

Stage of Purification	Yield of VIII:C % of Glycine Supernatant	Specific Activity VIII:C u/mg Protein
2 M Glycine Supernatant	100	1.1 ± 0.3
6.5 % PEG Precipitate	92.5 ± 9.3	1.4 ± 0.45

Cryos were subjected to glycine precipitation and the precipitate removed by centrifugation. The supernatants were brought to 6.5% PEG 6000 at 0°C and held for 30 minutes. The mixtures were then centrifuged and the precipitates were dissolved in glycine-dextrose buffer.

plasma prior to freezing was unnecessary (Chapter V) for optimal factor VIII yields, and so this part of the original technique (Blombäck and Thorell 1982) was omitted. In order to concentrate the factor VIII and remove glycine, the 2 M glycine supernatant was cooled to 0°C, as this procedure has been used to precipitate factor VIII in glycine-rich solutions (Shanbrom and Fekete 1971). However, no precipitate formed under these conditions. Concentration and further purification of factor VIII was achieved by precipitation of the glycine supernatant with 6.5% PEG at 0°C (Table 6-4). It was estimated that the average VIII:C yield using this procedure was about 83% from cryoprecipitate, with an average 2.1 fold purification over cryoprecipitate being achieved.

Direct extraction into glycine-containing buffer was attempted as an alternative to cryo solubilisation/reprecipitation. Using this approach however, lower VIII:C yields were obtained as considerable amounts of VIII:C were retained in the insoluble fibrinogen-rich residue.

(D) PURIFICATION USING ZINC

Preliminary experiments on zinc precipitation showed that addition of zinc acetate crystals to give 1 mM final concentration of zinc resulted in precipitation of most of the fibrinogen in cryo extract; however up to 40% of the VIII:C was also precipitated. Addition of the salt as a 5 mM solution led to near quantitative

TABLE 6-5A PURIFICATION OF CRYOPRECIPITATE USING ZINC

Stage of Purification	Yield in Fraction, % of Cryo Extract				Specific Activity VIII:C u/mg Protein
	VIII:C	VIIIR:Ag	Fibrinogen	Fibronectin	
Cryo Extract	100	100	100	100	0.44 ± 0.23
1 mM Zinc Acetate Supernatant	87 ± 6	84 ± 12	24 ± 4	76 ± 29	0.85 ± 0.17

Results show the mean and standard deviation of 4 experiments. Zinc acetate was slowly added as a 5 mM solution with constant stirring, to give a final concentration of 1 mM.

TABLE 6-5B PURIFICATION OF HES 40 PLASMA PRECIPITATES USING ZINC

Stage of Purification	Yield in Fraction, % of HES 40 Precipitate				Specific Activity VIII:C u/mg Protein
	VIII:C	VIIIR:Ag	Fibrinogen	Fibronectin	
HES 40 Precipitate	100	100	100	100	0.7 ± 0.18
1 mM Zinc Acetate Supernatant	89 ± 16	78 ± 12	21 ± 8	64 ± 22	1.3 ± 0.22

Results show the mean and standard deviation of 4 experiments. Zinc acetate was added as in Table 6-5A.

FIGURE 6-5

PROTEIN COMPOSITION OF FACTOR VIII CONCENTRATES

5% SDS-PAGE was done as described in Chapter II. The gels were dried and analysed by densitomery

A. Cryoprecipitate dissolved in buffer

B. 2 M glycine supernatant of cryoprecipitate

C. 4% HES 40 precipitate of plasma

D. 1 mM zinc acetate supernatant of 4% HES precipitate

E. Clinical intermediate-purity concentrate

F. 1 mM zinc acetate supernatant of intermediate-purity concentrate

G. Cryoprecipitate solubilised in supernatant plasma

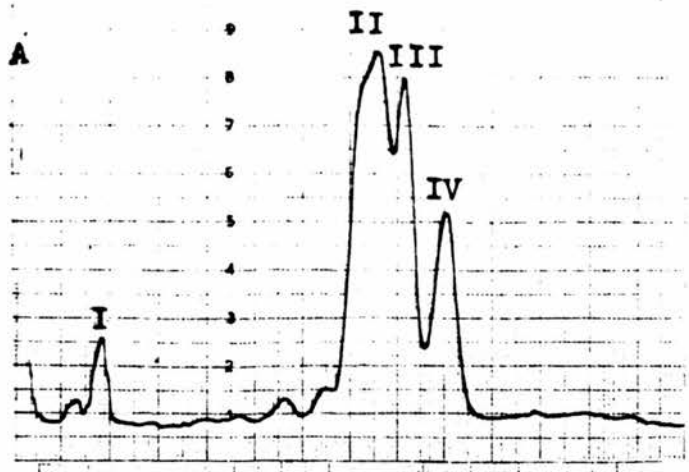
H. Cold insoluble fraction of cryoprecipitate

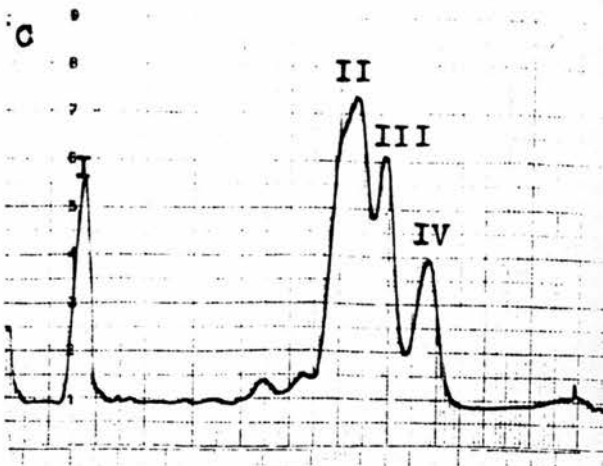
I - FIBRONECTIN

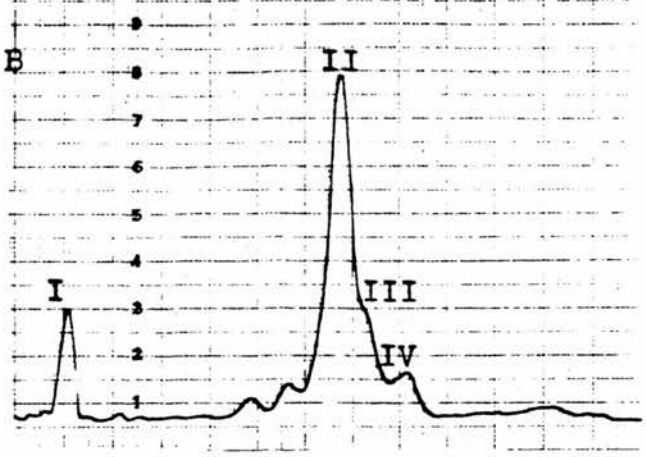
II - ALBUMIN AND α-CHAIN OF FIBRINOGEN

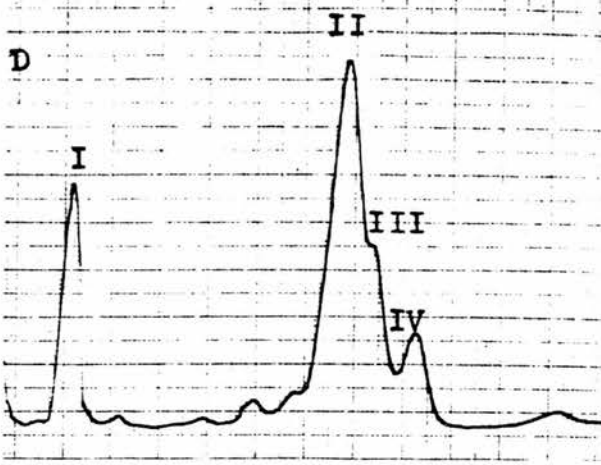
III - β-CHAIN OF FIBRINOGEN

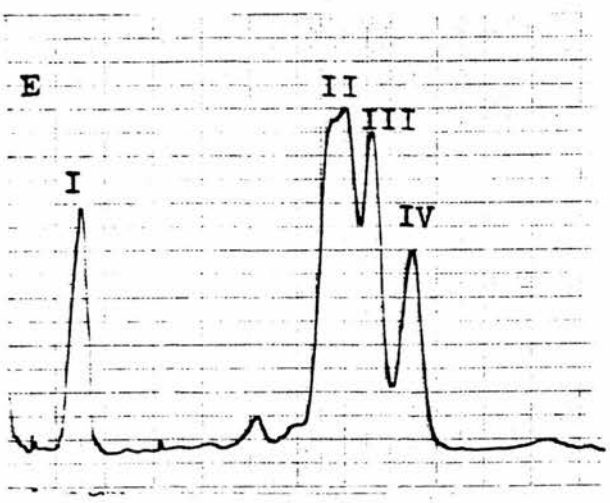
IV - γ-CHAIN OF FIBRINOGEN

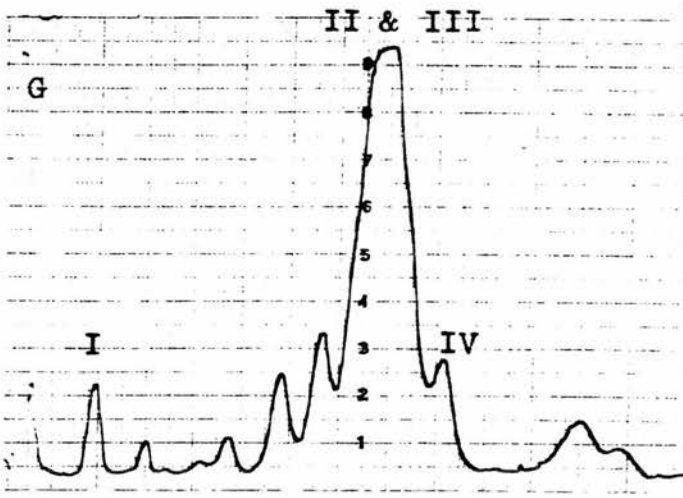

A
I
II
III
IV

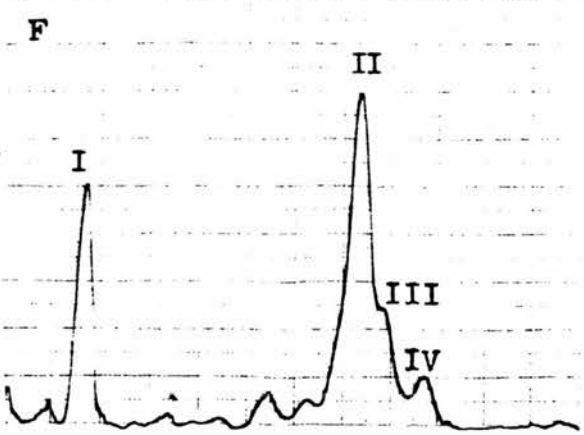

C
I
II
III
IV

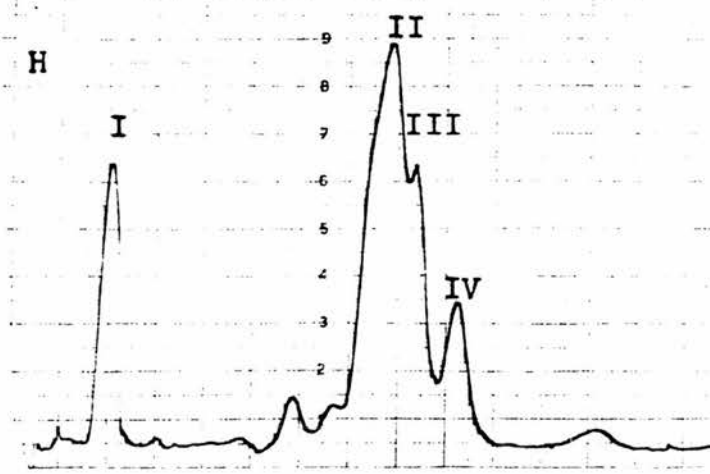

B
I
II
III
IV


D
I
II
III
IV


E
I
II
III
IV


G
I
II & III
IV


F
I
II
III
IV


H
I
II
III
IV

recovery of VIII:C and VIIIR:Ag in the supernatant, with over 70% of the fibrinogen being precipitated (Table 6-5A). Experiments using HES 40 precipitates (Chapter V) instead of cryo extracts produced similar results (Table 6-5B).

Direct extraction of cryo into 1 mM zinc acetate-20 mM Tris buffer did not give recoveries that were as good as the above precipitation technique. Assay of the partially dissolved residue indicated that factor VIII as well as fibrinogen was retained in the precipitate, resulting in lower yields in the extract.

(E) EFFECT OF PURIFICATION PROCEDURE ON CONCENTRATE PROTEIN COMPOSITION

Figure 6-5 shows the protein components of the various concentrates described in this Chapter determined by SDS-PAGE and densitometric scanning of the bands produced. It is apparent that glycine- and zinc-precipitation lead to a great reduction in fibrinogen, with the other main components - fibronectin and albumin - being relatively unaffected (Figure 6-5A and B, C and D, E and F). Figure 6-5G shows the pattern for cryoprecipitate solubilised in its own plasma. The protein composition is similar to that of normal plasma except that the fibrinogen and fibronectin bands are stronger, showing the concentration of these proteins in cryoprecipitate. Cold precipitation led to a further concentration of fibronectin and fibrinogen, with reduction of other proteins including albumin (Figure 6-5H).

FIGURE 6-6

TWO-DIMENSIONAL IMMUNOELECTROPHORESIS OF VIIIR:Ag IN FRACTIONS OF DIFFERENT PURITY

Electrophoresis was carried out at 200 V for 6 hours and 120 V for 18 hours for the first and second dimensions respectively. The anode was to the right in the first dimension and on top in the second dimension. Antiserum concentration in the second dimension was 0.3%. The precipitin arc produced by solubilised cryo was partly traced over with a marker pen to improve visual discrimination from background protein

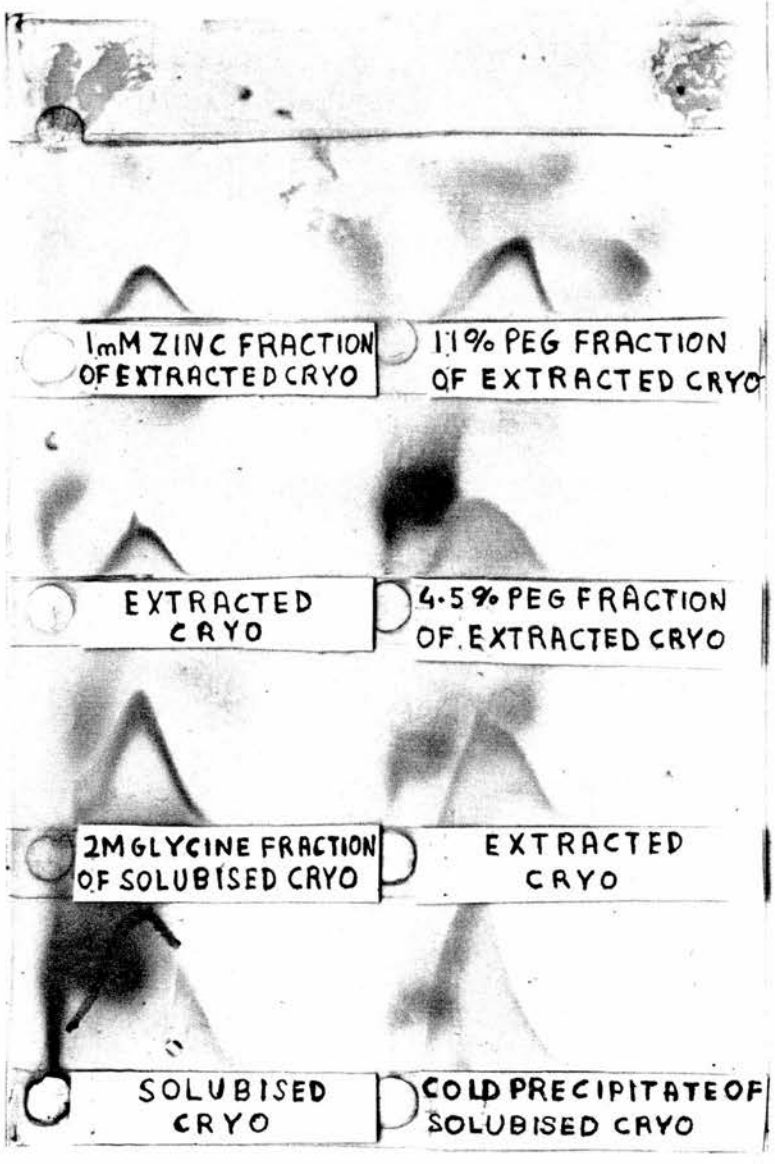

1mM ZINC FRACTION OF EXTRACTED CRYO
1·1% PEG FRACTION OF EXTRACTED CRYO
EXTRACTED CRYO
4·5% PEG FRACTION OF EXTRACTED CRYO
2M GLYCINE FRACTION OF SOLUBISED CRYO
EXTRACTED CRYO
SOLUBISED CRYO
COLD PRECIPITATE OF SOLUBISED CRYO

TABLE 6-6 ANALYSES OF VIIIR:Ag BY TWO-DIMENSIONAL IMMUNO-ELECTROPHORESIS

Sample	d_1	p	d_2
Solubilised Cryo	6	12	19
Cold Precipitate of Cryo	6	12	16
Glycine-Purified Cryo	7	12.5	17.5
Extracted Cryo	10	12	18
Zinc-Purified Extracted Cryo	9.5	12	16
4.5% PEG Fraction of Extracted Cryo	6	10.5	18
11% PEG Fraction of Extracted Cryo	11.5	17	19.5

Samples were analysed by 2DIEP as described in Methods. d_1, p and d_2 denotes the respective nigration distances in mm.

Thus, purification with this method involves mainly removal of albumin, while with the other techniques fibrinogen is the main component removed.

(F) FACTOR VIIIR:Ag IN PURIFIED FRACTIONS

Figure 6-6 shows the immunoprecipitin arcs of the various concentrates subjected to two-dimensional immunoelectrophoresis with antiserum to VIIIR:Ag. Table 6-6 summarises the electrophoretic data obtained, as described in Methods, from these arcs. The electrophoretic pattern of solubilised cryo is similar to that of plasma (see Figure 5-4) with slow-moving VIIIR:Ag forms that are conserved upon glycine - or cold - precipitation. Cold precipitation results in a decrease in the amount of fast moving VIIIR:Ag (as witnessed by a drop in d_2). Compared to solubilised cryo, extracted cryo shows a decrease in the slowest moving VIIIR:Ag forms (as seen by an increase in d_1), with no further decrease in slow moving VIIIR:Ag occurring as a result of zinc-precipitation. Fractional PEG precipitation leads to a loss of VIIIR:Ag of slow and medium mobility (as seen by increases in d_1 and p) in the final (11% PEG) precipitate, compared to the initial cryo extract. The first precipitation with this technique - 4.5% PEG, designed to remove fibrinogen - leads to loss of VIIIR:Ag in the fraction. The electrophoretic analysis indicates that this precipitate (usually a waste fraction in large-scale fractionation) is rich in slow and medium moving forms of VIIIR:Ag.

TABLE 6-7 SUMMARY OF CHARACTERISTICS OF HIGHER-PURITY FACTOR VIII CONCENTRATES

Product	Specific Activity VIII:C u/mg Protein	Fibrinogen % of Total Protein	Yield of VIII:C		Purification Over Intermediate Material*
			% of Intermediate Material	U/kg Starting Plasma	
Cold Insoluble Globulin Precipitate of Cryo	0.35	40	75	310	3.9
PEG Precipitate of Glycine-Purified Cryo	1.4	16	79	420	2.6
Zinc-Purified Cryo Extract	0.9	25	87	370	1.9
Zinc-Purified HES Precipitate	1.3	19	90	530	1.9
PEG-Purified Cryo Extract	0.9	12	50	210	2.3

* Defined as specific activity of final concentrate/specific activity of intermediate material.

Table 6-7 summarises the characteristics of the various concentrates produced by the methods described in this Chapter.

DISCUSSION

The growing realisation that despite intensive donor screening the majority of haemophiliacs develop liver abnormalities (Mannucci et al 1982) has made the inactivation of the probable viral contaminants in coagulation factor concentrates a priority. However, present methods of sterilisation require fibrinogen removal for the reasons outlined above. Since fibrinogen is also the major contaminant of extracted cryoprecipitate, its removal also results in a significant purification and allows higher solubility and potency.

Two recent studies (Rock et al 1980b, Smit-Sibinga et al 1981) have made use of a two-step method to produce a higher-purity factor VIII concentrate on a blood bank scale. These groups have claimed that collection of blood in heparin or heparin addition to CPD plasma allows factor VIII yields in cryoprecipitate to be significantly increased, and also permits the utilisation of a second precipitation at 0°C which precipitates and concentrates the factor VIII. They postulate that this second cold precipitation is possible because heparin induces precipitation at 0°C of fibronectin, which is often associated with factor VIII during fractionation (Chapter V). The results of the present study, however, do not support this hypothesis. As can be seen (Table 6-2) cooling of solubilised cryoprecipitate results in precipitation of fibronectin and factor VIII, together with some fibrinogen, irrespective of the

presence of heparin. These results indicate that what is necessary for cold precipitation to occur is not the presence of heparin, but a high protein concentration in the cryoprecipitate. This is shown by the relatively small precipitate and low factor VIII yields obtained from cryo totally drained of supernatant and dissolved in buffer, compared to cryo dissolved in its own supernatant plasma. These results are not surprising in view of the findings discussed in Chapters III and V regarding the spontaneous and polymer induced cold precipitation of plasma factor VIII fibrinogen and fibronectin. As I have shown, increasing the plasma protein concentration by albumin addition or simple concentration allows near quantitative precipitation of factor VIII and fibronectin in the cold. It is suggested that the cold insoluble precipitation reported by previous workers (summarised in Lane et al 1983) is a result of this phenomenon. The initial cryoprecipitation concentrates factor VIII and fibronectin to a level which results in a further albumin-induced precipitation when the cryo is dissolved in supernatant plasma and chilled. It is noteworthy that the other groups using cold precipitation (Lane et al 1983) all reported a protein concentration of about 60 mg/ml in the initial cryoprecipitate, suggesting that considerable plasma protein was left with the cryoprecipitate. The starch gel electrophoresis reported by one group (Welbergen 1981) also demonstrates a high albumin content in the initial cryo. This high

content of residual supernatant plasma protein is almost unavoidable using the plastic bag system (Chapter V). Using centrifugation and drainage (Chapter V) results in a cryo which when dissolved in buffer has a protein concentration of 20 to 30 mg/ml, compared to 60 to 70 mglml when dissolved in a similar volume of supernatant plasma. Heparin addition to this material increased the amount of fibronectin precipitated but did not affect factor VIII yield.

The use of hydrophilic polymers to effect further purification of cryoprecipitate did not give results as promising as those obtained with plasma (Chapter V). In general, it was found that factor VIII and fibrinogen precipitated together during polymer induced precipitation (Figure 6-1). Varying pH temperature or ionic strength did not improve separation. It is possible that aggregation of fibrinogen molecules during processing (Newman et al 1971) is responsible for the poor separation observed. Fractional precipitation with PEG at higher temperatures, although removing much of the fibrinogen, gave low VIII:C yields. Thus, it seems that with the classical PEG fractionation scheme that is used by a number of manufacturers, substantial fibrinogen removal is only possible at a considerable sacrifice in yield (Bidwell et al 1976).

The use of the recently described warm glycine precipitation technique (Blombäck and Thorell 1982) gave very promising results (Table 6-3). The precipitation

of fibrinogen with glycine was first noted by Edsall and Lever in 1951. Wagner et al (1964) showed that precipitation of plasma at 0°C with 2.3 M glycine gave factor VIII rich fractions. Subsequently this principle was utilised in Hyland's Method IV to purify cryoprecipitate (Shanbrom and Fekete 1971). In these early methods, precipitation was carried out at low temperatures resulting in co-precipitation of factor VIII and fibrinogen. It seems that utilisation of higher temperatures results in selective fibrinogen precipitation. Concentration of the supernatant factor VIII is necessary to remove glycine and produce a more potent solution. This can be readily achieved by subsequent PEG precipitation in the cold (Table 6-3). It is of interest that simple cooling to 0°C of the factor VIII rich glycine supernatant failed to precipitate factor VIII, thus highlighting the role of fibrinogen as a 'carrier' in such precipitations.

Foster et al (1983a) have utilised zinc ion precipitation of extracted bulk cryoprecipitate to remove fibrinogen from intermediate purity concentrate. Zinc ion fractionation of plasma proteins was described by Cohn et al (1950) and Maeda et al (1983) have investigated the mechanism of zinc-induced fibrinogen precipitation. The interaction between zinc and fibrinogen has also been studied by metal-chelate chromatography (Scully and Kakkar 1982). The results of the present study confirm that 1 mM zinc precipitates

fibrinogen selectively. Careful reagent addition as a dilute stock solution was found to be crucial for good results, as addition of the salt as crystals resulted in precipitation of VIII:C along with fibrinogen. It is probable that this is a result of local over-concentration of the reagent, as Foster et al (1983a) have shown that addition of amounts exceeding 1 mM also precipitate factor VIII. One minor disadvantage of this method is the insolubility of the fibrinogen-rich precipitate obtained, making it difficult to retrieve fibrinogen as a by-product.

An attempt was made to incorporate glycine and zinc directly into the buffer used in the 'Newman' procedure for extraction of factor VIII from cryo. It was hoped that this would result in retention of fibrinogen in the insoluble residue with factor VIII being recovered in the extract. This would eliminate the need for a second step to precipitate the fibrinogen. However, use of extracting buffers incorporating reagents at a concentration known to selectively precipitate fibrinogen led to much VIII:C being retained as well.

Analysis of VIIIR:Ag by two-dimensional immunoelectrophoresis (Figure 6-6 and Table 6-6) revealed that solubilised cryo had a population of VIIIR:Ag rich in slow moving forms. Slow moving forms of VIIIR:Ag are known to represent the higher molecular weight multimers of the protein (Zimmerman et al 1975b) and are associated with high VIIIR:RCF activity (Over et al 1978). It has

been suggested that the presence of these forms of VIIIR:Ag is necessary for the ability of concentrates to correct the bleeding time defect in von Willebrand's disease (Weinstein and Deykin 1979). Cryoprecipitate has this ability, whereas higher purity materials lacking higher molecular weight forms of VIIIR:Ag do not (Nilsson and Hedner 1977). It can be seen from my results that glycine precipitation of solubilised cryo preserved the latter's higher molecular weight forms of VIIIR:Ag and this product has been reported to correct the bleeding time defect of von Willebrand's disease (Thorell et al 1983). Extracted cryo can be seen to show some depletion of higher molecular weight VIIIR:Ag; this might be due to the use of low ionic strength extraction buffers, as VIIIR:Ag is known to be more susceptible to proteolysis under these conditions (Hellings 1981). Fractional PEG precipitation of extracted cryo, which is one of the commonest techniques in industrial factor VIII fractionation, removes substantial amounts of higher molecular weight VIIIR:Ag in the discarded 4.5% PEG precipitate. The VIIIR:Ag in the final 11% PEG fraction showed a marked anodal mobility. Thus it can be postulated that loss of higher molecular weight multimers having high VIIIR:RCF activity during fibrinogen removal is the cause for the inability of PEG-fractionated concentrates to correct the bleeding time in von Willebrand's disease. As has been pointed out, however, (Chapter I and references therein) correction of

the bleeding time is known to be more dependent on some other less well characterised activity than on VIIIR:RCF.

Table 6-7 summarises the options for further purification investigated in this Chapter. It is suggested that zinc precipitation under the described optimal conditions is the best alternative available. Cold insoluble globulin precipitation works through albumin removal and leaves considerable amounts of fibrinogen in the product (Table 6-7 and Figure 6-5). The final product is thus unsuitable for heat treatment. Fractional PEG precipitation although efficient for fibrinogen removal, gives low factor VIII yields. Both glycine and zinc precipitation result in fibrinogen removal with high VIII:C yields. The glycine technique however requires high concentrations of glycine and temperatures of 30°C are necessary. Zinc salts are cheaper than glycine and the addition can be carried out at room temperature. Furthermore, the residual zinc in the supernatant increases viral inactivation during subsequent heat treatment (Foster 1984: personal communication).

In summary this Chapter concludes:

1. Of the options investigated for removal of fibrinogen from cryoprecipitate, glycine or zinc precipitation under the conditions described results in adequate purification with high factor VIII yields.
2. Combination of the HES precipitation method (Chapter V) with subsequent zinc precipitation appears

to be the method of choice for producing a high-purity factor VIII concentrate.

CHAPTER VII

ADDITIONAL APPROACHES TO OPTIMAL USE OF DONATED FACTOR VIII

(A) PASTEURISATION OF FACTOR VIII CONCENTRATES

INTRODUCTION

As has been outlined (Chapter I) viral infection is the major hazard of factor VIII replacement therapy. Several studies have demonstrated asymptomatic liver damage in haemophiliacs treated with factor VIII concentrates (summarised by Mannucci 1981). The increased likelihood of viral contamination in large plasma-pool concentrates has led to some clinicians advocating the use of single-donor or small-pool materials such as cryoprecipitate (Gabra et al 1982). The use of accredited donors is an additional method of reducing such risks. However, single-donor materials cannot be standardised for their factor VIII content and precise dosage is thus impossible. Small-pool materials such as lyophilised cryoprecipitate necessitate a large proportion of the product being sacrificed for quality control if complete pharmaceutical testing is to be carried out. Moreover, some doubt exists as to whether such products reduce the risk of hepatitis viral infection as evinced by liver dysfunction (Levine et al 1977).

Studies employing classical plasma fractionation schemes show that contaminating hepatitis virus is concentrated in the albumin fraction, with other fractions including coagulation factor products being relatively less contaminated (Hoofnagle et al 1976). However, albumin solutions have an excellent record of non-

infectivity, while coagulation factor concentrates are high-risk products. This is undoubtedly due to the ability to subject albumin solutions to heat in the presence of stabilisers, thus inactivating contaminating viruses. The lability of coagulation factors has resulted in approaches other than heat treatment being attempted to remove or decrease potential viral contaminants. Thus, Einarrson et al (1981) have successfully decreased hepatitis virus contamination of prothrombin complex concentrates by hydrophobic affinity chromatography on alkylated agarose. These adsorbents selectively removed the virus while showing minimal affinity for coagulation factors II, VII, IX and X. Such an approach, however, is not applicable to factor VIII as Morgenthaler (1982) has shown that these adsorbents bind factor VIII irreversibly as well as the virus. Johnson et al (1976) have used PEG precipitation to remove hepatitis virus from albumin and prothrombin complex solutions. Precipitation with 20% PEG allowed removal of virus in the precipitate, thus utilising the large molecular weight difference between the proteins and the viral particles. The high molecular weight of factor VIII however would result in co-precipitation with the virus under these conditions.

Recently, Brummelhuis et al (1983) have shown that addition of specific human immunoglobulin to hepatitis B to blood products (including factor VIII concentrate) produced from deliberately contaminated

plasma neutralised the infectivity of these preparations. This approach, however, is specific to infectivity with hepatitis B virus. Similarly, Prince et al (1983,1984) have shown that lipid extraction techniques, such as detergent or ether treatment, result in a decrease in infectivity, an approach which is restricted to lipid-coated viruses.

As far as hepatitis B goes, the application of sensitive radioimmuno techniques in screening donations and final products can cut down considerably on the infectivity risk. However, given that the sensitivity of present day techniques is 3 or 4 orders of magnitude greater than the minimal infective dose, a similar reduction in viral titre is required to eliminate infectivity. This consideration determines the extent of viral inactivation/removal that a particular process has to effect.

The possibility that the agent responsible for the AIDS syndrome is a virus emphasises the need to develop general methods of viral inactivation. In this regard, the well tried heat treatment is an attractive option. Heat treatment has been used by one manufacturer to produce a factor VIII concentrate with reduced hepatitis infectivity, the factor VIII being stabilised during heating in a sucrose-glycine solution (Heimburger et al 1981). Although no stage yields were given, factor VIII losses of about 80% over the starting material (cryoprecipitate) occurred. Prior to heating, the

concentration of contaminating virus and other proteins, including fibrinogen, was decreased by glycine and salt precipitations. Losses of factor VIII in these steps are likely to have contributed to the low yields. The final concentrate had factor VIII antigen/activity ratios similar to the starting material, suggesting that physical loss of factor VIII molecules was occurring over the process. If such losses could be prevented, yields would be improved. This can be done by using a higher yielding method of removing fibrinogen (Chapter VI). Heat treatment would also require the use of stabilisers to effect selective preservation of factor VIII while allowing inactivation of contaminating viruses.

In this section, a new method of viral inactivation-heat treatment in the presence of sorbitol-glycine (Macleod et al 1983)- has been applied to produce - a factor VIII concentrate with decreased risk of viral infectivity. The characteristics of the product have been examined, both as regards its in vitro properties as well as its behaviour upon infusion to a haemophilic patient.

METHODS

Materials for heat treatment were cryoprecipitate extracts and HES 40 plasma precipitates prepared as described in Chapters V and VI. Fibrinogen content was reduced by zinc precipitation (Chapter VI).

Heat treatment was performed as follows: (Macleod et al 1983). The solution for heat treatment was placed in a plastic beaker on a magnetic stirrer/heater. With constant stirring, solid glycine was added to a final concentration of 50 g/litre of solution. The pH of the solution was monitored and kept above 7 by addition of 1 M NaOH as necessary. The final pH was adjusted to 7.5. Solid sorbitol was then added slowly to the solution, addition being made by scattering sorbitol lightly over the surface with constant stirring. As the sorbitol went into solution, the temperature of the mixture dropped. The temperature was kept above 30°C by heating with careful monitoring using a thermocouple. Care was taken to ensure that the temperature did not exceed 40°C. Sorbitol was added to a final concentration of 1850 g/litre of original solution. When the sorbital addition was complete, the mixture was transferred to a glass bottle and stoppered. The bottle was then placed in a water bath and heated under the conditions stated in Results. After heating, the mixture was treated (see Results) to remove the heat-stabilising additives. Samples from the various stages of the procedure were frozen at -40°C for subsequent assay.

Clinical evaluation of heat-treated factor VIII concentrate: A heat-treated factor VIII concentrate was produced at the Protein Fractionation Centre, Edinburgh, by the method of Newman et al (1971) (Chapter I) with fibrinogen reduction by zinc precipitation of the cryoprecipitate extract (Chapter VI). This material had the characteristics shown in Table 7-2 and passed all the quality control criteria for a clinical product. The concentrate was infused into a haemophilic patient in the Haematology Department of the Royal Infirmary of Edinburgh. Blood samples were taken before and at sequential time intervals after the infusion.

Recovery of infused factor VIII:C was calculated using the formula:

$$\text{recovery (\%)} = \frac{\text{amount of VIII:C recovered in plasma}}{\text{amount of VIII:C infused}}$$

To calculate the amount of VIII:C recovered in the patient's plasma, the VIII:C content 20 minutes after infusion was multiplied by the plasma volume, assuming 41 ml of plasma per kg body weight.

Half-life of infused VIII:C was calculated froma semi-logarithmic plot of plasma activity versus time, as shown in Figure 7-3.

All other assays and analytical methods were as described in Chapter II.

TABLE 7-1 RECOVERY OF FACTOR VIII CONCENTRATE PROTEINS AFTER HEAT-TREATMENT

Starting Material	Heating Conditions	Method of Reagent Removal	n	Recovery of Proteins (% of Starting Material)			
				VIII:C	VIIIR:Ag	Fibrinogen	Fibronectin
Cryo extract	12 h, 60°C	Dialysis*	2	56	89	97	92
HES-40 ppt.	12 h, 60°C	Dialysis	2	52	81	84	96
HES-40 ppt.	30 min 70°C	Dialysis	2	52	95	92	88
HES-40 ppt.	30 min 70°C	Precipitation+	1	71	82	61	52

Solutions were heated in the presence of glycine/sorbitol. Heating was carried out in a water bath adjusted to the appropriate temperature. Temperature measurement indicated that the solution reached water bath temperature in one minute.

* Solutions were dialysed against 100 volumes of 15 mM citrate, 150 mM NaCl pH 6.9 (citrate saline) for 7 hours. The dialysate was changed 3 times during the procedure.

\+ The heated solution was diluted with an equal volume of 20 mM citrate, 60 mM NaCl pH 6.9. The solution was then made 26% W/V with respect to NaCl and centrifuged for 60,000 g min. at 22°C. The precipitate was dissolved in citrate saline.

FIGURE 7-1

TWO-DIMENSIONAL IMMUNOELECTROPHORESIS OF FACTOR VIII CONCENTRATE PRE AND POST HEAT TREATMENT

Electrophoresis was carried out at 200 V for 6 hours and at 150 V for 18 hours in the first and second dimensions respectively. Antisera to VIIIR:Ag, fibrinogen and fibronectin were incorporated into the second dimension at concentrations of 0.2%, 2% and 2% respectively.

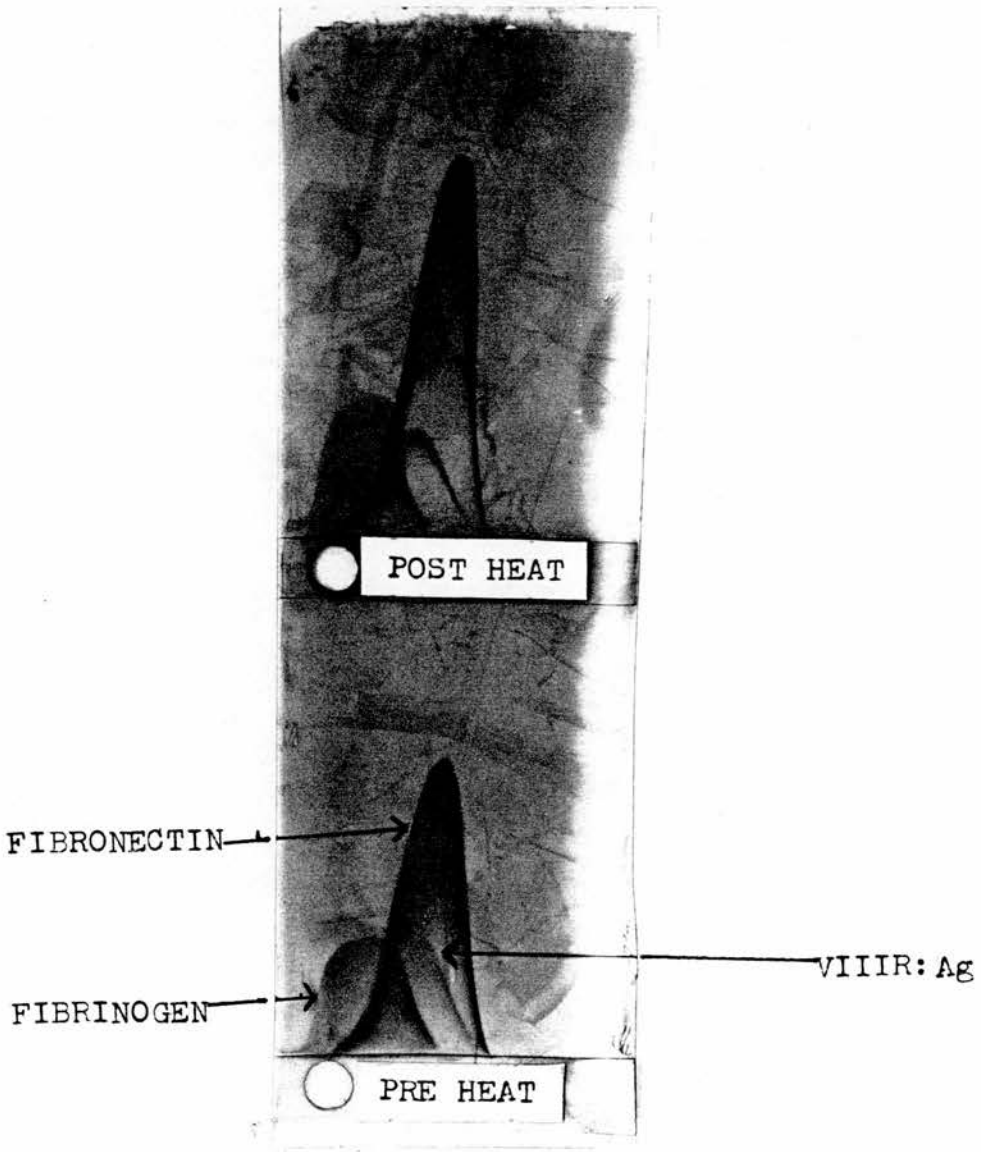
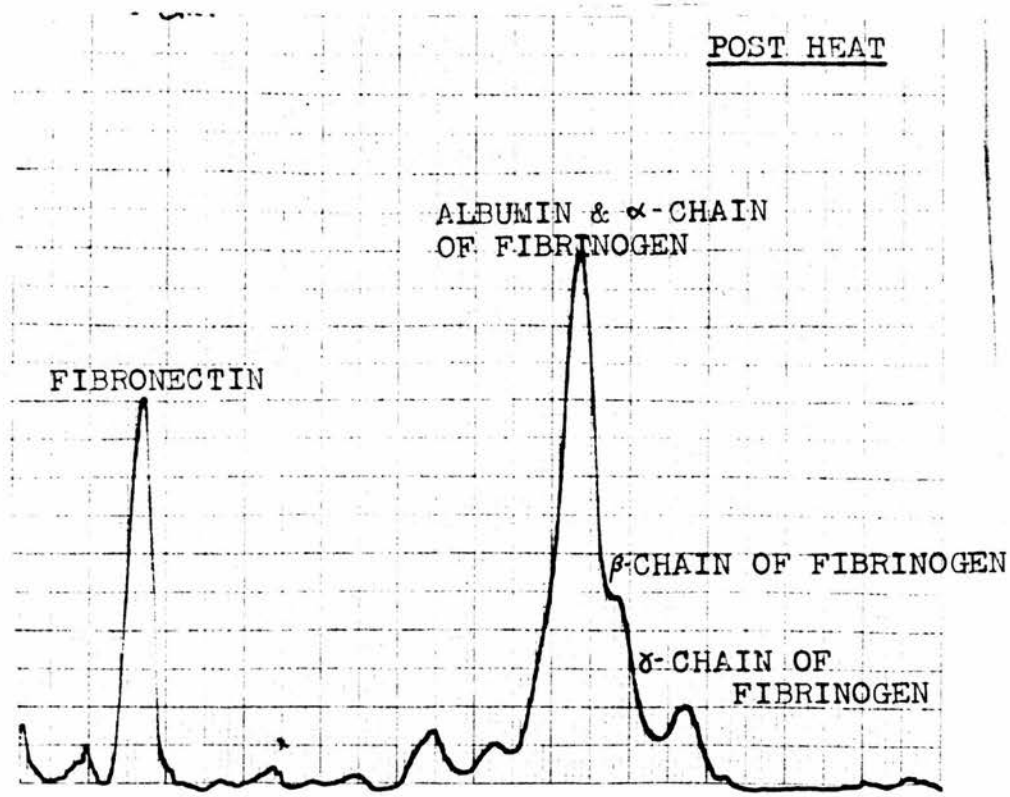

POST HEAT
FIBRONECTIN
VIIIR: Ag
FIBRINOGEN
PRE HEAT

FIGURE 7-2

SDS-PAGE OF FACTOR VIII CONCENTRATE PRE AND POST HEAT TREATMENT

Gels were 5% polyacrylamide. Electrophoresis was at 30 V for 18 hours.


POST HEAT
ALBUMIN & α-CHAIN
OF FIBRINOGEN
FIBRONECTIN
β-CHAIN OF FIBRINOGEN
δ-CHAIN OF
FIBRINOGEN

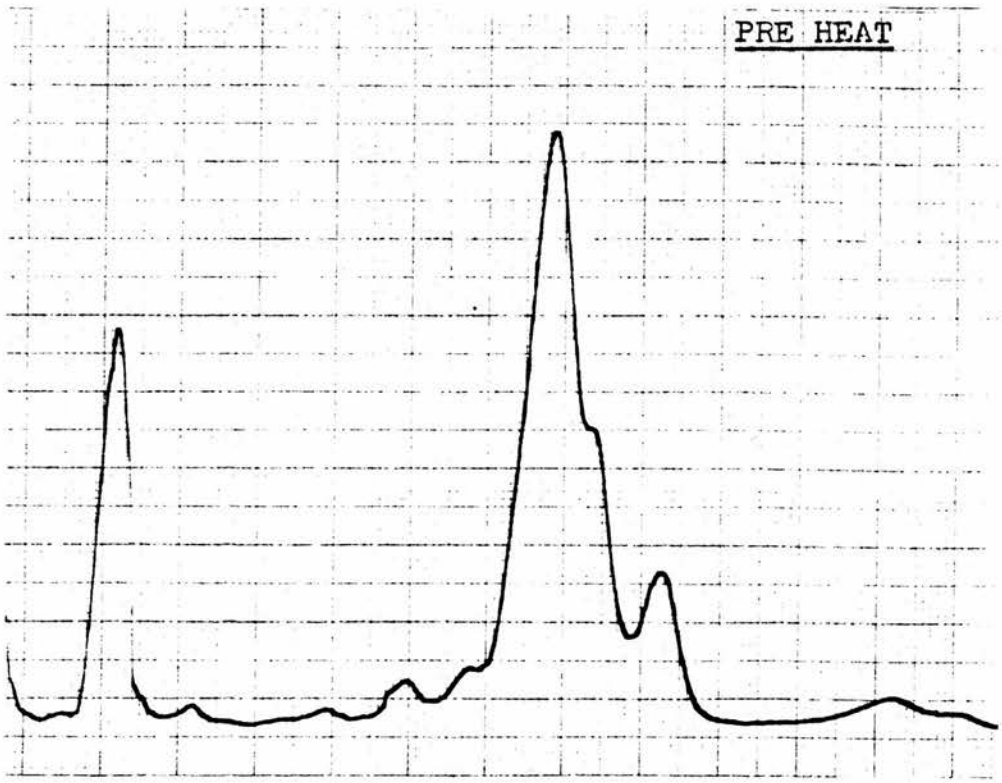

PRE HEAT

TABLE 7-2 ANALYTICAL PROFILE OF CLINICAL HEAT-TREATED FACTOR VIII CONCENTRATE

Factor VIII:C Content	145 i.u/vial
Total Protein	14.4 g/litre
Fibrinogen	5.2 g/litre
Sodium	40.4 mmol/litre
Potassium	0.01 mmol/litre
Chloride	11.9 mmol/litre
Citrate	12.3 mmol/litre
pH	6.8
Sorbitol	54 g/litre
Osmolality	509 mOsM
Zinc	3.6 ppm
Cellulose Acetate Electrophoresis	Normal
Rabbit Pyrogen Test	3.8°/6 rabbits
Limulus Pyrogen Test (Endotoxin equiv.)	≅ 0.5 ng.ml
Acute Toxicity	Pass
HBsAg RIA	Negative
Sterility Test	Pass
Isoagglutinin (Indirect Coombs Test)	A_1 A_2 B O 1/8 1/4 1/8 Negative

Data for vials reconstituted in 25 ml of distilled water.

Data supplied by Quality Control Department of the Protein Fractionation Centre, Edinburgh.

RESULTS

A. PASTEURISED FACTOR VIII CONCENTRATE: In vitro Characteristics.

Table 7-1 confirms the findings of Macleod et al (1983) regarding the stability of factor VIII heated in the presence of sorbitol-glycine. The use of 50% sucrose and 2 M glycine as stabilisers (Heimburger et al 1981) was also examined but the procedure resulted in low VIII:C yields and was not persued further. An attempt to stabilise factor VIII with citrate, as has been proposed for antithrombin III (Wickerhauser et al 1979) was unsuccessful as addition of citrate to 0.5 M caused immediate precipitation of the majority of the protein in the concentrate.

Figure 7-1 shows that heat-treatment did not affect the properties of the main proteins in the concentrate, as determined by two-dimensional immunoelectrophoresis. The primary structure of the proteins was also unaffected, as seen on SDS polyacrylamide gels (Figure 7-2).

B. PASTEURISED FACTOR VIII CONCENTRATE: In vivo Characteristics.

Table 7-2 lists the characteristics of the heat-treated factor VIII concentrate that was infused into a haemophilic patient. Table 7-3 summarises the in vivo results of this infusion, together with data for similar infusions using other factor VIII products.

TABLE 7-3 IN VIVO STUDIES WITH VARIOUS FACTOR VIII PRODUCTS

Preparation Infused	Recovery (%)	Half-Life (hours)
A) Frozen Cryoprecipitate*	100	8
B) Intermediate Purity Concentrate*	122	10.5
C) Heat-Treated Concentrate+	92	12.3

Data shows results of infusions to one patient (K.M.) given at different times.

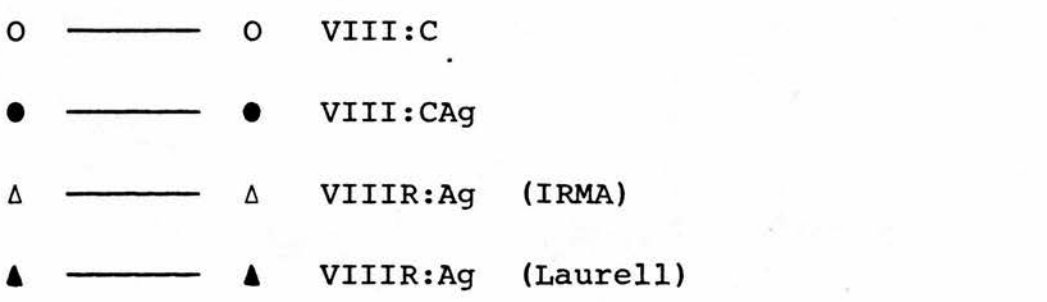
Recovery data for A & B were calculated using a concentrate standard, a method which gives higher recoveries than when using a plasma standard, as in C. Data for similar infusions of A & B to other patients were similar.

* Data taken from Toolis et al (1980)

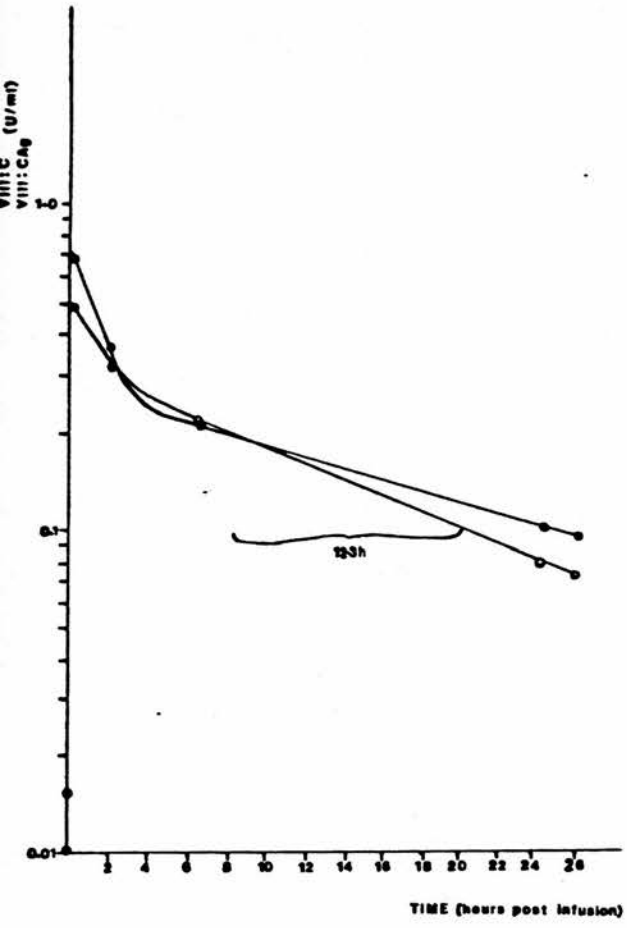
\+ Data from the present study

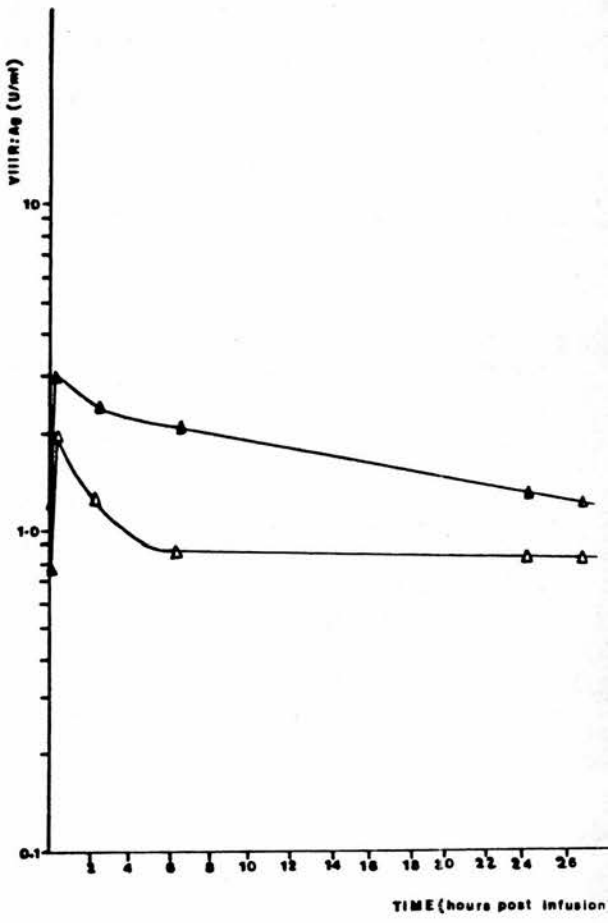
FIGURE 7-3

FACTOR VIII RELATED ACTIVITIES FOLLOWING INFUSION OF HEAT-TREATED CONCENTRATE



Half life was estimated by measuring the time it took for the VIII:C level to decline by half, as shown in the Figure.


VIII:C
VIII:CAg (U/ml)
1·0
0·1
0·01
12·3h
2 4 6 8 10 12 14 16 18 20 22 24 26
TIME (hours post infusion)


VIII:RAg (U/ml)
10
1·0
0·1
2 4 6 8 10 12 14 16 18 20 22 24 26
TIME (hours post infusion

In vivo recovery and half-life of the heat-treated material compared well with those obtained with materials that had not been heat-treated. Figure 7-3 shows the in vivo behaviour of various factor VIII related activities after infusion. VIIIR:Ag measured by Laurell assay tended to give higher values than when measured by IRMA, possibly due to antigenic breakdown with subsequent increased electrophoretic mobility, as has been noted for other, non-heated concentrates (Chapter I) (Prowse et al 1981). Figure 7-1 indicates that the heating step had no effect on the mobility of VIIIR:Ag.

TABLE 7-4 THE EFFECT OF HEATING ON VIII:C AND MODEL VIRUSES

Solution	Heat Treatment in Solution			
	(A) 60°C for 10 Hours		(B) As (A) Followed by 70°C for 30 Minutes	
	Before Heating	After Heating	Before Heating	After Heating
FACTOR VIII (Fibrinogen-Depleted Concentrate)				
Factor VIII:C (%, Mean & S.D. (m))	100	89 ± 22.5 (20)	100	76.9 ± 7.2 (5)
VIRUSES				
Vaccinia (pfu/ml)				
in Factor VIII (sorbitol/glycine)	$10^{8.5}$	10^{5}	$10^{7.5}$	$< 10^{1}$
in Albumin (sorbitol/glycine)*	$10^{8.5}$	$10^{4.5}$	N.D.	N.D.
in Albumin (standard solution)+	$10^{7.5}$	0	N.D.	N.D.
Mumps (pfu/ml)				
in Factor VIII (sorbitol/glycine)	$10^{5.5}$	$10^{3.5}$	$10^{5.5}$	$10^{2.5}$
in Albumin (standard solution)	$10^{5.5}$	0	N.D.	N.D.

TABLE 7-4 Cont THE EFFECT OF HEATING ON VIII:C AND MODEL VIRUSES

Solution	Heat Treatment in Solution			
	(A) 60°C for 10 Hours		(B) As (A) Followed by 70°C for 30 Minutes	
	Before Heating	After Heating	Before Heating	After Heating
Herpes Simplex (pfu/ml)				
in Factor VIII (sorbitol/glycine)	$10^{6 \cdot 5}$	$< 10^{1}$	$10^{6 \cdot 5}$	$< 10^{1}$
in Albumin (standard solution)	$10^{6 \cdot 5}$		N.D.	N.D.
Polio 2 (pfu/ml)				
in Factor VIII (sorbitol/glycine)	10^{6}	$< 10^{1}$	10^{6}	$< 10^{1}$
in Albumin (standard solutiion)	10^{6}	0	N.D.	N.D.

Data taken from Foster et al (1983c) and reproduced by permission

* Clinical albumin solution

\+ Stabilised as in routine production with sodium caprylate

N.D. Not determined

DISCUSSION

The present study confirms that heat-treatment is a practical option to effect sterilisation of a factor VIII concentrate. Use of the sorbitol-glycine stabilisers allows heating to be carried out with retention of VIII:C activity. The protection from thermal denaturation of proteins by sugars has been extensively studied (Lee and Timasheff 1981 and references within). Thermodynamic measurements have suggested the hypothesis that stabilisation is due to the increase in solvent surface tension by sugar addition, making protein unfolding with subsequent increased surface area thermodynamically unfavourable. The stabilisers also protect model viruses added to the system (Table 7-4) and the modified heating conditions (70°C for 30 minutes following the standard 60°C for 10 hours) are necessary to effect selective viral inactivation. The same workers have found that heating at 70°C for 30 minutes results in a viral kill equivalent to that obtained when this procedure is followed up by 60°C for 10 hours (Foster 1984, personal communication).

In vitro analyses of the product showed that no changes were induced in the proteins in factor VIII concentrate by heat-treatment. Whereas heat-treatment has been shown to result in changes in two-dimensional immunoelectrophoresis in antithrombin III concentrate (Wickerhauser et al 1979) no such changes were apparent

in the factor VIII concentrate. Other studies by Dawes et al (1983) also confirm the antigenic identity of the proteins pre- and post-heating, suggesting that formation of neo-antigens did not occur. Studies of in vivo behaviour are more important in demonstrating the suitability of the heat-treated material. In vivo recovery and half-life of VIII:C were similar to those obtained with other products not subjected to heat-treatment. Since heating appears to have no deleterious effects on the characteristics of the product, it can readily be used clinically, prior to confirmation of its decreased infectivity. As far as hepatitis B is concerned, a decrease in infectivity can be tested by challenging the concentrate with virus prior to the inactivation procedure, followed by infusion into chimpanzees (Brummelhuis et al 1983, Prince et al 1983). Since the heated product carries no greater risk of infectivity and is hopefully much safer than the current material, such studies are not mandatory prior to its issue for clinical use.

(B) ASSOCIATION OF FACTOR VIII WITH BLOOD GROUP

INTRODUCTION

It has long been known that persons of blood groups A and B have higher factor VIII levels than persons of group O (Preston and Barr 1964, Wahlberg et al 1980). This higher activity results in higher amounts of factor VIII being recovered in single donation cryoprecipitate from group A compared to group O donors (Regional Transfusion Directors' Committee 1978). One study has reported higher relative yields in thaw-siphon cryoprecipitate prepared from group A as compared to group O plasma (Prowse et al 1982). Oligosaccharide structures characteristic of blood group A, B/O have been shown to be associated with the factor VIII molecule, although the question of whether they are covalently linked is uncertain (Sodetz et al 1979, Samor et al 1982).

In this study, the association of factor VIII with blood group activity has been investigated using purified factor VIII concentrates prepared from group specific plasma. The aim was to define more closely the nature of the association.

METHODS

Factor VIII concentrates: Non-group specific intermediate purity concentrate was obtained from the Protein Fractionation Centre, Edinburgh. Group A- and group O-specific high purity concentrates were obtained from Kabi Vitrum, Sweden.

Solid phased antibodies: Rabbit anti-A substance, immunoglobulin (affinity purified on Synsorb A) and non-immune immunoglobulin were supplied by the Reagents Laboratory, Edinburgh Blood Transfusion Service, and were coupled to cyanogen bromide activated Sepharose 4B (Chapter II).

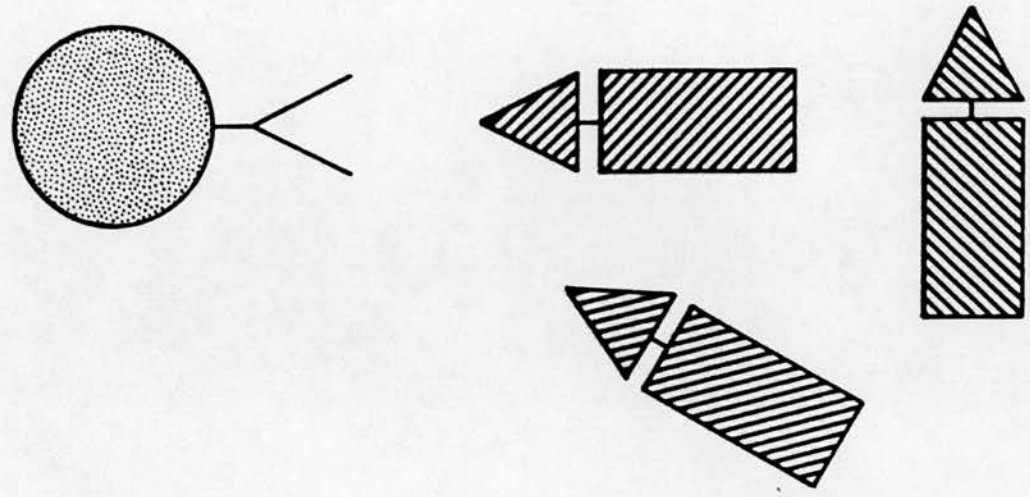
Factor VIIIR:Ag and factor VIII:CAg were isolated from the group specific concentrates by the method of Tuddenham et al (1979) using immunoadsorbent chromatography. Briefly, sheep anti-VIIIR:Ag immunoglobulin was coupled to Sephacryl S-1000 (Pharmacia) and the gel was mixed with group specific concentrate for 18 hours at room temperature. The gel was then packed into a column, was washed sequentially with 300 ml of 15 mM citrate, 150 mM NaCl pH 7.2 and 300 ml of 50 mM imidazole/HCl pH 7.2, 0.01% Tween 80. VIII:CAg was then dissociated from the bound VIIIR:Ag by elution with 300 mM $CaCl_2$, 50 mM imidazole/HCl pH 7.2, 0.01% Tween 80, 0.1% turkey serum. VIIIR:Ag bound to the antibody was eluted 3 M KSCN in imidazole buffer. The peak protein-containing fractions were then dialysed against two changes of 5 litres of 50 mM Tris, 150 mM NaCl pH 7.2, 0.01% Tween 80. Pools of VIII:CAg- and VIIIR:Ag-containing fractions

FIGURE 7-4

PRINCIPLE OF IMMUNOADSORBTION OF BLOOD GROUP ASSOCIATED FACTOR VIII

Immobilised anti-A antibody was incubated with factor VIII solutions. Group A substance present binds to the antibody, along with any associated factor VIII. The antibody beads are then removed and the supernatants assayed for residual factor VIII.

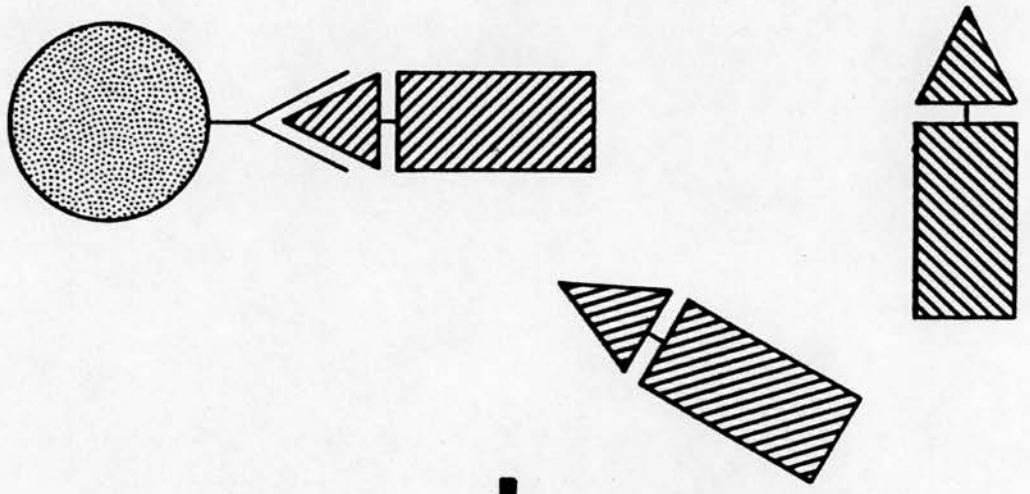
PRINCIPLE



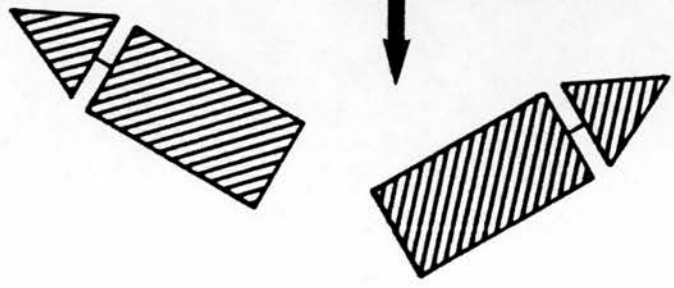
IMMOBILISED ANTIBODY

FACTOR VIII SOLUTION

INCUBATED AT ROOM TEMPERATURE WITH CONTINUOUS MIXING



BEADS SPUN OFF SUPERNATANTS COLLECTED



SUPERNATANTS ASSAYED FOR
FACTOR VIII R:AG
FACTOR VIII:CAG

were stored frozen for subsequent experiments.

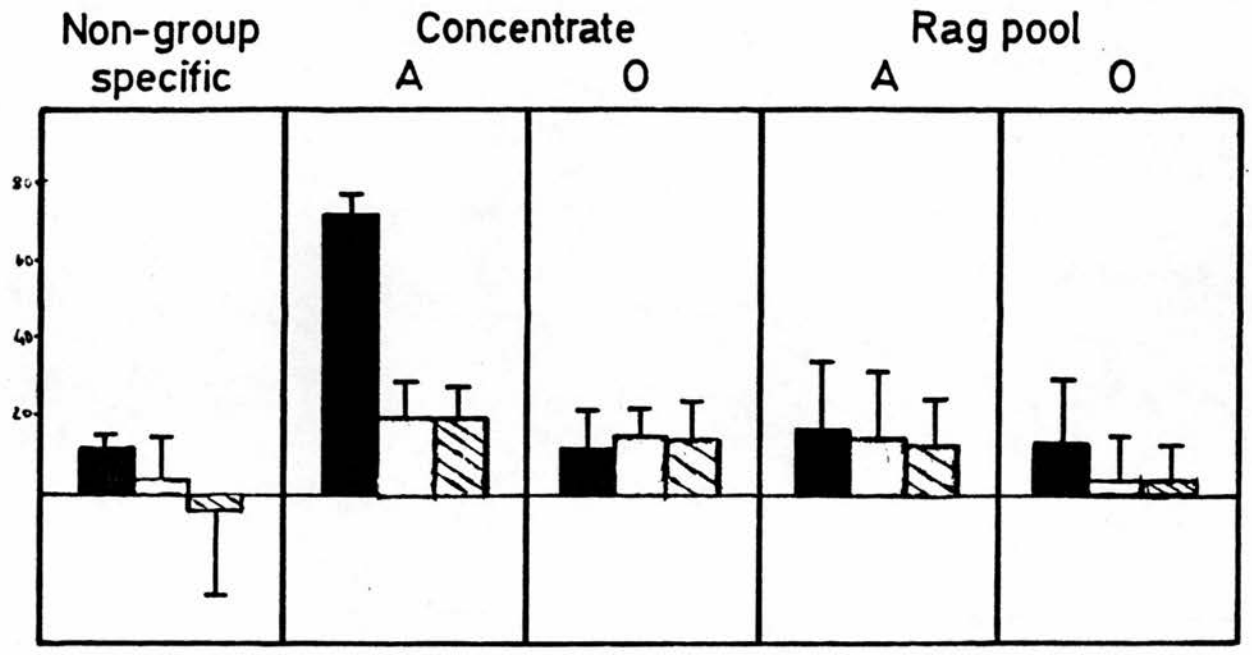
Reaction of factor VIII with immobilised antibodies:
The principle used is shown in Figure 7-4. Solutions of factor VIII were incubated with solid phased antibodies or buffer (50 mM Tris, 150 mM NaCl, pH 7.2). Incubations were performed at room temperature with continuous mixing. After one and four days of incubation, the beads were removed by centrifugation and the supernatants were collected and frozen for subsequent assay.

FIGURE 7-5

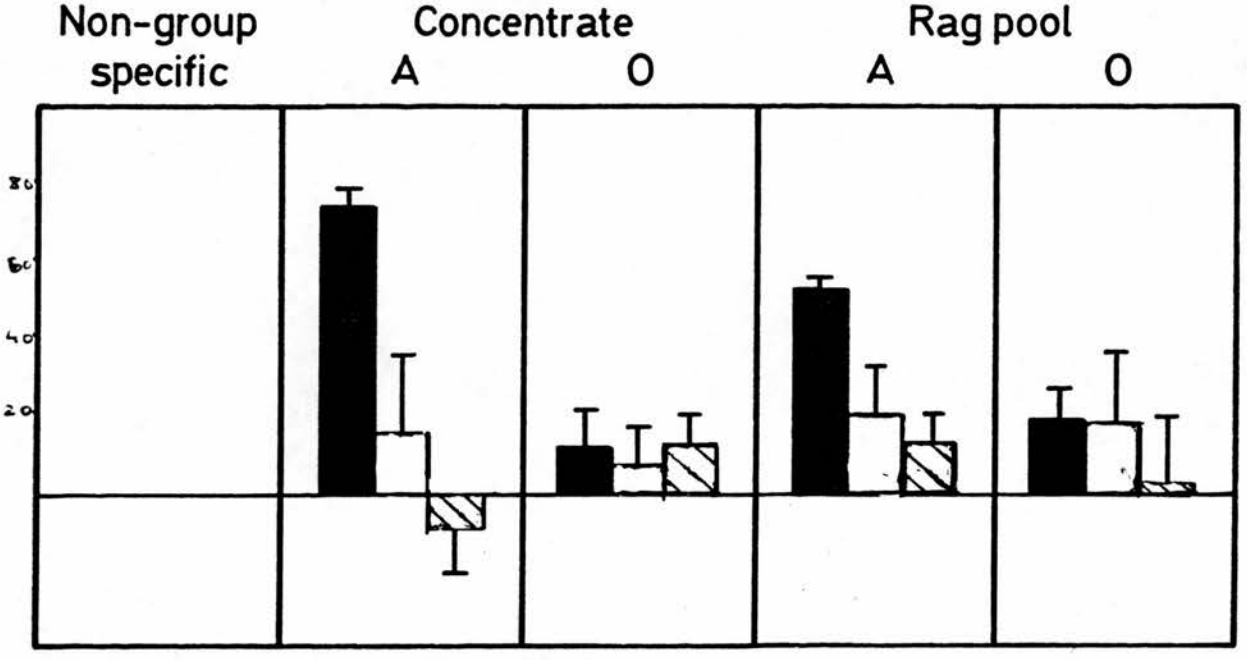
ADSORBTION OF FACTOR VIII BY IMMOBILISED ANTIBODIES
Results show the mean and standard deviations of 3 experiments. The percentage adsorbtion compared to the initial values was estimated by subtracting the residual activities from the starting activities.

RESULTS

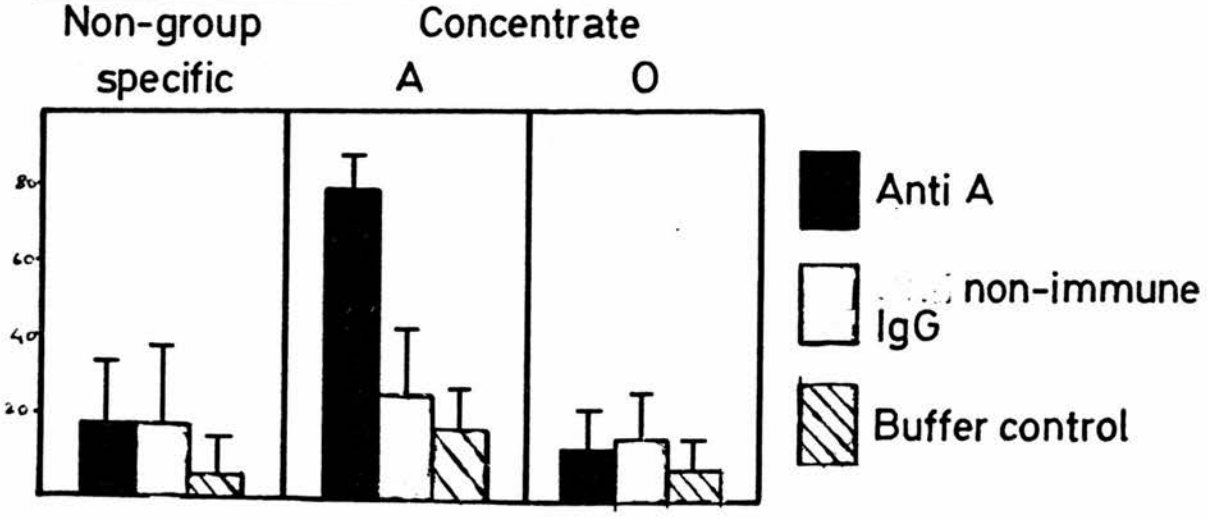
A. FVIIIR Ag - 1 DAY INCUBATION



B. FVIIIR Ag - 4 DAY INCUBATION



C. FVIII Cag - 1 DAY INCUBATION



RESULTS

Figure 7-5 summarises the results obtained.

A. GROUP A CONCENTRATE:

Anti-group A antibody adsorbed 75.2% of the VIIIR:Ag and 79.6% of the VIII:CAg from the group A concentrate, while non-immune rabbit IgG adsorbed 16.7% of the VIIIR:Ag and 25.9% of the VIII:CAg. The difference between incubations carried out with anti-A and non-immune IgG was significant, ($p < 0.001$) using an unpaired t-test, for both VIIIR:Ag and VIII:CAg.

B. GROUP O CONCENTRATE:

Anti-group A and non-immune IgG both adsorbed small amounts of VIIIR:Ag and VIII:CAg; the difference between the two antibodies were not significant.

C. NON-GROUP SPECIFIC CONCENTRATE:

Four day incubations resulted in clotting of the samples and thus no assays could be performed. Small amounts of VIIIR:Ag and VIII:CAg were adsorbed by both types of antibody after one day incubations; the difference between the two antibodies was not significant.

D. FACTOR VIIIR:Ag POOLS:

These were prepared from both types of group specific concentrates:

1. Group A pool.

   One day incubations produced adsorbtion of 17.2% by immobilised anti-A and 15% by the non-immune IgG compared to starting values; the difference was not

significant. Four day incubations led to adsorbtion of 54% by the anti-A and 22% by the non-immune IgG, a difference which was significant $p < 0.005$.

2. Group O pool.

For both incubation periods, the two antibodies adsorbed small amounts of VIIIR:Ag; the difference was not significant.

E. FACTOR VIII:CAg POOLS:

The VIII:CAg activity isolated proved very labile to freeze-thawing and no activity was measurable when the samples were assayed.

DISCUSSION

Previous studies on the association of blood group activity with factor VIII have relied on haem-agglutination inhibition techniques to demonstrate the association (Sodetz et al 1979, Samor et al 1982). Such an approach, however, will not distinguish between blood group substance associated with factor VIII or present as a contaminant in the factor VIII preparation. The use of immobilised specific antibody avoids this possibility, as any factor VIII removal must be through a close association with the blood group substance recognised by the antibody. It is acknowledged that this approach will not determine whether the association is covalent (Sodetz et al 1979) or is the result of adsorbtion of blood group substance to the factor VIII molecule during purification (Samor et al 1982). However, this study confirms that blood group A substance remains associated with the factor VIII molecule during the production of a high purity concentrate from group A plasma. Such plasma is known to have higher levels of factor VIII related activities than group O plasma. The reason for this is unknown at present, but could be related to a possible increased in vivo half-life for factor VIII due to incorporation of group A substance in the molecule. Confirmation of this hypothesis would require in vivo survival studies of factor VIII derived from group specific plasma into haemophilic patients.

Association of group A substance with

factor VIII might have been expected to result in some specific depletion from non-specific concentrate, which is made from plasma pools presumably containing a considerable number of group A donations. It is possible that this was not observed due to the lower purity of the non-specific concentrate, leading to blockage of the anti-A antibody with free group A substance in the preparation. The higher purification of the group specific concentrate would lead to removal of such un-associated blood group substance, allowing reaction of the antibody with blood group substance linked to factor VIII.

It has been suggested that group A plasma donations be preferentially used to derive cryoprecipitate, in order to increase the potency of this material (Tomasulo et al 1980). Such a practice, however, would be too restrictive. A more practical option would be to increase plasma levels of factor VIII using external stimuli, an aspect that is studied in Section C of this Chapter.

(C) RESPONSE OF FACTOR VIII TO DDAVP INFUSION, VENOUS OCCLUSION AND EXERCISE

INTRODUCTION

Factor VIII levels in blood are known to be increased by a number of external stimuli, including venous occlusion, physical exercise and infusion of hormonal agents such as vasopressin and its synthetic analogue 1-desaminocysteine (8-D-arginine) vasopressin (DDAVP) (Bloom 1977). DDAVP has been used clinically to treat patients with mild factor VIII deficiency, whose low factor VIII levels may be raised to levels that can support haemostasis by DDAVP infusion (Mannucci et al 1977). However, some concern has been expressed due to the anti-diuretic action of the analogue (Lowe et al 1977). DDAVP has also been used to raise VIII:C levels in blood donors prior to donation, the resulting high levels being recoverable in a concentrate and subsequently in vivo (Nilsson et al 1979). This approach has also been attempted in exploiting the increase in plasma VIII:C following exercise; cryoprecipitate produced from exercised donors however did not result in higher in vivo VIII:C levels when infused into haemophiliacs (Gastel et al 1973).

In this study, different factor VIII related activities were measured following DDAVP infusion, venous occlusion and exercise of male volunteers, with the aim of defining the factor VIII response in normal

individuals following these stimuli. The relative benefits of the 3 stimuli in raising factor VIII levels in blood donors can then be assessed.

METHODS

Studies were performed on 6 male volunteers, who were subjected to the 3 different stimuli as described by Prowse et al (1984b) (Appendix of this thesis). All assays were performed on frozen citrated plasma samples and were as described in Chapter II. The Laurell technique was used for measuring VIIIR:Ag.

FIGURE 7-6

CHANGES IN FACTOR VIII FOLLOWING DDAVP INFUSION

0.3 μg/kg was infused. Results are shown as mean ± standard deviation.

Significant ($p < 0.05$) increases are shown as filled symbols.

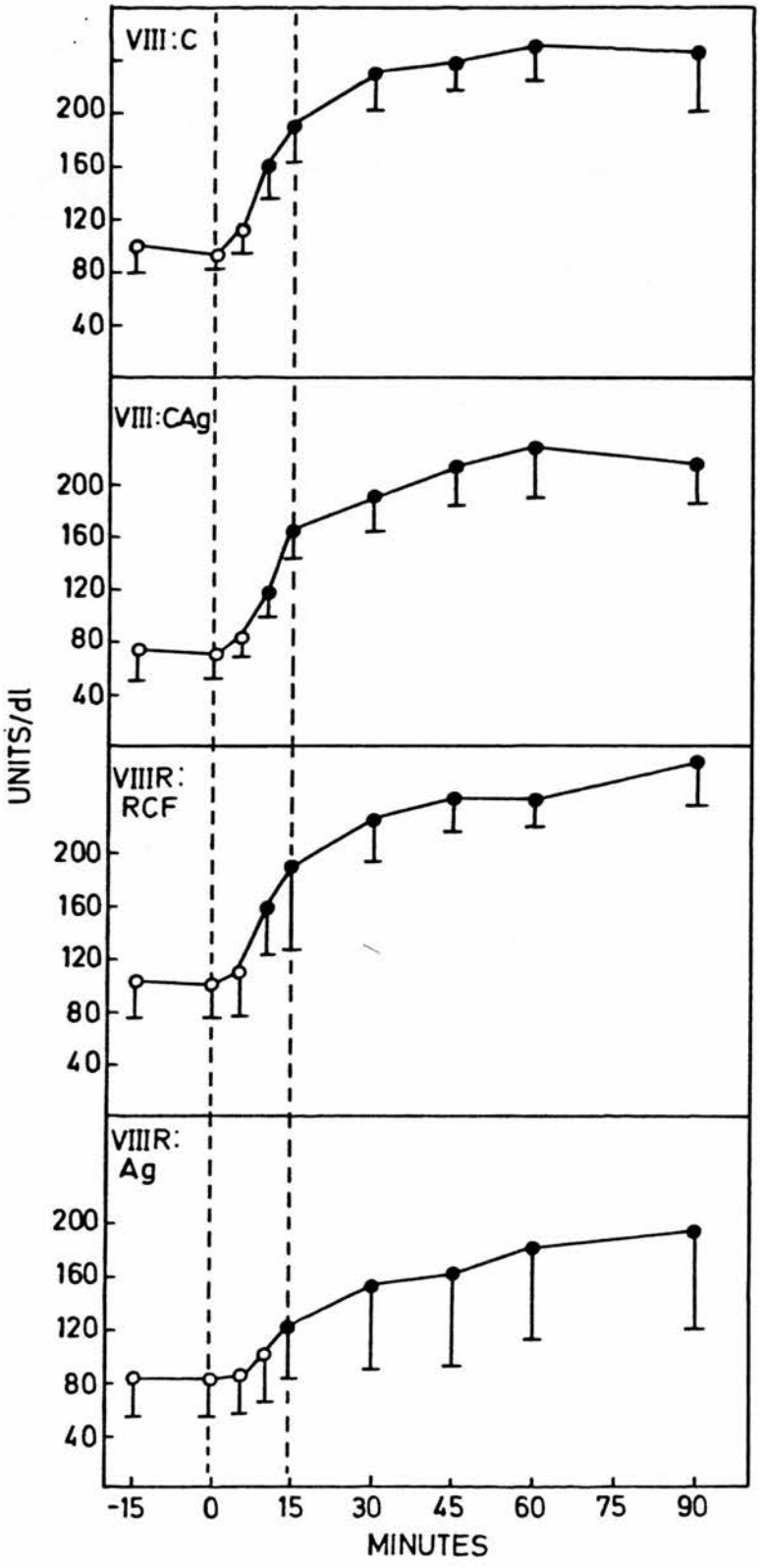
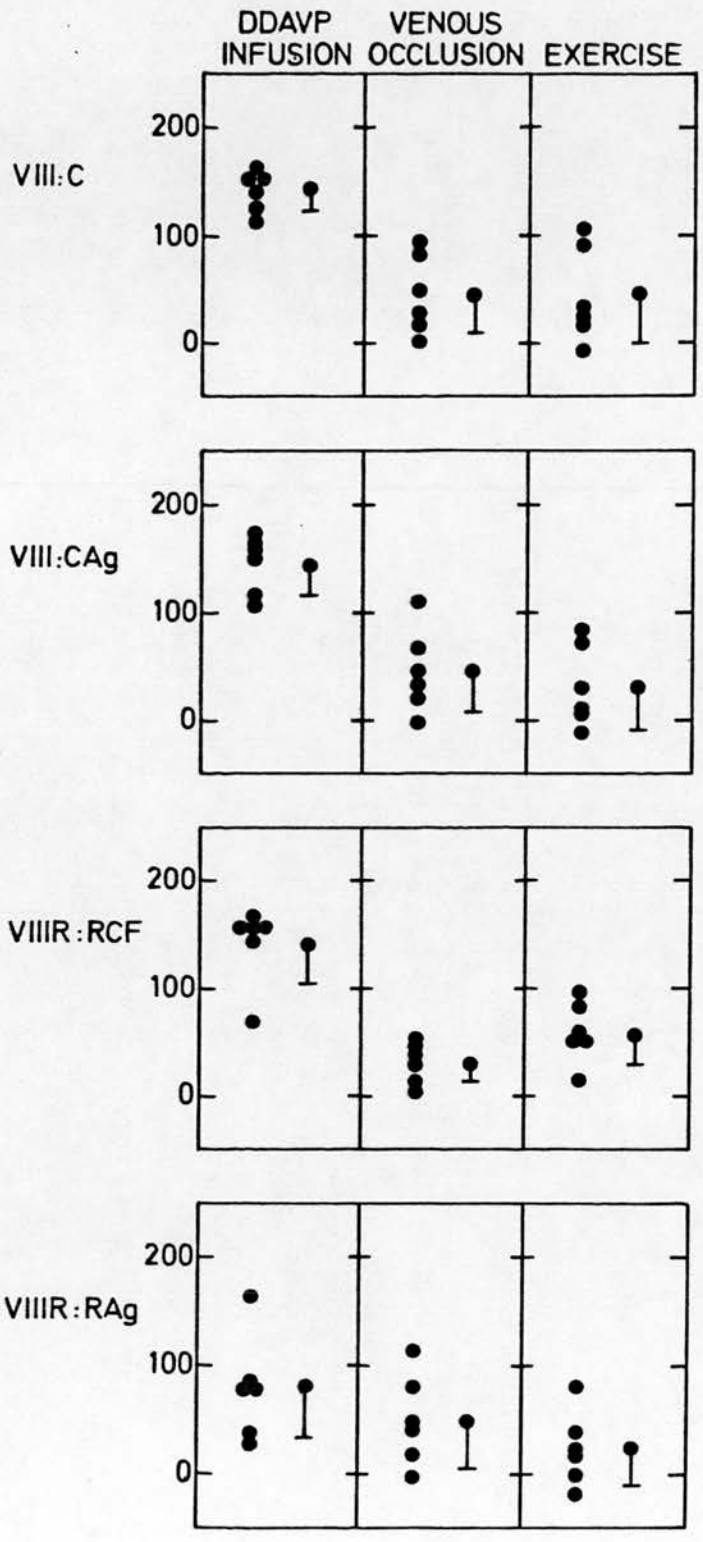

VIII:C
VIII:CAg
VIIIR:
RCF
VIIIR:
Ag
200
160
120
80
40
UNITS/dl
-15
0
15
30
45
60
75
90
MINUTES

FIGURE 7-7

INDIVIDUAL AND MEAN (± standard deviation) INCREASES IN THE VARIOUS FACTOR VIII ACTIVITIES 30 MINUTES AFTER DDAVP INFUSION AND IMMEDIATELY AFTER VENOUS OCCLUSION AND EXERCISE

Average basal levels of the activities were VIII:C 101, VIII:CAg 83, VIIIR:RCF 97 and VIIIR:Ag 98 u/dl.


DDAVP INFUSION
VENOUS OCCLUSION
EXERCISE
VIII:C
VIII:CAg
VIIIR:RCF
VIIIR:RAg
200
100
0

RESULTS

Figure 7-6 shows that during DDAVP infusion VIII:C rises significantly before VIIIR:Ag, although such differences were marginal. The rise of VIIIR:RCF following DDAVP infusion paralleled the increased in VIII:C more closely than that of VIIIR:Ag. Maximum levels of these activities did not occur until 30 minutes or more after infusion. All subjects showed at least a 2.3 fold increase in VIII:C 45 minutes after infusion (mean 2.63 fold; $p < 0.01$) and at least a 1.6 fold increase in VIIIR:Ag 75 minutes after DDAVP infusion (mean 2.49 fold; $p < 0.01$).

In contrast venous occlusion produced a lower response in both VIII:C (1.4 fold, $p < 0.05$) and VIIIR:Ag (1.48 fold; $p < 0.05$) and one subject gave no response at all. Similar results were obtained following exercise, although in this case the increase in VIIIR:Ag of 1.24 fold ($p < 0.05$) was less than that of VIII:C (1.47 fold; $p < 0.05$) and a different subject was a non-responder.

Figure 7-7 summarises the response of the different factor VIII related activities to all 3 stimuli tested. In each case, assay of VIII:CAg confirmed the results obtained by VIII:C measurement and in each case the rise in VIIIR:RCF was significant ($p < 0.05$).

DISCUSSION

The principle of administering an external agent to blood donors with the aim of increasing the procurement of a particular component is already established in blood transfusion practice. Thus, donors are injected with glucosteroids prior to leucapheresis in order to increase the blood level of granulocytes (French 1980). The immunisation of rhesus-negative volunteers with rhesus-positive cells to product anti-D immunoglobulin is also well known (O'Riordan 1973). The present study confirms that a similar principle can be used to increase the concentration of factor VIII in donor plasma. It was felt important to measure changes in all the different factor VIII related activities, in order to ensure that the VIII:C rises observed represented an actual increase in factor VIII, rather than a spurious increase through e.g. activation. It has been claimed that the VIII:C rise following exercise is a result of activation, possibly by thrombin (Kopitsky et al 1983) but the present study shows that following this and the other stimuli, VIII:CAg also increases, confirming an increase in the mass of this protein.

Of the 3 stimuli studied, DDAVP infusion produced the highest increase in plasma factor VIII related activities. It has been shown that the high factor VIII levels in plasma following DDAVP infusion can be recovered in low purity (Nilsson et al 1979) and high purity (Mikaelsson et al 1983b) factor VIII concentrates.

This high in vitro yield is reflected in the in vivo recovery of these products. In contrast, cryoprecipitate from exercised donors, while containing higher levels of factor VIII than normal cryoprecipitate, does not show the higher in vivo recoveries expected from the in vitro yield (Gastel et al 1973). It has been shown that intra-nasal administration of DDAVP can also increase factor VIII levels that are recoverable in concentrates (Mikaelsson et al 1982). Although the dosage required is an order of magnitude greater than the intra-venous dose, this might be a more practical way of increasing the factor VIII levels of plasma intended for concentrate production. Donors would have to inhale the analogue and then be bled 1-2 hours after, in order to allow a maximal rise in factor VIII levels. Good donor organisation would thus be required. Recently, another vasopressin analogue has been described which is claimed to increase factor VIII levels without the anti-diuretic effect of DDAVP (Cort et al 1981). If this effect can be confirmed in blood donors, it might be more acceptable in transfusion practice to use this agent as a means of increasing the factor VIII content of blood donations. It is suggested that administration of such substances, if found acceptable to donors, is a valuable way of improving factor VIII procurement.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

(A) INTRODUCTION

It is expected that the near future will see factor VIII concentrate overtaking albumin products as the driving force for plasma procurement in industrialised nations (Curling 1982). This ever increasing demand for factor VIII has resulted in local production in blood banks remaining a crucial factor in the overall supply. This is because such materials, mainly in the form of 'wet' cryoprecipitate, produce a much higher yield of factor VIII than concentrates from fractionation centres. This situation is even more accentuated in countries which depend totally on this form of production due to lack of fractionation facilities. Great efforts are continuously being made to increase the supply of plasma delivered to fractionation centres in a form that is suitable for factor VIII production (i.e. fresh frozen plasma) in order to phase out such local, non-standardised products. The recent introduction of a dedicated plastic pack for this specific purpose is a case in mind (Lane 1981). However, so long as yields by present day fractionation techniques remain below 300 u/kg of starting plasma, a voluntary blood donor system which produces 'recovered' plasma is unlikely to supply all the material needed. The question thus arises whether plasma needs to be treated in a special way for factor VIII production and whether small-scale, local and regional production of high yielding products can substitute for the low yielding large scale production of conventional fractionation.

The question of yield relating to plasma supply and quality, while ever predominant in state-backed voluntary blood programmes, is less relevant for commercial manufacturers of plasma fractions. In these concerns, "Source Plasma" from paid plasmapheresis donors is the raw material, in contrast to the "Recovered Plasma" which forms the bulk of the supply for national blood services. Product presentation and attractiveness to patients and clinicians are more important than yield, as the plasma source can be increased by buying more material and passing on the price to the consumer. Recently, it has also been shown that such high-potency concentrates may also be pasteurised, thus having the added attraction of a potentially reduced active virus content. It is not the purpose of this study to enter into a discussion of the ethics involved in the commercialisation of blood donation. However, the ever increasing price of these materials alone may be sufficient grounds to justify a programme of self-sufficiency. The limitation of the plasma supply to a pool of dedicated voluntary blood donors, with a fully documented and satisfactory medical history, also increases the safety of the product, compared to materials derived from large numbers of a continuously changing paid donor panel.

It has been the aim of this study to explore ways in which local or regional blood banks can generate enough factor VIII for self-sufficiency while retaining the product characteristics currently only obtained in

large-scale production. The results achieved in this study will be discussed in relation to work done by others in this area.

(B) IMPROVEMENT OF THE SUPPLY AND QUALITY OF PLASMA AS A RAW MATERIAL

Increasing the number of blood donors will obviously increase the potential supply of plasma available for factor VIII production. It has been estimated that an annual rate of 50,000 donations per million population is required to cover the needs for blood transfusion in an industrialised nation (Hässig and Lundsgaard-Hansen 1978). The same study reveals that proper management of a blood component programme should result in the generation of sufficient plasma to satisfy factor VIII needs in the form of intermediate-purity concentrate. In developing countries with a less sophisticated health service, the level of blood donation/requirement is much less, but can be expected to rise as general health care is improved. Achievement of the level of donation described above, coupled with the use of an 80% component programme, should lead to a balanced level of self-sufficiency with regards to factor VIII and red cells. However, recent developments, particularly progress in sterilising factor VIII concentrates, lead to decreased process yields and make it more difficult to achieve the necessary plasma input through a standard blood donation service. In this regard, rather than increasing the number of blood donors (and thus wasting red cells) several options

are possible:

1. Plasmapheresis of donors with the specific aim of increasing the amount of factor VIII produced by blood banks has been described (Andronescu et al 1983). This approach has also been used to procure plasma destined for large-scale fractionation - currently, such plasma costs about £46 per kg to produce (Robinson et al 1983). Although expensive, this option allows measures which cannot be achieved easily in whole blood collection e.g. use of different anticoagulants to improve VIII:C stability (Chapter III). However, it must be emphasised that procuring sufficient quantities of whole blood should be the first priority for a developing blood transfusion service.
2. Use of red cell concentrates instead of whole blood releases considerable amounts of plasma suitable for factor VIII production. The Swiss experience (Hässig and Lundsgaard-Hansen 1978) has shown that it is possible to issue up to 80% of blood requests in the form of red cell concentrates. An essential feature of this programme is the acceptance of the policy by clinicians and a judicious use of plasma substitutes as volume expanders (Mellstrand 1983). The preparation of such substitutes should thus be included in any programme aiming for self-sufficiency in plasma fractions.
3. Increasing the volume of plasma per blood donation.

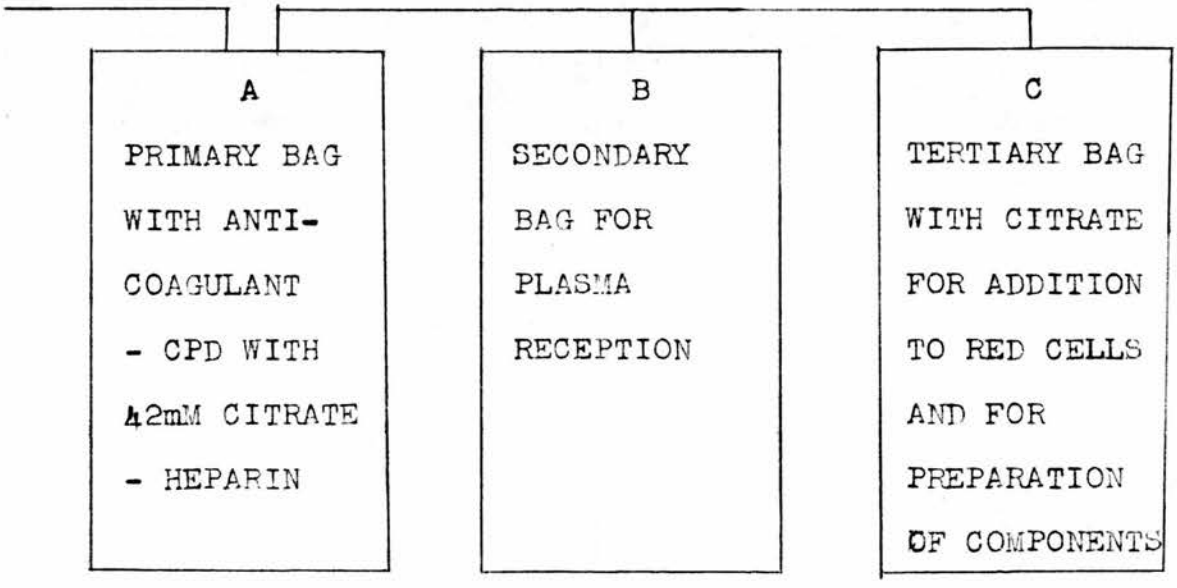
Use of the red cell additive SAGM (saline, adenine, glucose, mannitol) has allowed the volume of plasma obtainable from a 450 ml blood donation to be increased from 220 mls to 280 mls (Högmann et al 1983). The optimal additive system has now been incorporated into the standard multiple plastic bag systems for whole blood collection. The increase in cost relative to the old single or double bags is partially offset by the increased volume of plasma that can be harvested.

4. Increasing the VIII:C content of plasma destined for factor VIII production. It has been suggested that the long known association of blood group A with increased factor VIII levels (Chapter VIIB) be used in selecting plasma donations for cryoprecipitate production (Tomasulo et al 1980). This practice, however, must be considered unnecessarily restrictive. Treatment of donors to increase factor VIII levels, such as administration of the vasopressin analogue DDAVP (Chapter VIIC) is a more promising option and has been used successfully (Nilsson et al 1979, Mikaelsson et al 1982). Combining this option with a plasmapheresis programme on a relatively small number of dedicated donors could well reduce the necessary donor panel for factor VIII procurement to a few hundred donors. The benefits of this are numerous, particularly in the patient-safety aspect mentioned above.

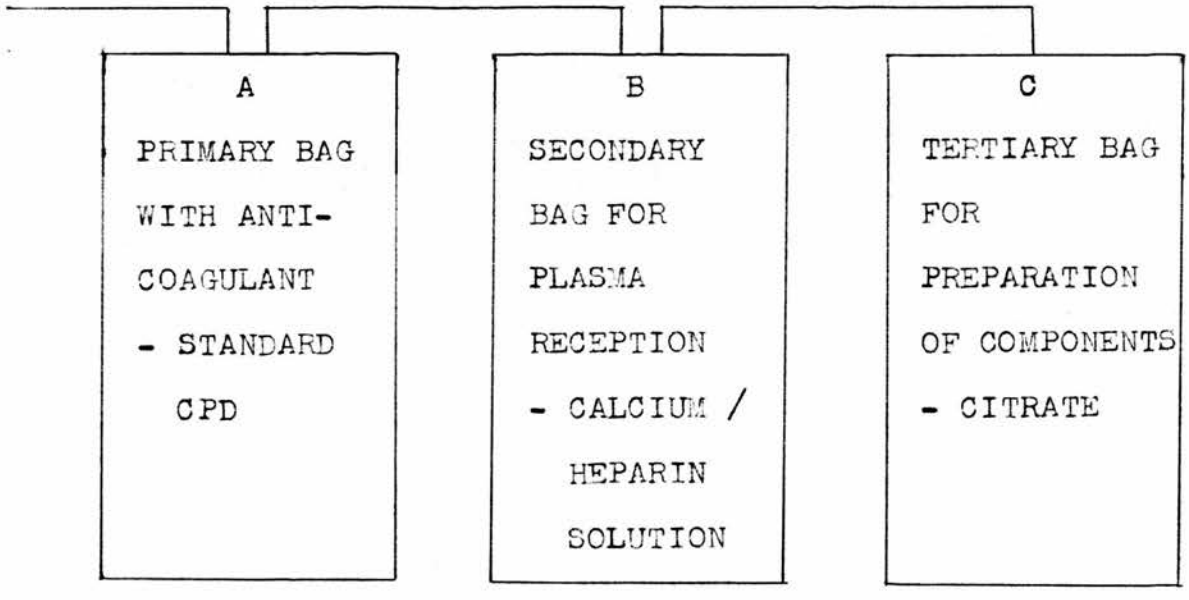
5. Improving plasma VIII:C stability. The lability of

FIGURE 8 - I : PLASTIC BAG SYSTEMS FOR OPTIMAL PRESERVATION OF DONATED FACTOR VIII

A.



B.



VIII:C in blood donations has resulted in many studies designed to improve VIII:C stability so that more plasma can be processed to factor VIII concentrate. On a blood bank scale recent attention has focused on the stability of VIII:C at physiological ionised calcium levels, achieved either by collection of blood in heparin or by recalcifying standard donations. The results of this study and others published recently indicate that a significant stabilisation of VIII:C in the crucial first few hours after donation is attainable by modification of the standard anti-coagulants so as to result in higher ionised calcium levels (Chapter III, Krachmalnicoff and Thomas 1983, Mikaelsson et al 1983a, Rock et al 1983b). The present day anticoagulant formulations have been based mainly on considerations of red cell viability and safety upon transfusion. Given the growing demand for factor VIII, an anticoagulant composition which is more compatible with VIII:C stability and which does not interfere with other components would be attractive. Two possible options, based on the results of the present study, for exploiting the calcium-induced stability of VIII:C by modifications of present day anticoagulants/multiple bag systems are shown in Figure 8-1.

a) Collection into anticoagulants of lower citrate concentration: In this approach, the standard CPD formulation would be altered so as to result in a

final plasma citrate of 10 mM, rather than the 25 mM attained by the usual anticoagulant. This citrate concentration would allow VIII:C stability to be preserved (Chapter III). In order to maintain anti-coagulation, this 'low-citrate CPD' could if necessary be supplemented with heparin, and if required additional citrate could be added to the red cells after plasma separation. A possible plastic bag system for this option is shown in Figure 8-1A. Although provision is made for citrate supplementation of red cells following plasma removal, further research is needed to determine whether 10 mM citrate is adequate to preserve red cell viability. Mishler et al (1978) have previously shown that a concentration of 14 mM citrate is sufficient for this purpose.

b) Recovery of plasma VIII:C by recalcification: Recalcification to 10 mM calcium within 4 to 6 hours following donation results in recovery of plasma VIII:C, which is stable for up to 18 hours (Chapter III). Separation of plasma can thus be delayed until transportation to the regional centre. Plasma can then be separated and expressed into a calcium/heparin solution. After allowing VIII:C to recover to higher levels (2 hours - Chapter III), plasma can be processed to concentrate the factor VIII. This option can also be carried out within an enclosed plastic bag system - Figure 8-1B. In this system,

additional citrate is not necessary for the red cells as collection is effected in standard CPD anti-coagulant. A tertiary pack with citrate is included for receiving residual plasma after factor VIII extraction, to allow continued anticoagulation after the heparin has degraded.

Both these approaches might result in heparin being present in the plasma being processed. The fractionation of plasma derived from heparin donations (about 12 u/ml of heparin in the plasma) has been shown in this study to result in processing difficulties. Smith (qu. Penny 1983; personal communication) has shown similar difficulties in processing such plasma to intermediate-purity concentrate. Further studies are necessary to determine whether the lower amounts of heparin resulting from the above options would result in similar difficulties.

It is suggested that the necessity for preserving VIII:C until plasma separation/processing can take place justifies the use of potential new collection systems as outlined in Figure 8-1. A similar system has already been commissioned by a Dutch blood bank for producing cryoprecipitate from heparin donations (Smit-Sibinga et al 1983). Further bag modifications would allow for subsequent extraction of the factor VIII (Figure 8-2). Stabilisation of VIII:C until regionally-collected donations can be processed at the main centre will have marked effects

on the amount of therapeutic material that can be derived. It can be estimated that an extra 300 units/kg of plasma may be obtained in plasma processed 18 hours after donation, an increase over present procedures of 50%.

(C) PROCESSING OF PLASMA TO BLOOD BANK FACTOR VIII CONCENTRATES

Cryoprecipitate:

The characteristics of blood bank cryo have been discussed (Chapters I, IV and V). Despite its inconvenience from the point of view of storage and administration, the material still enjoys widespread use, primarily because of the ease of preparation and the relatively high factor VIII yield which is obtained. Efforts to improve its quality have therefore continued and include the following:

1. Lyophilisation of small (5 to 20) pools of single-donation cryos by pooling aseptically into a stabilising buffer and then freeze-drying (Milligan et al 1981). The product thus prepared can be stored at 4°C instead of having to be deep-frozen. Because of the low purity, however, the potency is only about 5 u/ml when reconstituted, and syringe infusion is thus not possible.
2. Plasma additives have been proposed as a way of increasing yield. The present study does not support claims that PEG (Johnson et al 1979) or heparin

(Rock et al 1980b) increase factor VIII cryoprecipitability (Chapter V). It has been shown that the physical separation of cryoprecipitate is more important in this regard, fast centrifugation being necessary (Chapter V, Foster 1983b). Practical difficulties in centrifuging bags of frozen plasma might make transfer into bottles for centrifugation a better option, but this would involve loss of sterility.

3. Thaw-siphoning as introduced by Mason (1978) undoubtedly improves markedly the yield of the preparation, and if combined with a terminal centrifugation step can also greatly enhance the purity (Kang 1980, Chapter V). As has been found in this study, however, plasma freezing has to be rapid to effect maximum yield, a feature that does not seem as crucial when using standard thawing techniques (Rock and Tittley 1979). Care is required in storage of frozen plasma prior to processing, as temperature fluctuations result in increased fibrinogen deposition in the cryo, making subsequent processing difficult (Chapter IV).

4. Double precipitation of heparin cryoprecipitate has aroused much interest as a means of increasing the purity of blood bank cryo at high yield (Lane et al 1983). A variant of the technique employing a closed system throughout has been successfully tested in vivo (Smit-Sibinga et al 1984). Heparin collection has

been claimed to a) increase factor VIII cryo yields and b) permit utilisation of a second cold precipitation with a resultant increase in purity. The present study has examined in detail the behaviour of factor VIII in the cold in various components (plasma and cryoprecipitate, both in heparin and CPD anticoagulants) and can find no evidence of a specific heparin effect (Chapters III and VI). The increase in specific activity which is obtained upon chilling blood bank cryoprecipitate (irrespective of plasma anticoagulant) might be of some advantage, but this study shows that the final preparation has a fibrinogen content that precludes sterilisation by heat-treatment.

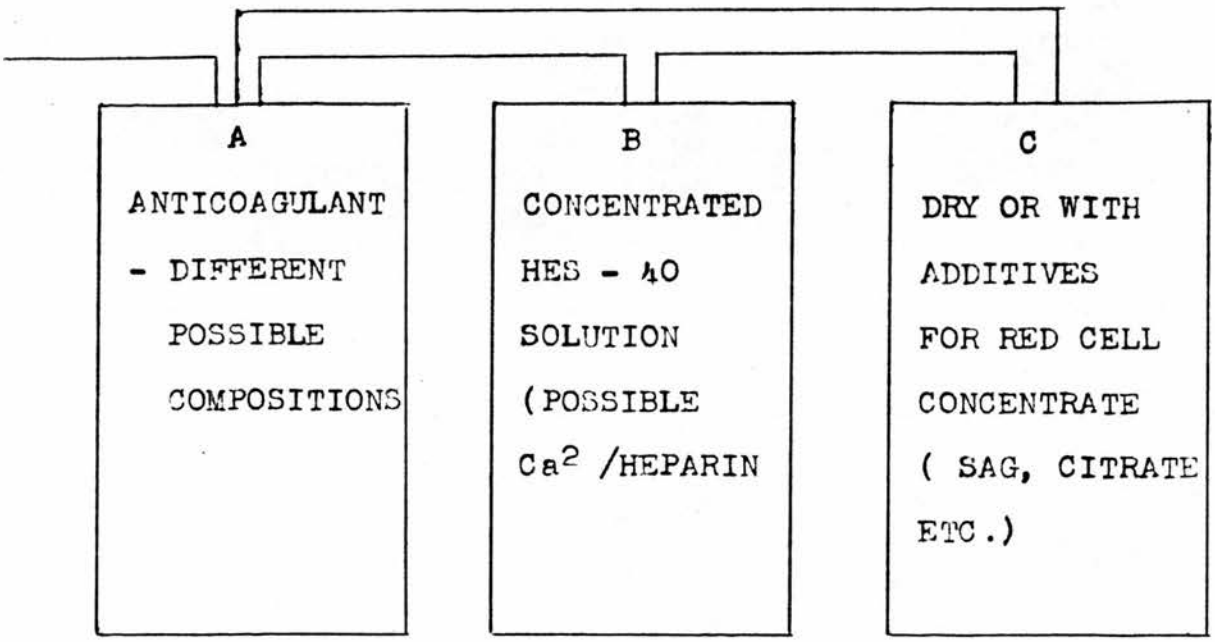
Alternatives to cryoprecipitate:

A large number of variables can influence the freezing and thawing of plasma, and thus the final cryo yield. Strict attention to every stage of the process is thus essential, requiring a high level of technical skill that might not be possible in a developing blood transfusion service. The processing of unfrozen plasma would avoid some of the variables affecting cryo yields. Rapid processing or VIII:C stabilisation would allow the use of liquid plasma as a raw material. This study has therefore investigated alternatives to cryoprecipitation as a means of concentrating factor VIII (Chapter V). Two options are suggested from the results:

1. Polymer induced cold precipitation. Chilling plasma

FIGURE 8 - 2 : PLASTIC BAG SYSTEMS FOR OPTIMAL HARVESTING OF BLOOD COMPONENTS

A.



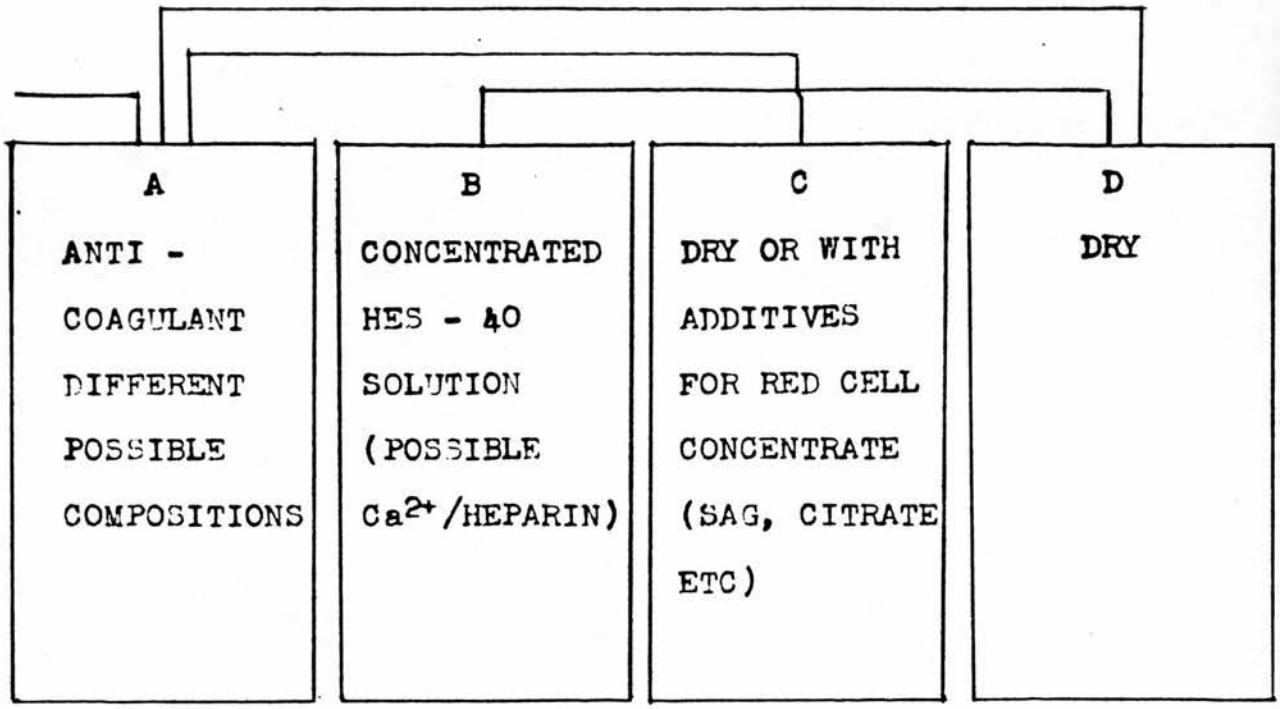
Scheme I - Triple bag system

Blood is collected into A, platelet-poor plasma is expressed into B, (red cell additives are expressed from C to A), the plasma is chilled and the factor VIII rich precipitate is harvested and the factor VIII-poor plasma is expressed from B to C.

Products: red cell concentrate (anaemia)
factor VIII-poor plasma (plasma expansion eg: shock)
factor VIII HES-precipitate (further processing)

FIGURE 8 - 2 Cont:

B.



Scheme II - Quadruple bag system

Blood is collected into A, platelet-rich plasma is expressed into D, (red cell additives are expressed from C into A), platelet concentrate is harvested in D, platelet-poor plasma is expressed into B, the plasma is chilled to produce the factor VIII rich precipitate and the factor VIII-poor plasma is expressed into C.

Products: red cell concentrate (anaemia)
factor VIII-poor plasma (shock)
factor VIII HES-precipitate (further processing)
platelet concentrate (thrombocytopenia)

Use of the additional bag allows harvesting of platelet-concentrate, but the volume of plasma available for Factor VIII production is less than for Scheme I as (1) production of platelet rich plasma does not allow hard centrifugation in

order to remove the maximum volume of plasma in the SAG procedure, (ii) a volume of about 40mls of plasma has to be left with the platelet concentrate.

in the presence of hydrophilic polymers results in precipitation of factor VIII and other proteins (Chapter V, Casillas and Simonetti 1982). By selecting the appropriate conditions, a selective concentration of factor VIII is achieved. Yield and purity of the resultant fraction are better than normal cryoprecipitate. The precipitate obtained by HES 40 precipitation has been further treated to obtain a freeze-dried concentrate (Chapter V). It is suggested that this simple technique can provide a superior alternative to cryoprecipitation as a means of concentrating factor VIII. Processing of in-house donations immediately after collection allows precipitation of the plasma to give a factor VIII rich fraction in less than 3 hours. A similar concentrate using PVP as a plasma precipitant has been shown to give good in vivo results upon infusion to haemophilic patients (Casillas et al 1983). This study suggests that HES is a preferable reagent in this regard, both in terms of the results obtained in plasma precipitation and the clinical acceptability. Figure 8-2 proposes a scheme for component production in a blood bank, incorporating polymer-precipitation of factor VIII and allowing other blood components to be harvested. The plastic bag system shown is based on the circular multiple bag configuration described by Lovric (1982) which is already available. Included are potential anticoagulant combinations in order to

preserve VIII:C as well as red cell additives such as SAGM, to increase the volume of plasma that can be harvested.

2. Plasma concentration and cold precipitation. Amongst other polymers, albumin is an effective selective precipitant of factor VIII (Chapter V). The same result can be achieved by concentrating the total plasma protein by water removal and chilling the concentrated plasma obtained. This process can easily be done in a blood bank using equipment which can rapidly concentrate litre volumes of plasma. A hollow fibre system capable of this task can be expected to cost about £10,000. The procedure would obviously involve abandoning the closed plastic bag system and thus any final products derived would have to be rendered sterile by filtration at the end of processing. The concentrated residual plasma would require treatment to allow its use as a volume expander. It is possible that adequate sterility precautions would allow production of uncontaminated products, although this method presents greater practical difficulties than polymer-induced precipitation. It is suggested that the method might be more applicable as a preliminary step in full-scale plasma fractionation, rather than in a programme for component production, as the final products derived can then all be subjected to sterile filtration as part of the fractionation procedure.

(D) FURTHER PROCESSING OF BLOOD BANK CONCENTRATES

1. Scale of operation. Single donation products such as 'wet' cryoprecipitate, suffer from the inherent disadvantage of lack of standardisation. The requirement of full-scale pharmaceutical quality control gives freeze-dried concentrates an advantage in this respect. Concentrates may be dispensed in multiple vial lots from a single resevoir of pooled material. If care is taken to ensure batch homogeneity, single vials from each pool can be assumed to be representative, and the characteristics of a batch can be defined by tests on individual vials. A proportion of each batch would thus be used for quality assurance. This factor argues against products such as small-pool lyophilised cryoprecipitate, as a large proportion of the product would have to be sacrificed for quality control. Although it has been argued that a restriction of pool size decreases the risk of viral exposure (Gabra et al 1982) this is likely to be true only for patients who are treated very infrequently. While pooling in industrial fractionation is done at the stage of preliminary extraction of factor VIII from plasma, blood bank procedures can effect pooling at the later stage when the preliminary concentrate is processed to the final solution. The high yield of the HES 40 precipitation technique developed in this study means that pooling on a 100-200 donation scale would produce an adequate

TABLE 8-1 MEASURES TO ENSURE STERILITY IN BLOOD BANK CONCENTRATE

Process in sterile room (filtered air, 4°C) with laminar flow cabinets (Hanratty 1983).

Sterilise all vessels and buffers used in processing (Gabra 1980).

Filter final solution through appropriate filters (0.22 µm) (Margolis and Rhoades 1981).

Lyophilise using sterile dryer (Myllylä 1983).

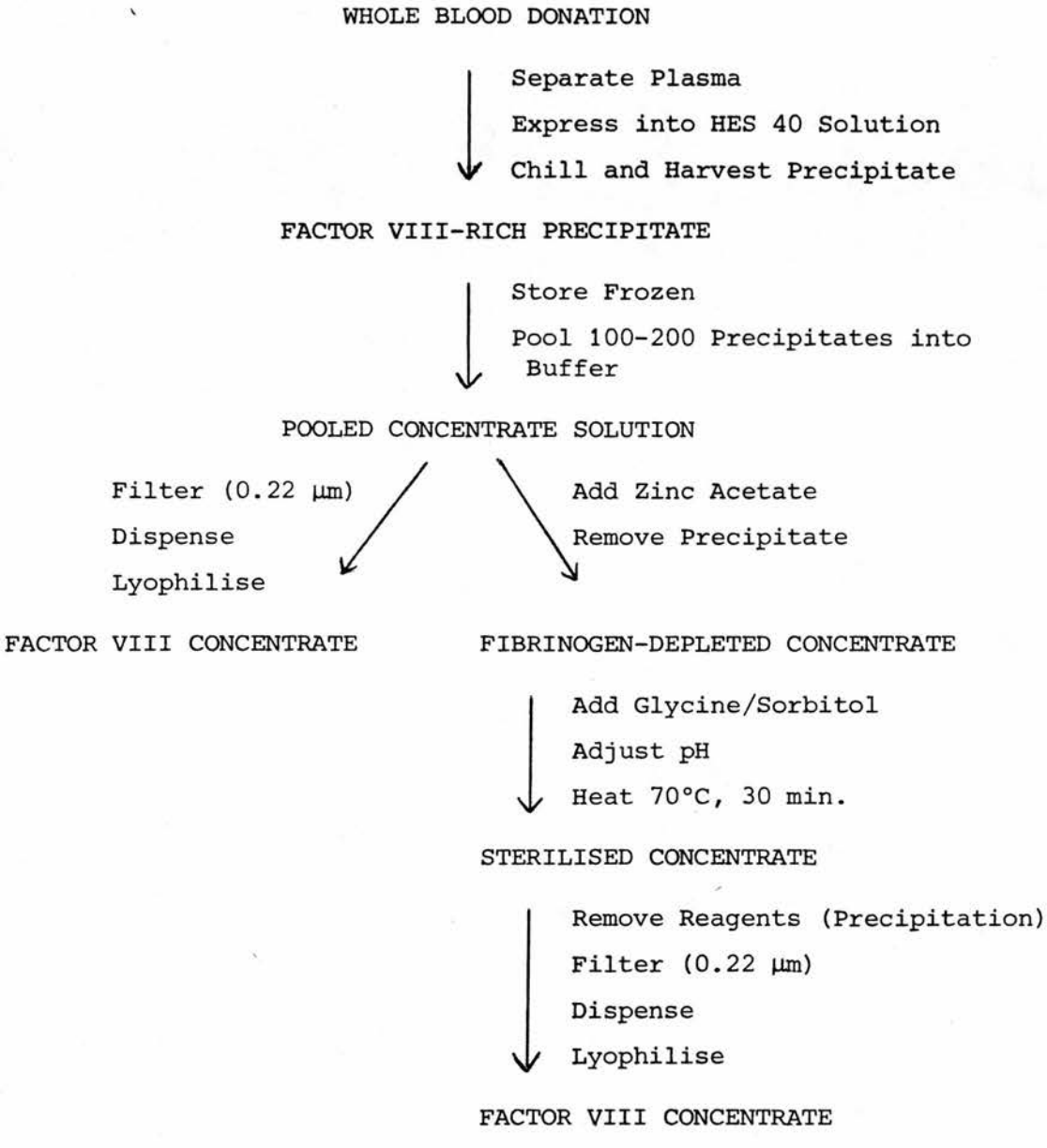
Quality assurance on final product - tests for sterility, toxicity and pyrogenicity (WHO, 1978).

batch size and enable the necessary tests to be done on representative samples. As described above, production to the preliminary concentrate stage can be done within a closed system. Further processing requires the use of an open system and additional measures are necessary to ensure sterility, Table 8-1 summarises these. Although these precautions were not taken during the present study, as the products were not destined for patient use, the HES 40 concentrate described in Chapter V could easily be produced using these precautions. In particular, sterile filtration should be possible due to the low fibrinogen content. In this regard, it is worth noting that the double cold precipitate obtained from heparinised plasma cannot be sterile-filtered (Hanratty 1982, personal communication) presumably due to its high level of fibrinogen (Chapter VI). Additional costs are obviously accrued by taking these measures. These would be proportional to the scale of the operation - Myllylä (1983) reports a cost of £80,000 per sterile dryer in the Finnish Red Cross Blood Transfusion Service plant, but these machines had to dry a whole range of fractions in addition to factor VIII concentrate. Precise prior costing for a blood bank starting a concentrate-producing programme is difficult, particularly when the equipment has to be imported. Against these costs has to be taken into account the consequence of a reliance on imported

material.

2. Further purification. The main reason for further purification is fibrinogen depletion with the aim of sterilising the material (Chapters VI and VIIA). Further purification on a blood bank scale has seldom been attempted, probably due to the low yields obtained using classical precipitation techniques. A chromatographic approach has been used successfully in blood banks in Australia and New Zealand, in which cryoprecipitate was produced from single donations, pooled and purified by passage through a column of controlled pore glass (CPG) (Margolis and Rhoades 1981, Woodfield et al 1983). The resulting fibrinogen-depleted material was obtained at a yield of 90% of the VIII:C relative to cryoprecipitate. From the results of the present study (Chapter VI) it is clear that separation methods based on molecular exclusion and cold precipitation, while very successful on plasma, are much less promising when further purifying concentrates. Selective removal of fibrinogen however can be rapidly achieved by glycine or zinc ion precipitation (Chapter VI, Blombäck and Thorell 1982, Foster et al 1983a). Of these options, zinc ion precipitation is to be preferred as glycine precipitation would require dilution of the factor VIII-rich fraction prior to heat treatment. Zinc ions in the factor VIII-rich fraction can be removed by PEG precipitation (Foster 1983, personal communication), but this is not

FIGURE 8-3 PRODUCTION OF A BLOOD BANK FACTOR VIII CONCENTRATE



necessary as subsequent heat-treatment would remove zinc in the reagent-removal operation (Chapter VII). Figure 8-3 describes the various stages for production of a factor VIII concentrate as described in this Thesis (Chapters V, VI and VII).

3. Product safety - sterilisation. No fractionation process can totally eliminate the risk of viral infection associated with products derived from human plasma. Heat-treatment (Chapter VII) appears to be the best option at present available, being a general viral inactivation method rather than a specific approach such as immuno-adsorption or lipid extraction. The data reproduced in Chapter VII (Foster et al 1983c) indicates that a range of viruses are inactivated using this technique. A heat-sterilised concentrate can therefore be expected to decrease significantly the risk of infection. Further measures in this regard include the restriction and characterisation of the donor pool as discussed above, the introduction of sensitive screening assays for viral markers and the holding of frozen plasma in quarantine to allow follow-up of donors (Lane 1981, Smith 1983a). This last option cannot be applied for processes, such as those described in this Thesis, relying on liquid plasma, and also assumes a level of organisation that is difficult to achieve in a developing blood transfusion service. Further research in viral screening/inactivation is necessary

as long as human plasma remains the raw material for factor VIII products.

(E) BLOOD TRANSFUSION AND FACTOR VIII IN DEVELOPING COUNTRIES

The main theme of this study has been the procurement of factor VIII in countries without access to modern technology. A recent forum recommended the following order of priorities for transfusion services in such countries:

(a) Provision of whole blood

(b) Provision of components

(c) Plasma fractionation (UNIDO 1982)

The adequate provision of whole blood for general hospital use is obviously the first priority of a transfusion service. The estimation of 50,000 donations annually per million inhabitants mentioned above is based on the level of hospital care in a major industrial nation and includes services such as cardiovascular surgery, transplants etc., which are not provided in developing countries. It can be expected that the development of the health service will include provision of some of these services, although not to the extent found in industrial nations. As an adequate blood donation rate is achieved, plasma will become available, through judicious use of red cell concentrates, for producing factor VIII. Staging the development of a blood transfusion service as recommended above would involve, using present day methods, the production of single donation frozen cryoprecipitate.

The unsuitability of this product as far as administration and storage are concerned, however, precludes its use as a substitute for imported concentrates. Cryoprecipitate production is a satisfactory first step in a situation where treatment was non-existent or was limited to whole blood or plasma transfusion. The product, however, would be unacceptable to a haemophiliac population used to adequate home-therapy with imported concentrates provided by the state health service. In this situation, which exists for example in the author's country, development of the transfusion service as far as factor VIII production goes must be geared to the production of concentrates with the same basic characteristics as the imported materials.

Use of the appropriate technology, as described in this study, should allow the provision of factor VIII concentrate in the required amounts. It has been estimated that the amount of factor VIII needed for haemophilia care in a population is 1-2 international units per capita per annum (Smit-Sibinga 1983). Assuming that a total blood intake of 30,000 donations per million population is achieved to cover red cell needs, then an 80% component programme will produce about 5,000 litres of plasma, although a proportion of this would be required for clinical use as fresh frozen plasma and platelet concentrate.

Rapid processing or VIII:C stabilisation will allow liquid plasma to be processed for factor VIII

production. Processing of 4,500 litres of fresh plasma by the HES 40 precipitation technique can be expected to yield about 2½ million units of factor VIII concentrate, which are adequate to cover needs. Processing by thaw-siphon cryoprecipitation can be expected to produce the same amounts, with the disadvantages associated with this non-standardised product. This, albeit simplistic, estimation does not take into account certain factors, e.g. loss of yield by sterilisation, inability to achieve the required level of red cell usage, which would decrease the amount of factor VIII produced. However, the additional options discussed, such as increasing the volume of plasma available per donation through the use of SAGM, pharmacological stimulation of donors etc., can all contribute towards offsetting these losses.

The technical problems of producing on a blood bank scale as opposed to the laboratory scale experiments conducted in this study are considerable. However, it is believed that the advantages of blood bank production of factor VIII concentrate can allow developing countries to attain self-sufficiency in this product within a comprehensive programme for the development of the blood transfusion service. The necessary capital expenditure is not excessive, considering the present cost of imported concentrates, and can be partly offset by aid programmes for this purpose (UNIDO 1982).

(F) CONCLUSIONS

This study has focused on optimising the procurement of factor VIII from donated blood. Other plasma fractions, at present available commercially through industrial fractionation, can also be derived in blood banks using the appropriate technology. The use of chromatographic techniques has already enabled the two main plasma fractions - albumin and immunoglobulin - to be prepared in regional or local blood banks (Curling 1983). Although the clinical safety of such products requires further verification, particularly as regards transmission of viral hepatitis, it is possible that the Cohn (alcohol precipitation) fractionation system, predominant in industrialised countries, will be substituted by such techniques in developing nations. The preliminary extraction of factor VIII from plasma, using the methods described in this study, should not interfere with the fractionation of other proteins using these alternative techniques. The possibility thus exists that small-scale processing on a local or regional level will allow developing nations to become self-sufficient in all blood products once the supply of raw material is assured.

Much interest has been generated recently on the use of genetic engineering for the production of plasma proteins, including factor VIII (Newswatch 1982, Maddox 1983). It is obvious that the commercial companies are acquiring a monopoly on the expertise and the technology involved. Although vast problems must be

surmounted before factor VIII can be successfully produced with this technology, the commercial stimulus will doubtless produce a clinical product in a few years time. This will alleviate greatly many problems presently hampering the procurement of factor VIII, particularly the risk of viral contamination of the product. However, the need for purifying, concentrating and sterilising the product will still exist, and techniques to perform these operations will still be needed. It is suggested that the use of processing methods similar to those described in this Thesis for small-scale processing of plasma will be more relevant in this regard than the Cohn system for fractionating bulk plasma. Transfusion services also need to examine the possibilities for such non-plasma derived production methods. Thus it seems that studies in the field of factor VIII fractionation are going to be of relevance to the future as much as they are today.

REFERENCES

Abildgaard C.F., Penner J.A. and Watson Williams E.J. (1980) Anti-inhibitor coagulant complex (Autoplex) for treatment of factor VIII inhibitors in hemophilia. Blood 56, 978-984.

Addis T. (1911) The pathogenesis of hereditary haemophilia. Journal of Pathology and Bacteriology 15, 427-452.

Aledort L.M. (1982) Current concepts in diagnosis and management of hemophilia. Hospital Practice 17, 77-92.

Alexander B. (1978) Effects of plasma expanders on coagulation and hemostasis: dextran, hydroxyethyl starch and other macromolecules revisited. In 'Blood Substitutes and Plasma Expanders', 293-326, Alan R. Liss Inc. N.Y.

Alexander B. and Goldstein R. (1953) Dual hemostatic defect in pseudohemophilia. Journal of Clinical Investigation 32, 551.

Alexander B., Odake K., Lawler D. and Swanger M. (1975) Coagulation, hemostasis and plasma expanders: a quarter century enigma. Federation Proceedings 34, 1429-1440.

Allain J.P., Verroust F. and Soulier J.P. (1980) In vitro and in vivo characterisation of factor VIII preparations. Vox Sanguinis 38, 68-80.

American Association of Blood Banks. Technical Manual 1970 and 1978.

Amrani D.L., Mosseson M.W. and Hoyer L.W. (1982) Distribution of plasma fibronectin (cold-insoluble globulin) and components of the factor VIII complex after heparin-induced precipitation of plasma. Blood 59, 657-663.

Andronescu S., Apateanu V. and Iancu C. (1983) Plasmapheresis and the production of cryoconcentrate. XVth Congress of the World Federation of Hemophilia Abstract Book, Abstract 70.

Austen D.E.G. and Smith J.K. (1982) Factor VIII fractionation on aminohexyl Sepharose with possible reduction in hepatitis B antigen. Thrombosis and Haemostasis 48, 46-48.

Avoy D.R., Ellison S.S., Nolan N.J., Cox R.S., Franco J. A., Harbury C.A., Schrier S.L. and Pool J.G. (1978) The effect of delayed refrigeration on red blood cells, platelet concentrates and cryoprecipitable AHF. Transfusion 18, 160-168.

Bangham D.R., Biggs R., Brozovic M., Denson K.W.E. and Skegg L. (1971) A biological standard for measurement of blood coagulation factor VIII activity. Bulletin of the World Health Organisation 45, 339-351.

Barrowcliffe T.W., Kemball-Cook G., Morris G., Holt J.C., Furlong R.A. and Peake I.R. (1981) Factor VIII-related activities in therapeutic concentrates. Journal of Laboratory and Clinical Medicine 77, 429-438.

Barrowcliffe T.W., Kemball-Cook G. and Gray E. (1983) Binding to phospholipid protects factor VIII from inactivation by human antibodies. Journal of Laboratory and Clinical Medicine 101, 34-43.

Beck E.A., Tranqui-Poult L., Chapel A., Perret B.A., Furlan M., Hudry-Clergeon G. and Suscillon M. (1979) Studies on factor VIII-related protein. I. Ultrastructural and electrophoretic heterogeneity of human factor VIII-related protein. Biochimica et Biophysica Acta 578, 155-163.

Biggs R. (1978) Plasma concentrations of factor VIII and factor IX and treatment of patients who do not have antibodies directed against these factors. In: 'The Treatment of Haemophilia A and B and von Willebrand's Disease'. Ed: R. Biggs. Blackwell Scientific Publications, Oxford.

Bidwell E. (1955) The purification of antihaemophilic globulin from animal plasma. British Journal of Haematology 1, 386-389.

Bidwell E, Dike G.W.R. and Snape T.J. (1966) Therapeutic materials. In: 'Human Blood Coagulation, Haemostasis and Thrombosis'. Ed: R. Biggs. Blackwell Scientific Publications, Oxford.

Birch C. (1937) Haemophilia, clinical and genetic aspects. University of Illinois, Urbana.

Blatt P.M., Brinkhous K.M., Culp H.R., Krauss J.S. and Roberts H.R. (1976) Antihaemophilic factor concentrate therapy in von Willebrand's disease. Dissociation of bleeding-time factor and ristocetin cofactor activities. Journal of the American Medical Association 236, 2770-2772.

Blombäck E.G.B. and Thorell L.G. (1982) Process in purification and concentration of the factor VIII complex. U.S. Patent No. 4,348,315.

Blombäck M. (1958) Purification of antihaemophilic globulin. I. Some studies on the stability of the antihaemophilic globulin activity in fraction I-O and a method for its partial separation from fibrinogen. Arkiv für Kemi 12, 387-396.

Bloom A.L. (1977) Physiology of factor VIII. In: 'Recent Advances in Blood Coagulation'. Volume 2. Ed: L. Poller. Churchill Livingstone, Edinburgh.

Bloom A.L. (1982) Introduction. In: 'The Haemophilias'. Methods in Haematology Volume 5. Ed: A.L. Bloom. Churchill Livingstone, Edinburgh.

Bloom A.L., Giddings J.C., Bevan B., Letton M. and Drummond R.J. (1969) Comparison of quick and slow methods of producing cryoprecipitate antihaemophilic factor from fresh and 24 hour old blood. Journal of Clinical Pathology 22, 447-452.

Bloom A.L., Giddings J.C. and Wilks C.J. (1973) Factor VIII on the vascular intima: possible importance in haemostasis and thrombosis. Nature New Biology, 241, 217-219.

Brinkhous K.M. (1939) A study of the clotting defect in haemophilia: the delayed formation of thrombin. American Journal of Medical Science 198, 509-516.

Brinkhous K.M. (1954) Plasma anti-hemophilic factor. Biological and clinical aspects. Sang 25, 738-741.

Britten A. (1983) Worldwide view of plasma procurement and fractionation. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 30.

Britten A. and Grove-Ramussen M. (1966) Stability of factor VIII in the frozen state. Transfusion 6, 230-233.

Brown D.L., Hardisty R.M., Koroy M.H. and Bracken C. (1967) Antihaemophilic globulin: preparation by an improved cryoprecipitation method and clinical use. British Medical Journal 2, 79-85.

Brummelhuis H.G.J., Over J., Duiris-Vorst C.C., Wilson de Sturler L.A., Ates G., Hock P.J. and Deerink-Brogens E.E. (1983) Contributions to the optimal use of human blood. IX. Elimination of hepatitis B transmission by (potentially) infectious plasma derivates. Vox Sanguinis 45, 205-216.

Cable R.J., Hoyer L., Marchesi S., Mukharji B., Morse E.E. and Saxton P. (1983) Influence of plasma source on T-lymphocyte subpopulations in haemophiliacs using factor VIII concentrate. (Letter). New England Journal of Medicine 309, 1057-1058.

Carlebjörk G. and Blombäck M. (1983) Improvement of plasma quality as raw material for FVIII:C concentrates. Freezing of plasma. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 10.

Carlebjörk G, Blombäck M and Åkerblom O. (1983) Improvement of plasma quality as raw material for factor VIII:C concentrates. Storage of whole blood and plasma and interindividual plasma levels of fibrinopeptide A. Vox Sanguinis 45, 233-242.

Casillas G. and Simonetti C. (1982) Polyvinylpyrrolidone (PVP): a new precipitating agent for human and bovine factor VIII and fibrinogen. British Journal of Haematology 50, 665-672.

Casillas G., Simonetti C., De los Santos J.C., Farlas C. and Kempfer C. (1983) Preparation and clinical application of a new factor VIII concentrate. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 213.

Centres for Disease Control (1982) Pneumocystitis carinii pneumonia among persons with hemophilia A. Morbility and Mortality Weekly Reports 31, 365-367.

Cohn E.J., Strong L.E., Hughes W.L., Mulford D.J., Askworth J.N., Melin M. and Taylor H.L. (1946) Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. Journal of the American Chemical Society 68, 459-475.

Cohn E.J., Gurd F.R.N., Surgenor D.M., Barnes B.A., Brown R.K., Derouaux G., Gillespie J.M., Kahnt J.M., Lever W.F., Liu C.H., Mittelman D., Mouton R.F., Shmid K. and Uroma E.J. (1950) A system for the separation of the components of human blood: quantitative procedures for the separation of the protein components of human plasma. Journal of the Americal Chemical Society 72, 465-474.

Cort J.H., Fishman A.J., Dodds W.J., Rand J.H. and Schwartz I.L. (1981) New category of vasopressin receptor in the brain. International Journal of Peptide and Protein Research 17, 14-22.

Cosgriff T.M., Hodgson L.A. and West J.V. (1983) The anti-thrombin III content of cryoprecipitate prepared from blood collected with and without heparin. Vox Sanguinis 44, 98-101.

Curling J.M. (Editor) Methods of Plasma Protein Fractionation. 1980. Academic Press.

Curling J.M. (1982) Current practice and future possibilities in plasma protein fractionation. XVIIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract PL-1-7.

Curling J.M. (Editor) Separation of Plasma Proteins. 1983. Pharmacia Fine Chemicals

Davie E.W. and Ratnoff O.D. (1964) Waterfall sequence for intrinsic blood clotting. Science 145, 1310-1312.

Dawes J.R., Foster P.R., Griffin B. and Macleod A.J. (1983) The use of immunoassays to examine the effect of pasteurisation on the immunological integrity of FVIII concentrate. British Society for Thrombosis and Haemostasis Meeting Abstract Book, p.24.

Denson K.W.E. (1967) The use of antibodies in the study of blood coagulation. Blackwell Scientific Publications.

De Vreker R.A. (1980) The fractionator's point of view. In: 'Management of the Hemophilias'. Ed: P. Jones, J. Martin-Villan, R. De Vreker and R. Taub. Scandinavian Journal of Haematology Supplement 35.

Deykin D. (1974) Factor VIII inhibitors. New England Journal of Medicine 291, 205.

van Dieijen G., Tans G., Rosing J. and Hemker H.C. (1981) The role of phospholipid and factor VIIIa in the activation of bovine factor X. Journal of Biological Chemistry 256, 3433-3442.

Edsall J.T. and Lever W.F. (1951) Effects of ions and neutral molecules on fibrin clotting. Journal of Biological Chemistry 191, 735-756.

Einarsson M., Kaplan, L., Nordenfelt E. and Miller E. (1981) Removal of hepatitis B virus from a concentrate of coagulation factors II, VII, IX and X by hydrophobic interaction chromatography. Journal of Virological Methods 3, 213-228.

Ekert H. and Chavin S.I. (1977) Changes in electrophoretic mobility of human factor VIII-related antigen: evidence for subunit structure. British Journal of Haematology 36, 271-279.

Ellis B.C. and Stransky A. (1961) A quick and accurate method for the determination of fibrinogen in plasma. Journal of Laboratory and Clinical Medicine 58, 477-488.

Enck R.E., Betts R.F., Brown M.R. and Miller G. (1979) Viral serology (hepatitis B virus, cytomegalovirus, Epstein-Barr virus) and abnormal liver function tests in transfused patients with hereditary haemorrhagic disease. Transfusion 19, 32-38.

Exner T. and Rickard K.A. (1983) Anamnestic response to high purity porcine A.H.F. (Letter). Thrombosis and Haemostasis 50, 623.

Fay P.J., Chavin S.I., Schroeder D., Young F.E. and Marder V.J. (1982) Purification and characterization of a highly purified human factor VIII consisting of a single type of polypeptide chain. Proceedings of the National Academy of Sciences USA 79, 7200-7204.

Fekete L.F., Holst S.L., Peeton F. and Deveber D.L. (1972) 'Auto'-factor IX concentrate: a new therapeutic approach to treatment of hemophilia A patients with inhibitors. XIVth International Congress of Hematology Abstract Book, Abstract 295.

Fekete L.F., Mesa C. and Holst S.I. (1974) Stabilization of A.H.F. using heparin. U.S. Patent No. 3,803,115.

Fiets G. and Feitsma C. (1982) Effect of freezing rate on clotting factor VIII activity in fresh frozen plasma. Internal Report - Red Cross Blood Bank, Groningen.

Foster P.R. (1983a) Freezing, storage and transport. Lecture in Scottish National Blood Transfusion Service Seminar.

Foster P.R. (1983b) Improving yield in the manufacture of factor VIII concentrates. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 7.

Foster P.R., Dickson A.J., McQuillan T.A., Dickson I.H., Keddie S. and Watt J.G. (1982) Control of large-scale plasma thawing for recovery of cryoprecipitate factor VIII. Vox Sanguinis 42, 180-189.

Foster P.R., Dickson I.H., Macleod A.J. and Bier M. (1983a) Zinc fractionation of cryoprecipitate. IXth International Congress on Thrombosis and Haemostasis Abstract Book, Abstract 348.

Foster P.R., Dickson I.H., McQuillan T.A. and Dawes J. (1983b) Factor VIII stability during the manufacture of a clinical concentrate. IXth International Congress on Thrombosis and Haemostasis Abstract Book, Abstract 349.

Foster P.R., Macleod A.J., Cuthbertson B., Dickson I.H. and Dawes J. (1983c) Towards a high-yield, high quality factor VIII concentrate. Inaugural Meeting, British Blood Transfusion Society Abstract Book, Abstract 1.

French J.E. (1980) Leukapheresis practice in the U.S. In: 'The Conference of Leukapheresis Donor Safety', Bethesda; 1980.

Froebel K.S., Madhok R., Forbes C.D., Lennie S.E., Lowe G.D.O. and Sturrock R.D. (1983) Immunological abnormalities in haemophilia: are they caused by American factor VIII concentrate? British Medical Journal 287, 1091-1093.

Fulcher C.A. and Zimmerman T.S. (1982) Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. Proceedings of the National Academy of Sciences USA 79, 1648-1652.

Funk C., Gmür J., Herold R. and Straub P.W. (1971) Reptilase-R. A new reagent in blood coagulation. British Journal of Haematology 21, 43-52.

Furlong R.A. and Peake I.R. (1983) Studies on the stability of factor VIII coagulant antigen (VIII:CAg) in the presence of VIII:C antibodies. British Journal of Haematology 53, 55-63.

Gabra G.S. (1980) Production of lyophilised cryoprecipitate. Internal Report, West of Scotland Blood Transfusion Centre.

Gabra, G.S., Crawford R.J. and Mitchell R. (1982) Factor VIII cryoprecipitate and hepatitis risk. (Letter). Lancet ii, 1220.

van Gastel C., Sixma J.J., Borst Eilers E., Leautaud M., Moes M., van der Plas M., Bouma B.N. and Sybesma J.D. (1973) Preparation and infusion of cryoprecipitate from exercised donors. British Journal of Haematology 25, 461-466.

Goldstein R., Bunker J.P. and McGovern J.J. (1964) The effect of storage of whole blood and anticoagulants upon certain coagulation factors. Annals of the New York Academy of Sciences 115, 422-442.

Gornall A.G., Bardswill C.J. and David M.M. (1949) Determination of serum proteins by means of the Biuret reaction. Journal of Biological Chemistry 177, 751-766.

Govaerts P. and Gratia P. (1931) Contribution a l'etude de l'hemophilie. Revue Belge Sciences Medic 3, 689-695.

Greenwood F.C., Hunter W.M. and Glover J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific activity. Biochemical Journal 89, 114-123.

Hanratty S. (1982) Personal communication.

Hanratty S. (1983) The use of heparin to improve the yield of factor VIII from fresh frozen plasma. In: 'Development Systems for Efficient Separation of Factor VIII from Human Fresh Frozen Plasma'. Ed: R.S. Lane, G.A. Rock and C.Th. Smit-Sibinga.

Hao Y.C., Ingham K.C. and Wickerhauser M. (1980) Fractional precipitation of proteins with polyethylene glycol. In: Methods of Plasma Protein Fractionation'. Ed: J.M. Curling. Academic Press.

Hardisty R.M. and Macpherson J.C. (1962) A one-stage factor VIII (antihaemophilic globulin) assay and its use on venous and capillary plasma. Thrombosis et Diathesis Haemorrhagica 7, 215-229.

Hässig A. and Lundsgaard-Hansen P. (1978) The procurement of blood and plasma for the production of components and derivatives within the frame of an integrated national blood program. Vox Sanguinis 34, 257-260.

Hayashi M. and Yamada K.M. (1982) Divalent cation modulation of fibronectin binding to heparin and to DNA. Journal of Biological Chemistry 257, 5263-5267.

Heimburger V.N., Schwinn H., Gratz P., Lüben G., Kumpe G. and Herchenkan B. (1981) Faktor VIII - konzentrat, hochgereinigt und in lösung erhitzt. Arzneimittel-Forschung 4, 619-622.

Hellings J. (1981) On the structure and function of human factor VIII-von Willebrand factor. The effect of proteases. Thesis. University of Amsterdam.

Hershgold E.J., Pool J.G. and Pappenhagen A.R. (1966) The potent antihemophilic globulin concentrate derived from a cold insoluble fraction of human plasma: characterization and further data on preparation and clinical trial. Journal of Laboratory and Clinical Medicine 67, 23-32.

Hogmann C.F., Åkerblom O., Hudlund K., Rosén I. and Wiklind L. (1983) Red cell suspensions in SAGM medium. Further experience of in vivo survival of red cells, clinical usefulness and plasma-saving effects. Vox Sanguinis 45, 217-223.

Holmberg L., Borger L. and Nilsson I.M. (1981) Factor VIII:C and factor VIII:CAg response in patients with haemophilia A and von Willebrand's disease after administration of different factor VIII concentrates or plasma. British Journal of Haematology 47, 587-596.

Hondow J.A., Russel W.J., Duncan B.M. and Lloyd J.V. (1982a) The stability of coagulation factors in stored blood. Australian and New Zealand Journal of Surgery 52, 265-269.

Hondow J.A., Russel W.J., Tunbridge L.J. and Lloyd J.V. (1982b) Stability of von Willebrand factor in blood stored at 4°C. Thrombosis Research 27, 125-130.

Hoofnagle J.H., Gerety R.J., Thiel J. and Barker L.F. (1976) The prevalence of hepatitis B surface antigen in commercially prepared plasma products. Journal of Laboratory and Clinical Medicine 88, 102-113.

Hoyer L.W. (1972) Immunologic studies of antihaemophilic factor (AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. Journal of Laboratory and Clinical Medicine 80, 822-833.

Hoyer L.W. (1973) Immunologic properties of antihemophilic factor. In: 'Progress in Haematology'. Volume 8.

Hoyer L.W. (1981) The factor VIII complex: structure and function. Blood 58, 1-13.

Hoyer L.W. and Shainoff J.R. (1980) Factor VIII-related protein circulates in normal human plasma as high molecular weight multimers. Blood 55, 1056-1059.

Hruby M.A. and Schauf V. (1978) Transfusion related short-incubation hepatitis in hemophilic patients. Journal of the American Medical Association 240, 1355-1357.

Ikkala E., Helske T., Myllylä G., Nevanlinna H.R., Pitkänen P. and Rasi V. (1982) Changes in the life-expectancy of patients with severe haemophilia A in Finland in 1930-79. XVIIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract F-123.

Ingram G.I.C. (1976) The history of haemophilia. Journal of Clinical Pathology 29, 3-13.

Jakab T., Pflugshaupt R., Furlan M. and Beck E.A. (1978) Variable degradation of factor VIII-related protein in lyophilised concentrates of antihaemophilic factor (AHF). Vox Sanguinis 35, 36-40.

James H.L. and Wickerhauser M. (1972) Development of large-scale fractionation methods. III. Preparation of a factor VIII concentrate of intermediate purity. Vox Sanguinis 23, 402-412.

Johnson A.J., Karpatkin M.H. and Newman J. (1969) Preparation of and clinical experience with antihemophilic factor concentrates. Thrombosis et Diathesis Haemorrhagica Supplement 35, 49-55.

Johnson A.J., Semar M., Newman J., Brandt D. and Middleton S. (1976) Removal of hepatitis B surface antigen (HB_SAg) from plasma fractions. Journal of Laboratory and Clinical Medicine 88, 91-101.

Johnson A.J., Macdonald V.E., Semar M., Fields J.E., Schuck J., Lewis C. and Brind J. (1978) Preparation of the major plasma fractions by solid-phase polyelectrolytes. Journal of Laboratory and Clinical Medicine 92, 194-210.

Johnson A.J., Macdonald V.E. and Brind J. (1979) Enhanced yield of antihemophilic factor and von Willebrand factor by cryoprecipitation with polyethylene glycol. Vox Sanguinis 36, 72-76.

de Jonge J., Smit-Sibinga C.T. and Das P.C. (1983) Metabolic aspects and viability of heparin - CPDA-1 - stored red cell concentrate as a by-product of a high-yield factor VIII production method. Haemostasis 13, 214-218.

Juckes I.R.M. (1971) Fractionation of proteins and viruses with polyethylene glycol. Biochimica et Biophysica Acta 229, 535-546.

Kahn R.A., Johnson R.K. and Heaton W.A.L. (1979) Effects of prolonged room temperature holding of whole blood intended for preparation of components. Transfusion 19, 539-541.

Kang E.P. (1980) An improved thaw-siphon method for the cryoprecipitate preparation. Vox Sanguinis 38, 172-177.

Kasper C.K., Myhre M.J., McDonald J.D., Nakasako Y. and Feinstein D.I. (1975) Determinants of factor VIII recovery in cryoprecipitate. Transfusion 15, 312-322.

Kernoff P.B.A., Thomas N.D., Lilley P.A. and Tuddenham E.G.D. (1981) Polyelectrolyte fractionated porcine factor VIII concentrate in the treatment of haemophiliacs with antibodies to factor VIIIC. IXth Congress of the International Society for Thrombosis and Haemostasis Abstract Book, Abstract 589.

Kekwick R.A. and Wolf P. (1957) A concentrate of human antihaemophilic factor. Its use in six cases of haemophilia. Lancet i, 647.

Koerner K. and Stampe D. (1982) Stability of factors of the coagulation system in fresh frozen plasma during storage at -40°C and -20°C. Blut 45, 76.

Kopitsky R.G., Switzer M.E.P., Williams R.J. and McKee P.A. (1983) The basis for the increase in factor VIII procoagulant activity during exercise. Thrombosis and Haemostasis 49, 53-57.

Krachmalnicoff A. and Thomas D.P. (1983) The stability of factor VIII in heparinized plasma. Thrombosis and Haemostasis 49, 224-227.

Lane R. (1981) The single plasma pack:IPP. Talk during symposium. Advances in Blood Transfusion Practice. Cambridge, May 1981.

Lane R., Rock G.A. and Smit-Sibinga C.T. (Eds) (1983) Development systems for efficient separation of factor VIII from human fresh frozen plasma. Proceedings of a Symposium held in Groningen, November 1981.

Lane S. (1840) Successful transfusion of blood. Lancet i, 185.

Laurell C.B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analytical Biochemistry 15, 45-52.

Laurent T.C. (1963) The interaction between polysaccharides and other macromolecules. The solubility of proteins in the presence of dextran. Biochemical Journal 89, 253-257.

Lazarchick J. and Hoyer L.W. (1978) Immunoradiometric measurement of the factor VIII procoagulant antigen. Journal of Clinical Investigation 62, 1048-1052.

Leavis P.C. and Rothstein F. (1974) The solubility of fibrinogen in dilute salt solutions. Archives of Biochemistry and Biophysics 161, 671-682.

Lederman M.M., Ratnoff O.D., Scillian J.J., Jones P.K. and Schacter B. (1983) Impaired cell-mediated immunity in patients with classic hemophilia. New England Journal of Medicine 308, 79-83.

Lee J.C. and Timasheff S.N. (1981) The stabilization of proteins by sucrose. Journal of Biological Chemistry 256, 7193-7201.

Legaz M.E., Schmer G., Counts R.B. and Davie E.W. (1973) Isolation and characterization of human factor VIII. Journal of Biological Chemistry 248, 3946-3955.

Levi Y., Dupêchez T., Rimmélé D., Allary M., Boschetti E. and Saint-Blancard J. (1983) New chromatographic method for the purification of factor VIII:C. C.R. Acad. Sc. Paris t.296, 257-259.

Levine P.A., McVerry B.A., Attock B. and Dormandy K.M. (1977) Health of the intensively treated hemophiliac, with special reference to abnormal liver chemistries and splenomegaly. Blood 50, 1-9.

Liu D.T.H., Irwin J.F., LeFave R.O., Grzenczyk B. and Pai R.C. (1980) An improved method for the preparation of intermediate purity antihemophilic factor concentrate for therapeutic usage. Vox Sanguinis 38, 216-221.

Lovric V.A. (1982) The 'circle' pack system. XVIIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract S-16-3.

Lowe G., Pettigrew A., Middleton S., Forbes C.D. and Prentice C.R.M. (1977) DDAVP in hemophilia. (Letter). Lancet ii, 614-615.

Ludlam C.A., Peutherer J.F., Stirling M.L. and Murray J. (1982) Incidence of hepatitis B virus infection in 55 patients with haemophilia A 1971-79. XVIIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract F-125.

Lusher J.M., Blatt P.M., Penner J.A., Aledort L.M., Levine P.H., White G.C., Warrier A.I. and Whitehurst D.A. (1983) Autoplex versus proplex; a controlled, double-blind study of effectiveness in acute hemarthroses in hemophiliacs with inhibitors to factor VIII. Blood 62, 1135-1138.

Macfarlane D.E., Stibbe J., Kirby E.P., Zucker M.B., Grant R.A. and McPherson J. (1975) A method for assaying von Willebrand factor (ristocetin cofactor). Thrombosis et Diathesis Haemorrhagica 34, 306-308.

Macfarlane R.G. (1938) M.D. Thesis, University of London.

Macfarlane R.G. (1964) An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier. Nature 202, 498-499.

Macfarlane R.G. (1976) The theory of blood coagulation. In: 'Human Blood Coagulation, Haemostasis and Thrombosis'. Ed: R. Biggs. Blackwell Scientific Publications, Oxford.

Macfarlane R.G. and Barnett B. (1934) The haemostatic possibilities of snake venom. Lancet ii, 985.

Mackenzie A. (1982) Data communicated to Foster P.R., presented by Foster P.R. (1983a).

Macleod A.J., Dickson I.H. and Foster P.R. (1983) Pasteurisation of coagulation factor concentrates. IXth International Congress on Thrombosis and Haemostasis Abstract Book, Abstract 1371.

Maddox J. (1983) Factor VIII cloning. Nature 306, 528.

Maeda H., Kishi T. and Ikeda S. (1983) The precipitation of human fibrinogen by zinc chloride. Bulletin of the Chemical Society of Japan 56, 1351-1356.

Mannucci P.M. (1981) Hemophilia diagnosis and management: progress and problems. In: 'Recent Advances in Blood Coagulation'. Volume 3. Ed: L. Poller. Churchill Livingstone, Edinburgh.

Mannucci P.M., Ruggeri Z.M., Pareti F.J. and Capitanio A. (1977) 1-Desamino-8-D-arginine vasopressin: a new pharmacological approach to the management of haemophilia and von Willebrand's disease. Lancet i, 869-872.

Mannucci P.M., Colombo M. and Rizzetto M. (1982) Non-progressive course of non-A, non-B chronic hepatitis in multitransfused hemophiliacs. Blood 60, 655-658.

Margolis J. and Rhoades P. (1979) Preparation of stable intermediate purity factor VIII concentrate with a note on high-purity factor VIII. Vox Sanguinis 36, 369-374.

Margolis J. and Rhoades P.H. (1981) Preparation of high-purity factor VIII by controlled pore glass chromatography. Lancet ii, 446-449.

Martin S.E., Francis C.W. and Marder V.J. (1983) Potentiation of wheat germ agglutinin aggregation of platelets by von Willebrand protein and by a 116,000 molecular weight tryptic fragment. Thrombosis Research 31, 437-449.

Marx J. (1982) New disease baffles medical community. Science 217, 618-621.

Mason E.C. (1978) Thaw-siphon technique for production of cryoprecipitate concentrate of factor VIII. Lancet ii, 15-17.

Mason E.C., Pepper D.S. and Griffin B. (1981) Production of cryoprecipitate of intermediate purity in a closed system thaw-siphon process. Thrombosis and Haemostasis 46, 543-546.

Masure R. (1969) Human factor VIII prepared by cryo-precipitation. Vox Sanguinis 16, 1-9.

Mazurier C., Samor B., Lefevre A., Seghatchian J. and Goudemand M. (1983) Influence of fibronectin and fibrinogen in the FVIII/VWF cryoprecipitation process. IXth International Congress on Thrombosis and Haemostasis Abstract Book, Abstract 347.

McKusick V.A. (1965) The royal hemophilia. Scientific American 213, 88-95.

McLellan D.S., Pelly C., McLellan H.G., Jones P. and Aronstam A. (1982) The 'in vivo' survival characteristics of factor VIII procoagulant antigen (VIII:CAg) in haemophilia A subjects. Thrombosis Research 25, 33-39.

Mellstrand T. (1983) Electrolyte solutions and plasma substitutes. In: 'Proceedings from Seminar on National Self-Reliance in Blood and Blood Fractions for Developing Countries'. Swedish Pharmaceutical Press, Stockholm.

Menitove J.E., Aster R.H., Casper J.T., Lauer S.J., Gottschall J.L., Williams J.E., Gill J.C., Wheller D.V., Piaskowski V., Kirchner P. and Montgomery R.R. (1983) T-lymphocyte subpopulations in patients with classic hemophilia treated with cryoprecipitate and lyophilized concentrates. New England Journal of Medicine 308, 83-86.

Mikaelsson M., Nilsson I.M., Vilhardt H. and Wiechel B. (1982) Factor VIII concentrate prepared from blood donors stimulated by intranasal administration of a vasopressin analogue. Transfusion 22, 229-233.

Mikaelsson M., Forsman N. and Oswaldson V.M. (1983a) Human factor VIII: a calcium linked protein complex. Blood 62, 1006-1015.

Mikaelsson M. and Williams J.M. (1983b) The use of desmopressin (DDAVP) in the preparation of improved factor VIII concentrate. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 6.

Milligan G., Graham R., Hanratty S., Muir W. and Mitchell R. (1981) Production of freeze-dried human antihaemophilic cryoprecipitate. Journal of Clinical Pathology 34, 1091-1093.

Minot G.R. and Taylor F.H.L. (1947) Hemophilia; the clinical use of antihemophilic globulin. Annals of Internal Medicine 26, 363-367.

Mishler J.M. (1982) Pharmacology of hydroxyethyl starch. Use in therapy and blood banking. Oxford University Press.

Mishler J.M., Darley J.H., Cederholm-Williams S. and Wright G. (1978) Whole blood storage in citrate and phosphate solutions containing half-strength trisodium citrate: cellular and biochemical studies. Journal of Pathology 124, 125-129.

Mitra G. and Lundblad J.L. (1981) Studies on ultrafilration and antihaemophilic factor. Vox Sanguinis 40, 109-114.

Mollison P.L. and Young I.M. (1942) In vivo survival in the human subject of transfused erythrocytes after storage in various preservative solutions. Quarterly Journal of Experimental Physiology 31, 359-366.

Morawitz P. (1905) Die chemie der blutgerinnung. Ergenbisse der Physiologie 4, 307-416.

Morgenthaler J.J. (1982) Chromatography of antihemophilic factor on diaminoalkane - and aminoalkane - derivatized sepharose. Thrombosis and Haemostasis 47, 124-127.

Mosesson M.W. and Amrani D.L. (1980) The structure and biologic functions of plasma fibronectin. Blood 56, 145-158.

Mustard J.F. (1957) A study of changes in platelets, antihaemophilic globulin, factor V and factor VII during blood collection and storage by different techniques. British Journal of Haematology 3, 202-214.

Mustard J.F. (1958) Some in vitro effects of various concentrations of disodium ethylenediamine tetraacetate, potassium oxalate and sodium citrate on coagulation of blood. American Journal of Clinical Pathology 30, 498-506.

Myllylä G. (1983) Case study of a production plant. In: 'Proceedings from Seminar on National Self-Reliance in Blood and Blood Fractions for Developing Countries'. Swedish Pharmaceutical Press, Stockholm.

Neal G.G. and Chavin S.I. (1979) The role of factors VIII and IX in the activation of bovine blood coagulation factor X. Thrombosis Research 16, 473-484.

Newman J. Johnson A.J., Karpatkin M. and Puszkin S. (1971) Methods for the production of clinically effective intermediate and high purity factor VIII concentrate. British Journal of Haematology 21, 1-20.

Newswatch (1982) Genentech, Speywood close ranks in move to clone factor VIIIC for $400-million antihemophilic market.

Nilsson I.M., Blombäck M. and von Francken I. (1957) On an inherited autosomal hemorrhagic diathesis with anti-hemophilic globulin (AHG) deficiency and prolonged bleeding time. Acta Medica Scandinavica 159, 35-57.

Nilsson I.M. and Hedner U. (1977) Characteristics of various factor VIII concentrates used in treatment of haemophilia A. British Journal of Haematology 37, 543-557.

Nilsson I.M., Walter H., Mikaelsson M. and Vilhardt T. (1979) Factor VIII concentrate prepared from DDAVP stimulated blood donor plasma. Scandinavian Journal of Haematology 22, 42-46.

Nilsson I.M., Holmberg L., Stenberg P. and Henriksson P. (1980) Characteristics of the factor VIII protein and factor XIII in various factor VIII concentrates. Scandinavian Journal of Haematology 24, 340-349.

Nilsson L., Hedner U., Nilsson I.M. and Robertson B. (1983) Shelf-life of bank blood and stored plasma with special reference to coagulation factors. Transfusion 23, 377-381.

Nyman D. (1970) The preparation of an artificial reagent for the one-stage factor VIII assay. Thrombosis et Diathesis Haemorrhagica 23, 306-312.

Ogata K., Saito H. and Ratnoff O.D. (1983) The relationship of the properties of antihemophilic factor (factor VIII) that support ristocetin-induced platelet agglutination (Factor VIIIR-RC) and platelet retention by glass beads as demonstrated by a monoclonal antibody. Blood 61, 27-35.

Olson J.D., Brockway W.J., Fass D.N., Bowie E.J.W. and Mann K.G. (1977) Purification of human and bovine von Willebrand factor. Journal of Laboratory and Clinical Medicine 89, 1278-1294.

Onder O. and Hoyer L.W. (1979) Factor VIII coagulant antigen in factor IX complex concentrates. Thrombosis Research 15, 569-572.

O'Riordan J.P. (1973) Scientific advances in transfusion. Journal of the Irish Medical Association 66, 27-36.

Orringer E.P., Koury M.J., Blatt P.M. and Roberts H.R. (1976) Hemolysis caused by factor VIII concentrates. Archives of Internal Medicine 136, 1018-1020.

Over J., Bouma B.N., van Mourik J.A., Sixma J.J., Vlooswijk R. and Bakker-Woudenberg I. (1978) Heterogeneity of human factor VIII. I. Characterization of factor VIII present in the supernatant of cryoprecipitate. Journal of Laboratory and Clinical Medicine 91, 32-46.

Owen W.G. and Wagner R.H. (1972) Antihemophilic factor. A new method for purification. Thrombosis Research 1, 71-88.

Patek A.J. and Taylor F.A.L. (1937) Hemophilia. II. Some properties of a substance obtained from normal human plasma effective in accelerating the coagulation of hemophilic blood. Journal of Clinical Investigation 16, 113-124.

Paulssen M.M.P., van der Graaf-Wildschut M., Kolhorn A. and Planje M.C. (1975) Radioimmunoassay of anti-haemophilic factor (factor VIII) antigen. Clinica Chimica Acta 63, 349-353.

Peake I.R. (1982) Immunoradiometric assays of factor VIII. In: 'The Hemophilias' Methods in Haematology Volume 5. Ed: A.L. Bloom. Churchill Livingstone, Edinburgh.

Penick G.D. and Brinkhous K.M. (1956) Relative stability of plasma antihaemophilic factor (AHF) under different conditions of storage. American Journal of the Medical Sciences 232, 434-441.

Penny A.F. (1983) Anticoagulation in cell separation procedures. Apheresis Bulletin 1, 164-171.

Pepper M.D., Learoyd P.A. and Rajah S.M. (1978) Plasma factor VIII, variables affecting stability under standard blood bank conditions and correlation with recovery in concentrates. Transfusion 18, 756-760.

Perkins H.A., Rolfs M.R. and Acra D.J. (1962) The stability of factor VIII (antihemophilic globulin) in fresh-frozen blood bank plasma. Transfusion 2, 313-320.

Pflugshaupt R. and Kurt G. (1983) FPA content - a criterion of quality for plasma as factor VIII source. Vox Sanguinis 45, 224-232.

Polson A. (1972) Mechanism of cryoprecipitation. Preparative Biochemistry 2, 53-59.

Polson A., Potgieter G.M., Largier J.F., Mears G.E. and Joubert F.J. (1964) The fractionation of protein mixtures by linear polymers of high molecular weight. Biochimica et Biophysica Acta 82, 463-495.

Polson A. and Ruiz-Bravo C. (1972) Fractionation of plasma with polyethylene glycol. Vox Sanguinis 23, 107-118.

Pool J.G. (1967) The effect of several variables on cryoprecipitated factor VIII concentrates. Transfusion 7, 165-167.

Pool J.G. and Robinson J. (1959) Observations on plasma banking and transfusion procedures for haemophilic patients using a quantitative assay for antihaemophilic globulin (AHG). British Journal of Haematology 5, 24-30.

Pool J.G. and Shannon A.E. (1965) Production of high-potency concentrates of antihaemophilic globulin in a closed-bag system; assay in vitro and in vivo. New England Journal of Medicine 273, 1443-1447.

Preston A.E. (1967) The factor VIII activity in fresh and stored plasma. British Journal of Haematology 13, 42-49.

Preston A.E. and Barr A. (1964) The plasma concentration of factor VIII in the normal population. II. The effects of age, sex and blood group. British Journal of Haematology 10, 238-245.

Prince A.M., Stephen W., Kotitschke R. and Brotman B. (1983) Inactivation of hepatitis B and non-A, non-B viruses by combined use of Tween 80, β-propiolactone and ultraviolet irradiation. Thrombosis and Haemostasis 50, 534-536.

Prince A.M., Horowitz B., Brotman B., Huima T., Richardson L. and Ende M.C.van den (1984) Inactivation of hepatitis B and Hutchinson strain non-A, non-B hepatitis viruses by exposure to Tween 80 and ether. Vox Sanguinis 46, 36-43.

Prowse C.V. (1984) Summarised data on quality control of fresh frozen plasma. Personal communication.

Prowse C. and McGill A. (1979) Evaluation of the 'Mason' (continuous-thaw-siphon) method for cryoprecipitate production. Vox Sanguinis 37, 235-243.

Prowse C.V., Griffin B., Pepper D.S., Dickson A.J., McQuillan T.A., Dickson I.H. and Foster P.R. (1981) Changes in factor VIII complex activities during the production of a clinical intermediate purity factor VIII concentrate. Thrombosis and Haemostasis 46, 597-601.

Prowse C.V., Taylor E., Boulton F., McClelland D.B.L. and Griffin B. (1982) Effects of blood group on the properties of factor VIII. XVIIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract F-83.

Prowse C.V., Bessos H., Farrugia A., Smith A. and Gabra J. (1984a) Donation procedure, fibrinopeptide A and factor VIII. (Letter). Vox Sanguinis 46, 55-57.

Prowse C.V., Farrugia A., Boulton F.E., Tucker J., Ludlam C.A., McLaren M., Belch J.J.F., Prentice C.R.M., Dawes J. and MacGregor I.R. (1984b) A comparative study using immunological and biological assays of the haemostatic responses to DDAVP infusion, venous occlusion and exercise in normal men. Thrombosis and Haemostasis 51, 110-114.

Rapaport S.I., Ames S.B. and Mikkelson S. (1959) The levels of antihemophilic globulin and proaccelerin in fresh and bank blood. American Journal of Clinical Pathology 31, 297-304.

Rapaport S.I., Schiffman S., Patch M.J. and Ames S.B. (1963) The importance of activation of antihemophilic globulin and proaccelerin by traces of thrombin in the generation of intrinsic prothrombinase activity. Blood 21, 221-236.

Ratnoff O.D., Kass L. and Lang P.D. (1969) Studies on the purification of antihemophilic factor (factor VIII). II. Separation of partially purified antihemophilic factor by gel filtration of plasma. Journal of Clinical Investigation 48, 957-962.

Ratnoff O.D., Slover C.C. and Poon, M.C. (1976) Immunological evidence that the properties of human antihemophilic factor (factor VIII) are attributes of a single molecular species. Blood 47, 657-667.

Regional Transfusion Directors' Committee (1978) Variables involved in cryoprecipitate production and their effect on factor VIII activity. British Journal of Haematology 43, 287-295.

Rickard K.A., Dority P., Campbell J., Batey R.G., Johnson S. and Hodgson J. (1982) Hepatitis and haemophilia therapy in Australia. Lancet ii, 146-148.

Rizza C.R. (1976a) The clinical features of clotting factor deficiencies. In: 'Human Blood Coagulation, Haemostasis and Thrombosis'. Ed: R. Biggs. Blackwell Scientific Publications, Oxford.

Rizza C.R. (1976b) The management of patients with coagulation factor deficiencies. In: 'Human Blood Coagulation, Haemostasis and Thrombosis'. Ed: R. Biggs. Blackwell Scientific Publications, Oxford.

Rizza C.R. (1981) Management of patients with inherited blood coagulation defects. In: 'Haemostasis and Thrombosis'. Ed: A.L. Bloom and D.P. Thomas. Churchill Livingstone, Edinburgh.

Rizza C.R. and Mathews J.M. (1982) Effect of frequent factor VIII replacement on the level of factor VIII antibodies in haemophiliacs. British Journal of Haematology 52, 13-24.

Rizza C.R. and Rhymes I.L. (1982) Coagulation assay of VIIIC and IXC. In: 'The Hemophilias'. Methods in Haematology Volume 5. Ed: A.L. Bloom. Churchill Livingstone, Edinburgh.

Rizza C.R. and Spooner R.J.D. (1983) Treatment of haemophilia and related disorders in Britain and Northern Ireland during 1976-80: report on behalf of the directors of haemophilia centres in the United Kingdom. British Medical Journal 286, 929-933.

Robinson A.E., Penny A.F., Smith J. and Tovey D.L. (1983) Pilot study for large-scale plasma procurement using automated plasmapheresis. Vox Sanguinis 44, 143-150.

Rock G.A. (1982) Stabilization of factor VIII activity in whole blood or blood plasma. U.S. Patent No. 4,359,463.

Rock G.A. (1983a) Factor VIII:C one stage assay of plasma containing heparin. In: 'Development Systems for Efficient Separation of Factor VIII from Human Fresh Frozen Plasma'. Ed: R.S. Lane, G.A. Rock and C.Th. Smit-Sibinga.

Rock G.A. (1983b) In: 'International Forum on Production and Quality Control of Fresh Frozen Plasma'. Vox Sanguinis 44, 246-259.

Rock G.A. and Tittley P. (1977) Variations in cryo-precipitate production. Transfusion 17, 50-53.

Rock G.A. and Tittley P. (1979) The effect of temperature variations on cryoprecipitate. Transfusion 19, 86-89.

Rock G.A., Cruickshank W.H., Tackaberry E.S. and Palmer D.S. (1979) Improved yields of factor VIII from heparin-ised plasma. Vox Sanguinis 36, 294-300.

Rock G.A., Cruickshank W., Tackaberry E. and Palmer D. (1980a) Stability of CPD plasma factor VIII procoagulant activity in the presence of various protease inhibitors. XVIth Congress of the International Society of Blood Transfusion Abstract Book, Abstract 1170.

Rock G.A. and Palmer D.S. (1980b) Intermediate purity factor VIII production utilizing a cold-insoluble globulin technique. Thrombosis Research 18, 56-60.

Rock G.A., Ganz P. and Tackaberry E. (1983a) The relationship of biological and immunological activities of factor VIII. Biochemical and Biophysical Research Communications 115, 981-987.

Rock G.A., Cruickshank W.H., Tackaberry E.S., Ganz P.R. and Palmer D.S. (1983b) Stability of VIII:C in plasma: the dependence on protease activity and calcium. Thrombosis Research 29, 521-535.

Rosner F. (1969) Hemophilia in the T lmud and Rabbinic writings. Annals of International Medicine 70, 833-837.

Rotblat F. and Tuddenham E.G.D. (1981) Immunological studies of factor VIII coagulant activity (VIII:C). 1. Assays based on a haemophilic and an acquired inhibitor to VIII:C. Thrombosis Research 21, 431-445.

Rothschild (1882) qu.Bullock W. and Fildes P. (1911) Treasury of human inheritance, parts V and VI, section XIVa, Haemophilia.

Rubenstein A. (1981) Heated lyophilized factor VIII concentrate - in vitro studies. VIIIth International Congress on Thrombosis and Haemostasis Abstract Book, Abstracts 1051 and 1054.

Sakariassen K.S., Bolhuis P.A. and Sixma J.J. (1979) Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. Nature 279, 636-639.

Salzman E.W. (1963) Measurement of platelet adhesiveness: a simple in vitro technique demonstrating an abnormality in von Willebrand's disease. Journal of Laboratory and Clinical Medicine 62, 724-735.

Samor B., Mazurier C., Goudemand M., Debeire P., Fournet B. and Montreuil J. (1982) Preliminary results on the carbohydrate moiety of factor VIII/von Willebrand factor (FVIII/VWf). Thrombosis Research 25, 81-89.

Sandler S.G. and Katz A.J. (1984) Impact of AIDS on blood services in the United States. Vox Sanguinis 46, 1-7.

Scully M.F. and Kakkar V.V. (1982) Structural features of fibrinogen associated with binding to chelated zinc. Biochimica et Biophysica Acta 700, 130-133.

Seeler R.A. (1976) In: 'Unsolved Therapeutic Problems in Haemophilia'. DHEW Publication No. (NIH) 77-1089. Ed: J.C. Fratantoni and D.L. Aronson.

Self L.B. and Hoofnagle J. (1976) Acute and chronic liver disease in hemophilia. In: 'Unsolved Therapeutic Problems in Hemophilia'. DHEW Publication No. (NIH) 77-1089. Ed: J.C. Fratantoni and D.L. Aronson.

Sgouris J.T. and Wickerhauser M. (1973) Use of frozen cryoprecipitate for the preparation of clinical factor VIII concentrate. Transfusion 13, 399-404.

Shanberge J.N., Gruhl M.C., Ikemori R., Inoshita K., Chalos M.K. and Aster R.H. (1972) A comparison of factor VIII activity in cryoprecipitates prepared from ACD and CPD plasma. Transfusion 12, 251-258.

Shanbrom E. and Fekete L.F. (1971) Production of stable high-potency human AHF using polyethylene glycol and glycine to fractionate a cryoprecipitate of AHF concentrate. U.S. Patent No. 3,631,018.

Simonetti C., Casillas G., Pavlovsky A. and Bachman A.E. (1969) Concentrate of factor VIII (FI-O-Ta) for clinical use. Thrombosis et Diathesis Haemorrhagica Supplement 35, 245-251.

Simonetti C., Casillas G., Farias C. and De Los Santos J.C. (1981) Un nuevo concentrado de factor VIII humano. Preparacion y applicacion clinica. Medicina (Buenos Aires) 41, 249-252.

Sixma J.J., Sakariassen K.S. and Bolhuis P.A. (1981) The relationship between the multimeric structure of FVIII-VWF and the facilitation of platelet adhesion to human subendothelium (von Willebrand factor activity vWF-A). VIIIth International Congress on Thrombosis and Haemostasis Abstract Book, Abstract 625.

Slichter S.J., Counts R.B., Henderson R. and Harker L.A. (1976) Preparation of cryoprecipitated factor VIII concentrates. Transfusion 16, 616-626.

Smith C.M., Miller G.E. and Breckenridge R.T. (1972) Factor VIII concentrates in outpatient therapy. Journal of the American Medical Association 220, 1352-1354.

Smith J.K. (1983a) Qualitative aspects of fresh plasma procurement with particular reference to factor VIII production. Contribution during Scottish National Blood Transfusion Service Seminar.

Smith J.K. (1983b) In: 'Development Systems for Efficient Separation of Factor VIII from Human Fresh Frozen Plasma'. Ed: R.S. Lane, G.A. Rock and C. Th. Smith-Sibinga.

Smith J.K., Snape T., Bidwell E., Edwards R. and Gunson H. (1977) Preparation of factor IX concentrate from platelet-rich plasma. Thrombosis and Haemostasis 37, 363-366.

Smith J.K., Snape T.J., Haddon M.E., Gunson H.H. and Edwards R. (1978) Methods of assessing factor VIII content of stored fresh frozen plasma intended for preparation of factor VIII concentrates. Transfusion 18, 530-537.

Smith J.K. and Bidwell E. (1979) Therapeutic materials used in the treatment of coagulation defects. Clinics in Haematology 8, 183-206.

Smith J.K., Evan D.R., Stone V. and Snape T.J. (1979) A factor VIII concentrate of intermediate purity and higher potency. Transfusion 19, 299-306.

Smith J.K., Bowell P.J., Bidwell E. and Gunson H.H. (1980) Anti-A haemagglutinins in factor VIII concentrates. Journal of Clinical Pathology 33, 954-957.

Smit - Sibinga C.Th. (1983) A system analysis for improved collection of factor VIII: the Groningen system. In: 'Development Systems for Efficient Separation of Factor VIII from Human Fresh Frozen Plasma'. Proceedings of a workshop, Groningen 1981. Ed: R.S. Lane, G.A. Rock and C.Th. Smit-Sibinga.

Smit-Sibinga C.Th., Welbergen H., Das P.C. and Griffin B. (1981) High yield method of production of freeze-dried purified factor VIII by blood banks. Lancet ii, 449-450.

Smit-Sibinga C.Th., Das P.C., van Imhoff G.W. and Daenen S. (1983) Heparin and factor VIII. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 8.

Smit-Sibinga C.Th., Daenen S.M.G.J., van Imhoff G.W., Maas A. and Das P.C. (1984) Heparin small pool high yield purified factor VIII: in vivo recovery and half-life of routinely produced freeze-dried concentrate. Thrombosis and Haemostasis 51, 12-15.

Snyder E.L., Hezzey A., Warick F., Mosher D.F., Davisson W. and Buchloz D.H. (1983) Stability of red cell antigens and plasma coagulation factors in new formulation plastic bag containers. Transfusion 23, 49-53.

Sodetz J.M., Paulson J.C. and McKee P.A. (1979) Carbohydrate composition and identification of blood group A, B and H oligosaccharide structures on human factor VIII/von Willebrand factor. Journal of Biological Chemistry 254, 10754-10760.

Soulier J.P. (1959) Séparation du fibrinogène et du facteur anti-hémophilique A. II. A l'aide de la bentonite. Path. Biol. Paris 7, 2451-2454.

Spaet T.H. and Garner E.S. (1955) Studies on the storage lability of human antihemophilic factor. Journal of Laboratory and Clinical Medicine 46, 111-119.

Stel H.V., van der Kwast Th.H. and Veerman E.C.J. (1983) Detection of factor VIII coagulant antigen in human liver tissue. Nature 303, 530-532.

Stibbe J., Hemker H.C. and van Creveld S. (1972) The inactivation of factor VIII in vitro. Thrombosis et Diathesis Haemorrhagica 27, 43-58.

Suomela H. (1980) An ion exchange method for immunoglobulin production. In: 'Methods of Plasma Protein Fractionation'. Ed: J.M. Curling. Academic Press.

Suomela H., Blombäck M. and Blombäck B (1979) The activation of factor X evaluated by using synthetic substrates. Thrombosis Research 10, 267-281.

Sussman I.I. and Weiss H.J. (1978) Dissociation of factor VIII in the presence of proteolytic inhibitors. Thrombosis and Haemostasis 40, 316-325.

Switzer M.E. and McKee P.A. (1976) Some effects of calcium on the activation of human factor VIII/von Willebrand factor protein by thrombin. Journal of Clinical Investigation 60, 819-828.

Tabor E., Snoy P., Gerety R.J., Wickerhauser M., Menache D. and Seeff L.B. (1983) Transmission of agent of post-transfusion non-A, non-B hepatitis by cryoprecipitate prepared from plasma of symptomless carrier. (Letter). Lancet i, 63-64.

Thode J., Fogh-Andersen N., Wimberly P.D., Møller Sorensen A. and Siggard-Andessen O. (1983) Relation between pH and ionized calcium in vitro and in vivo in man. Scandinavian Journal of Clinical and Laboratory Investigation Supplement 165, 79-82.

Thompson A.R. and Counts R.B. (1976) Removal of heparin and protamine from plasma. Journal of Laboratory and Clinical Medicine 88, 922-929.

Thorell L., Blombäck M and Blombäck B. (1983) An in vivo study of a new factor VIII high purity preparation. Thrombosis Research 31, 375-385.

Tomasulo P.A., Richards W., Bailey M., Gatewski M., Aster R.H. and Lazerson J. (1980) Preselection of donors to improve the quality of cryoprecipitate. American Journal of Haematology 8, 191-196.

Toolis F., McKay G. and Prowse C.V. (1980) In vivo characteristics of thaw-siphon cryoprecipitate compared to other factor VIII preparations. Thrombosis and Haemostasis 43, 25-27.

Törmä E. and Myllylä G. (1983) Parameters affecting the fractionation of the FVIII:C activity in production of very high purity AHF concentrate. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 9.

Tuddenham E.G.D. (1983) Innovative alternatives to human factor VIII. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 37.

Tuddenham E.G.D., Trabold N.C. and Hoyer L.W. (1979) The properties of factor VIII coagulant activity prepared by immunoadsorbent chromatography. Journal of Laboratory and Clinical Medicine 93, 40-53.

Tuddenham E.G.D., Lane, R.L., Rotblat F., Johnson A.J., Snape T.J., Middleton S. and Kernoff P.B.A. (1982) Response to infusions of polyelectrolyte fractionated human factor VIII concentrate in haemophilia A and von Willebrand's disease. British Journal of Haematology 52, 259-267.

Unger P., Blombäck M., Manella P., Pflugshaupt R. and Stampfli K. (1983) A new blood collection device for proportional admixture of blood and anticoagulant. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 110.

UNIDO (1982) Final declaration in seminar on national self-reliance in blood and blood fractions for developing countries. Swedish Pharmaceutical Press, Stockholm.

Vermeer C., Soute B.A.M., Ates G. and Brummelhuis H.G.J. (1976) Contributions to the optimal use of human blood. VII. Increases in the yield of factor in four-donor cryo-precipitate by an improved processing of blood and plasma. Vox Sanguinis 30, 1-22.

Wagner R.H., McLester W.D., Smith M. and Brinkhous K.M. (1964) Purification of antihemophilic factor (factor VIII) by amino-acid precipitation. Thrombosis et Diathesis Haemorrhagica 11, 64-74.

Wahlberg T.B., Savidge G.F., Blombäck M. and Wiechel B. (1980) Influence of age, sex and blood groups on 15 blood coagulation variables in a reference material composed of 80 blood donors. Vox Sanguinis 39, 301-308.

Ware A.G., Guest M.M. and Seegers W.H. (1947) Fibrinogen, with special reference to its preparation and certain properties of the product. Archives of Biochemistry 13, 231-236.

Watt J.G. (1976) Plasma fractionation. Clinics in Haematology 5, 95-112.

Watt J.G. (1982) Personal communication.

Weaver R.A., Gabrill D.A. and Langdell R.D. (1967) Concentrated anti-hemophilic factor (AHF) from outdated blood. Transfusion 7, 168-173.

Weber K. and Osborne M. (1969) The reliability of molecular weight determinations by dodecyl sulphate-poly-acrylamide gel electrophoresis. Journal of Biological Chemistry 244, 4406-4412.

Weinstein M. and Deykin D. (1979) Comparison of factor VIII-related von Willebrand factor proteins prepared from human cryoprecipitate and factor VIII concentrate. Blood 53, 1095-1105.

Weinstein M., Chute L. and Deykin D. (1981) Analysis of factor VIII coagulant antigen in normal, thrombin-treated and hemophilic plasma. Proceedings of the National Academy of Sciences USA 78, 5137-5141.

Weiss A.E., Webster W.P., Strike L.E. and Brinkhous K.M. (1976) Survival of transfused factor VIII in hemophilic patients treated with epsilon aminocaproic acid. Transfusion 16, 209-214.

Weiss H.J. (1965) A study of the cation and pH-dependent stability of factors V and VIII in plasma. Thrombosis et Diathesis Haemorrhagica 14, 32-51.

Weiss H.J. and Hoyer L.W. (1973) Von Willebrand factor dissociation from antihemophilic factor procoagulant activity. Science 182, 1149-1151.

Weiss H.J., Baumgartner H.R., Tschopp T.S., Turitto V.T. and Cohen D. (1978) Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. Blood 51, 267-279.

Welbergen H. (1981) Possibilities and limitations for routine production of a high recovery intermediate purity factor VIII concentrate in a regional blood bank. Thesis, University of Groningen.

Wensley R.T. and Snape T.J. (1980) Preparation of improved cryoprecipitated factor VIII concentrate. A controlled study of three variables affecting the yield. Vox Sanguinis 38, 222-228.

WHO (1981) The collection, fractionation, quality control and uses of blood and blood products. World Health Organisation Publication, 1981.

Wickerhauser M., Mercer J.E. and Eckenrode J.W. (1978) Development of large-scale fractionation methods. VI. An improved method for preparation of anti-hemophilic factor. Vox Sanguinis 35, 18-31.

Wickerhauser M., Williams C. and Mercer J.E. (1979) Development of large scale fractionation methods. VII. Preparation of antithrombin III concentrate. Vox Sanguinis 36, 281-293.

Woodfield D.G., Benny G. and Berry E. (1983) Factor VIII concentrate production using controlled pore glass chromatography. XVth World Federation of Hemophilia Congress Abstract Book, Abstract 69.

Yang H.C. and Duffy C. (1978) Factor VIII procoagulant antigen content of antihemophilic factor (AHF) concentrates. Transfusion 18, 747-749.

Zimmerman T.S., Ratnoff O.D. and Powell A.E. (1971) Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. Journal of Clinical Investigation 50, 244-254.

Zimmerman T.S., Hoyer L.W., Dickson L. and Edgington T.S. (1975a) Determination of the von Willebrand's disease antigen (factor VIII-related antigen) in plasma by quantitative immunoelectrophoresis. Journal of Laboratory and Clinical Medicine 86, 152-159.

Zimmerman T.S., Roberts J. and Edgington T.S. (1975b) Factor VIII-related antigen: multiple molecular forms in human plasma. Proceedings of the National Academy of Sciences USA 72, 5121-5125.

Zimmerman T.S. and Ruggeri Z.M. (1983) Von Willebrand's disease. Clinics in Haematology 12, 175-200.

Zuber T. amd Morgenthaler J.J. (1982) Polyvinylpyrrolidone as a precipitating agent for factor VIII and fibrinogen. British Journal of Haematology 52, 517-518.

PUBLISHED WORK

I Prowse C.V., Bessos H., Farrugia A., Smith A. and Gabra J. (1984a) Donation procedure, fibrinopeptide A and factor VIII. Vox Sanguinis 46, 55-57.

II Prowse C.V., Farrugia A., Boulton F.E., Tucker J., Ludlam C.A., McLaren M., Belch J.J.F., Prentice C.R.M., Dawes J. and MacGregor I.R. (1984b) A comparative study using immunological and biological assays of the haemostatic responses to DDAVP infusion, venous occlusion and exercise in normal men. Thrombosis and Haemostasis 51, 110-114.

III Farrugia A., Griffin B., Pepper D. and Prowse C. (1984) Studies on the procurement of coagulation factor VIII: Selective precipitation of factor VIII with hydrophilic polymers. Thrombosis and Haemostasis 51, (In Press)

Copies of the above publications are included at the end of this Thesis as an Appendix. Paper III is appended as a final corrected printer's proof.

APPENDIX

PUBLISHED WORK

Vox Sang. 46: 55–57 (1984)

0042–9007/84/0461–0055 $2.75/0

Donation Procedure, Fibrinopeptide A, and Factor VIII

C. V. Prowse[a], *H. Bessos*[a], *A. Farrugia*[a], *A. Smith*[a], *J. Gabra*[b]

[a] Edinburgh and South-East Scotland Blood Transfusion Service, Royal Infirmary, Edinburgh;
[b] Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, UK

The consensus of a recent forum [1] showed a limited donation time and adequate mixing were regarded as important parameters in the collection of good quality plasma. Evidence for this is provided in terms of plasma levels of factor VIII [2] and, more recently, fibrinopeptide A (FpA) [3]. More stringent control of the donation procedure is required to ensure a low plasma FpA than a normal factor VIII level, and it has been suggested that regular mixing during donation is necessary to ensure low FpA levels [3, 4]. High FpA levels have been associated with difficulties in processing plasma to factor VIII products [3].

We have recently determined the effect of donation procedure on plasma quality using these two parameters. In the first study, three groups of ten 420-ml donations were collected in CPD anticoagulant: (1) *mixed:* with an inverted pack, so blood entered the pack through the anticoagulant, with continuous gentle manual mixing; (2) *standard:* with an inverted pack and manual mixing two or three times during donation, and (3) *unmixed:* with an upright pack and mixing only at the end of donation.

All donations were collected in under 10 min, the mixed blood was centrifuged immediately after donation and cell-free plasma frozen for the subsequent assay of FpA and factor VIII [5, 6]. In these donations samples for donor grouping were obtained by reverse flow through the donor line before sampling for the assay of fibrinopeptide.

The results showed no significant difference in plasma FpA level for these three donation procedures (table I). Nor was any difference in factor VIII found (overall mean 0.89 IU/ml), even if group O and group A donations were analyzed separately.

There was some indication in study 1 of a correlation between plasma FpA and donation time. To clarify this and to confirm the original observations, a second study was undertaken. In this case 450 ml blood was collected from group A donors, in CPD anticoagulant using the previous *standard* and *unmixed* procedures. In addition, a third group of ten donations was taken with a reduced arm-cuff pressure and careful selection of venesection site to give donation times between 10 and 20 min. In all three groups, particular care was taken that the

Table I. Fibrinopeptide A in donor plasma collected in different ways

	Unmixed	Standard	Mixed	All
Study 1	8 (3 – 27)	15.5 (3 – 258)	14 (6 – 50)	12 (3–258)
Study 2				
< 10 min donation	4 (2 – 7)	5 (2 – 11)	–	5 (2 – 11)
> 10 min donation	–	27 (15 – 400)	–	–

Results are expressed as median (range) in nanograms per milliliter. Each group consists of ten donations. In study 1 83% of donations contained <30 ng FpA/ml, and in study 2 (<10 min donation) 85% contained <7 ng/ml. Data were analyzed using non-parametic statistical tests.

donation line was stripped into anticoagulant immediately after donation. The results confirmed that mixing during donation has no significant effect on plasma FpA or factor VIII and that prolonged donation can result in an elevated FpA. By combining both studies, the overall correlation of donation time with FpA was significant ($r = 0.3295$; $p < 0.02$). This was more obvious, if the two studies were considered separately, but it should be noted that the longest donation taken (20 min) only contained 29 ng FpA/ml. More importantly, a highly significant difference ($p < 0.01$ for 50 donations collected in less than 10 min) was found between study 1 and study 2 plasma FpAs which we ascribe to the rapid stripping of donation lines in the latter case.

During this work we have also noted that the addition of 100 U/ml heparin and trasylol to CPD plasma prior to freezing is not necessary to stabilize FpA levels, except possibly in unmixed blood, and that storage of CPD blood or plasma at 20 °C for 20 h resulted in only a minor increase of FpA: from 14 to 20 ng/ml. Since the above results relate to specially treated donations we have also assessed the FpA level in plasma derived from 216 donations collected unter the conditions of study 1/*standard* mixing with similar results (median 13 ng/ml). Such plasma has been used in preparing cryoprecipitate and intermediate purity factor VIII concentrate for the last decade.

On the basis of these results we conclude that care in stripping the donation line is an important part of the donation procedure in ensuring low plasma FpA levels. If attention is paid to this, good quality plasma may be obtained from donations collected in under 10 min with occasional manual mixing during donation, and more rigorous donation protocols result in little, if any, improvement.

References

1 International Forum: What are the critical factors in the production and quality control of frozen plasma intended for direct transfusion or for fractionation to provide medically needed labile coagulation factors? Vox Sang. *44:* 246–259 (1983).

2 Kaspar, C. K.; Myhre, B. A.; McDonald, I. D.; Nakasako, Y.; Feinstein, D. I.: Determinants of factor VIII recovery of cryoprecipitate. Transfusion *15:* 312–322 (1975).

3 Pflugshaupt, R.; Kurt, G.: FpA content – a criterion of quality for plasma as factor VIII source. Vox Sang. *45:* 224–232 (1983).

4 Carlebjörk, G.; Blomback, M.; Akerblom, O.: Improvement of plasma quality as raw material for factor VIII: C concentrates. Vox Sang. *45:* 233–242 (1983).

5 Nossel, H. L.; Yudelman, I.; Canfield, R. E.; Butler, V. P.: Measurement of fibrinopeptide A in human blood. J. clin. Invest. *54:* 45–53 (1974).

6 Prowse, C. V.; Griffin, B.; Pepper, D. S.; Dickson, A. J.; McQuillan, T. A.; Dickson, I. H.; Foster, P. R.: Changes in factor VIII complex activities during the production of a clinical intermediate purity factor VIII concentrate. Thromb. Haemostasis *46:* 597–601 (1981).

Dr. C. V. Prowse,
Regional Blood Transfusion Centre,
Royal Infirmary,
Edinburgh EH3 9HB (UK)

A Comparative Study Using Immunological and Biological Assays of the Haemostatic Responses to DDAVP Infusion Venous Occlusion and Exercise in Normal Men

C. V. Prowse, A. Farrugia, F. E. Boulton, J. Tucker*, C. A. Ludlam*, M. McLaren**, J. J. F. Belch**, C. R. M. Prentice**, J. Dawes*** and I. R. MacGregor****

From the Edinburgh and South-East Scotland Blood Transfusion Service, and the Department of Haematology*, Royal Infirmary, Edinburgh; the Department of Medicine**, Royal Infirmary, Glasgow; the MRC/SNBTS Blood Components Assay Group***, Forrest Road, Edinburgh, and the Scottish National Blood Transfusion Service****, Headquarters Laboratory, Forrest Road, Edinburgh, Great Britain

Key words

Plasminogen activator – Factor VIII – Prostaglandin – DDAVP – Venous occlusion – Exercise

Summary

In a group of six normal male volunteers, infusion of DDAVP, venous occlusion and exercise were shown to increase plasma levels of factor VIII and plasminogen activator, activity and antigen, to different extents and at differing rates. Any mechanisms suggested to explain release of these proteins by various stimuli should account for such differences.

All three stimuli could also increase plasma levels of prostacyclin metabolites, although this was only significant for high doses of DDAVP. Other potential endothelial markers, such as fibronectin and thrombospondin, showed no specific increase after any of the stimuli.

Introduction

The original observation that the vasopressin analogue, 1-desamino-8-D-arginine vasopression (DDAVP), causes a rise in the plasma level of factor VIII and plasminogen activator (1) is now well established (2, 3, 4, 5). A similar response occurs following a variety of acute stimuli (6). Intravenous or intranasal DDAVP has been used to prevent bleeding in factor VIII deficiency (7, 8) and to determine fibrinolytic capacity in patients (9). Indirect evidence suggests the rise in plasminogen activator (PA) following DDAVP infusion and other stimuli is due to tissue-type PA (t-PA; 3, 10, 11).

Previous work from this laboratory (5), and others, has established a discrepancy in the procoagulant factor VIII (VIII:C) and VIII-related antigen (VIIIR:Ag) responses to DDAVP infusion, and has also suggested that DDAVP does not act directly on endothelium, the putative source of PA and VIIIR:Ag. Recently direct immunoassays for t-PA (12), as well as for the antigens of the factor VIII complex, VIIIC:Ag and VIIIR:Ag (13), have been established. Here we report results obtained using these assays and compare them with results of the corresponding bioassays following stimulation by DDAVP infusion, venous occlusion and exercise, in studies designed to detect discrepancies in their mode of release by different stimuli. In addition, changes in other potential markers of endothelial including prostacyclin metabolites (14, 15) were determined.

Correspondence to: C. V. Prowse, Blood Transfusion Centre, Royal Infirmary, Edinburgh EH3 9HB, Great Britain

Materials and Methods

Studies were performed on 6 male volunteers, fully informed of the aims of the study, starting in the morning after a light breakfast and at least 20 min rest. Each volunteer was subjected to three stimuli, at intervals of at least 7 days, in the following order:

DDAVP Infusion

50 ml saline was infused from −15 to 0 min into an antecubital vein via a butterfly needle. From 0 to 15 min 0.3 ug DDAVP/Kg in 50 ml saline was infused through the same needle. Blood samples were obtained from the contralateral arm by separate venepuncture at −15, 0, 5, 10, 15, 30, 45, 60 and 90 min.

Venous Occlusion (VO)

VO was achieved by application of a sphygmomanometer cuff, at a pressure midway between systolic and diastolic pressure, to an arm for 20 min. Blood samples were obtained from the occluded arm at the end of this period (+20 min), control samples being obtained from the contralateral arm at −20, 0 and + 20 min.

Exercise

Exercise was performed on a bicycle ergometer, each subject cycling for successive 1 min intervals at 120, 150, 180, 210 and 240 watts sequentially. This 5 min programme was chosen as being the maximum that all six subjects were able to complete.

In addition, 6 female and 3 male volunteers were infused with 0.4 ug/Kg DDAVP as above, during studies of prostacyclin metabolites.

Assays

Fresh citrated plasma was used to determine PA by euglobulin clot lysis and fibrin plate assays, and frozen citrated plasma assessed for procoagulant factor VIII:C (one-stage) and factor VIIIR:Ag as described previously (16). In addition frozen citrated plasma samples were used in the determination of ristocetin cofactor (VIII:RCF) (17), VIIIC:Ag (18), fibronectin by the Laurell method (19) using antisera from Behringwerke, antithrombin III using the Protopath kit (Dade), fibrinogen (20), total protein (21) factor XII by one-stage assay corresponding to that used for factor VIII:C; t-PA was assayed by radioimmunoassay (22) in plasma and in serum, obtained by clotting blood at 37° in glass tubes for one hour, in the absence of any additives.

Samples were also obtained in EDTA-theophylline-prostaglandin E1 for assay of beta-thromboglobulin, platelet factor 4 and thrombospondin (23, 24), and in 0.013 M citrate, 3×10^{-5} M indomethacin, 10^{-5} M adenosine (final concentrations) for radioimmunoassay of prostacyclin metabolites. The latter assay was performed directly on plasma without organic solvent extraction (15). For comments on this assay, see Discussion.

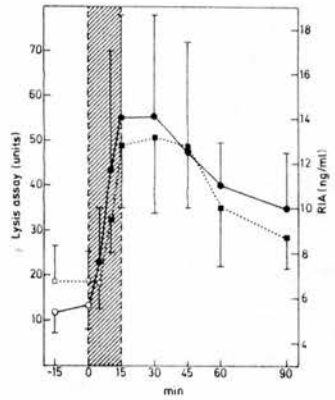


Fig. 1 Change in plasminogen activator following DDAVP infusion. 0.3 ug DDAVP/Kg was infused to 6 subjects from 0 to 15 min and PA levels determined by euglobulin lysis time assay (○-----○) and t-PA immunoassay (□-----□). Results are shown as mean (± SD) and significant ($p < 0.05$) increases are shown by filled symbols.

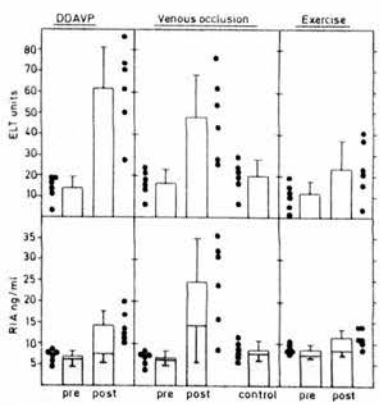


Fig. 2 Plasminogen activator levels following different stimuli. Basal and maximum levels of PA determined by euglobulin lysis time assay and radioimmunoassay. Individual and mean (± SD) results are shown for DDAVP infusion, venous occlusion and exercise. For the immunoassay mean serum levels are also shown by the shaded portion of the histogram. Results for the unoccluded arm following venous occlusion are also shown (control). Post-DDAVP infusion results represent a mixture of results from 15 and 30 min as the maximum levels achieved were taken. They therefore differ slightly from results shown in Fig. 1. All post-stimulation levels were significantly greater ($p < 0.05$) than the corresponding basal values.

Results are expressed as mean and standard deviations, the significance of differences between groups being assessed by the Student paired t-test and correlations between the increment in PA and Factor VIII determined using the Pearson correlation coefficient.

Results

Plasminogen Activator-Activity

PA, determined by clot lysis assay, increased 4.5 fold in plasma euglobulin following infusion of DDAVP at 0.3 ug/Kg. The rise was maximal immediately after infusion (Figs. 1 and 2) and all subjects showed at least a 3 fold rise in PA activity. Following VO the rise in PA activity averaged 3.4 fold (Fig. 2) although two volunteers, classified as low responders, gave less than 2 fold increases. A small, but significant (average 18%) rise in PA activity was also noted in the unoccluded arm. The mean rise in PA activity following exercise was 2.3 fold (Fig. 2) and again 2 subjects gave less than a 2 fold rise, only one of these being a low responder to VO. For all three stimuli the peak response of activity was significant ($p < 0.01$). Corresponding results (not shown) were obtained using the fibrin plate assay.

Plasminogen Activator-Antigen

By radioimmunoassay basal levels of t-PA antigen of 7 ng/ml were found. This rose 1.95 fold ($p < 0.01$) following DDAVP infusion, 4.20 fold ($p < 0.05$) after VO and 1.33 fold ($p < 0.05$) after exercise (Figs. 1 and 2). This assay also confirmed the significant rise in PA in the unoccluded arm following VO (1.31 fold, $p < 0.05$). Fig. 2 also shows the proportion of t-PA antigen which can bind fibrin, and is thus not found in serum, differs for each stimulus.

Factor VIII

Fig. 3 shows that during DDAVP infusion VIII:C rises significantly before VIIIR:Ag, although such differences are marginal. The rise in VIIIR:RCF after DDAVP infusion parallelled the increase in VIII:C more closely than that of VIIIR:Ag. Maximum levels of these activities did not occur until 30 min or more after infusion. All subjects showed at least a 2.3 fold increase in VIII:C 45 min after infusion (mean 2.63 fold, $p < 0.01$) and at least a 1.6 fold increase in VIIIR:Ag 75 min after DDAVP infusion (mean 2.49 fold, $p < 0.01$).

In contrast VO produced a lower response in both VIII:C (1.4 fold, $p < 0.05$) and VIIIR:Ag (1.48 fold, $p < 0.05$) and one subject gave no response at all. Similar results were obtained following exercise, although in this case the increase in VIIIR:Ag of 1.24 fold ($p < 0.05$) was less than that of VIII:C (1.47 fold; $p < 0.05$), and a different subject was a non-responder (Fig. 4).

For all three stimuli assay of VIIIC:Ag confirmed the results obtained by VIII:C measurement and in each case the rise in VIIIR:RCF was significant ($p < 0.05$, Fig. 4). Comparison of PA activity and VIII:C revealed a significant correlation ($p < 0.05$) between the increments in these two proteins following VO and exercise, but not DDAVP infusion.

Other Assays

Plasma concentrations of fibrinogen, antithrombin III, beta-thromboglobulin, platelet factor 4, thrombospondin and total protein did not change after DDAVP infusion or exercise. VO resulted in some increase but these could be accounted for in terms of haemoconcentration as shown by a 19% increase in total protein content of plasma obtained from the occluded arm. In the two volunteers who gave the highest factor VIII:C response to DDAVP no change in factor XII was found following any of the three stimuli.

Direct radioimmunoassay of plasma prostacyclin metabolites showed a basal level of 22 ± 6 pg/ml. This rose, but not significantly, after DDAVP infusion (17 ± 45%, n = 3), VO (40 ± 53%, n = 4) and exercise (9 ± 36%, n = 5).

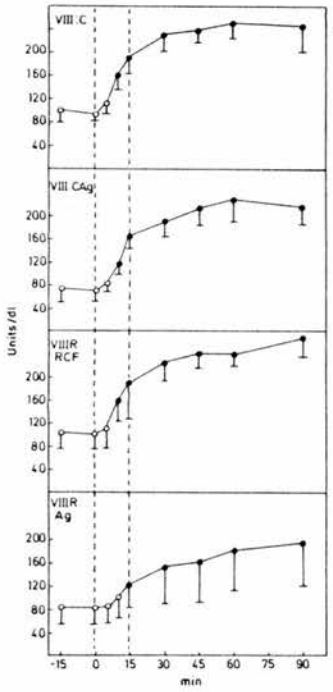


Fig. 3

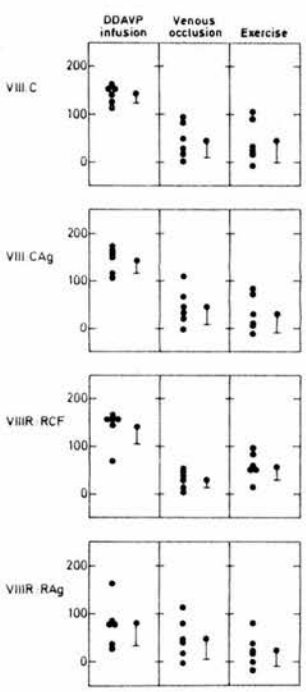


Fig. 4

Fig. 3 Changes in factor VIII following DDAVP infusion. Factor VIII (u/dl) determined as VIII:C, VIIIC:Ag, VIIIR:RCF and VIIIR:Ag after infusion of 0.3 ug DDAVP/Kg. See Fig. 1 for details.

Fig. 4 Factor VIII increments following different stimuli. Individual and mean (± SD) increases (u/dl) in the various factor VIII activities 30 min after DDAVP infusion, and immediately after venous occlusion and exercise. Average basal levels of the activities were VIII:C 101, VIII:CAg 83, VIIIR:RCF 97 and VIIIR:Ag 98 u/dl. See text for significance of these results.

However, in nine volunteers, who all flushed, infused at a higher (0.4 ug/Kg) dose of DDAVP, the 66 ± 54% increase in plasma prostacyclin metabolites was significant ($p < 0.01$). At this same dose a 24% rise was noted in a patient with severe von Willebrand's disease who had normal basal levels of t-PA antigen (5.5 ng/ml) and activity (4 ELT units) but exhibited no factor-VIII or PA response to DDAVP.

Preliminary studies in 3 subjects showed that infusion of 0.4 ug/Kg DDAVP had no effect on the PA or factor VIII response to VO determined 2 hr later. In 2 of these a rise in plasma prostacyclin metabolites following VO was demonstrated the previous day, but this was abolished by DDAVP infusion prior to VO.

Discussion

The three stimuli used in this study were chosen to produce a maximal response within a short period, although two of the six subjects were not exhausted by the standard exercise. Venous occlusion for longer than 20 min produces no further increase in factor VIII (25). Using these stimuli the increase in plasma PA and factor VIII differed in each case and discrepancies were noted between the change in activity and antigen levels for each protein. Thus DDAVP and VO produced a rapid rise in PA activity while exercise had less effect. In contrast t-PA antigen was increased most by VO. For each stimulus the proportion of t-PA antigen found in serum also differed. This material is apparently t-PA-inhibitor complexes which, unlike the free enzyme (26), is inactive and unable to bind fibrin (22, 27). In the present study, the proportion of antigen bound to fibrin during clotting varied from 8% under basal conditions, to 45% following VO. The formation of t-PA-inhibitor complexes explains the discrepancies between activity and antigen assays since the stimuli used here have no effect on intrinsic or urokinase-like PA (28, 29).

Factor VIII was released more slowly than PA following DDAVP infusion, but despite this, DDAVP produced a greater increase in plasma factor VIII than VO or exercise. In each case, except VO, an excess of factor VIII:C over factor VIII:RAg was observed after stimulation. This has also been reported for submaximal VO (30). The rapid increase in factor VIIIR:RCF, relative to VIIIR:Ag, after DDAVP infusion may be explained by the release of the higher multimers of VIIIR:Ag, which have been associated with VIIIR:RCF activity (31).

Since PA and factor VIIIR:Ag are produced by endothelial cells (32, 33), DDAVP, VO and exercise might also be expected to affect other potential endothelial markers. In fact, no change in plasma levels of fibronectin (34), thrombospondin (35), antithrombin III (36) or platelet factor 4 (37) was observed after any of these stimuli, although high doses of DDAVP produced a significant rise in plasma prostacyclin metabolites. This is unrelated to changes in PA or factor VIII since a patient with severe von Willebrand's disease who gave no PA or factor VIII response following DDAVP infusion retained some prostacyclin metabolite response. Furthermore, prior DDAVP infusion had no effect on the PA or factor VIII response to VO in normal subjects but abolished the previously observed prostacyclin metabolite response.

Previous studies on changes in plasma 6-keto prostaglandin F_1 during, or following DDAVP infusion have produced contrasting results (14, 15, 38, 39, 40). Some of these discrepancies may arise from differences in the specificity of the various antibodies used in the radioimmunoassays (41). The assay used here was performed on unextracted plasma samples and had a sensitivity of 5 pg/ml, detecting 95 ± 9 per cent of added 6-keto prostaglandin F_1. Equivalent results are obtained following addition of hydrolised prostacyclin to the assay system. The assay detects a dose-dependent increase in plasma antigen during infusion of prostacyclin to patients, and an undetectable level of antigen in the plasma of patients taking non-steroidal anti-inflammatory drugs (Belch – in preparation). The assay thus detects metabolites of prostacyclin. Although it was originally described as an assay for 6-keto prostaglandin F_1, in view of the fact that only 40 per cent of prostacyclin is metabolised to this product (42) and mass spectrometry reveals no change in 6-keto prostaglandin F_1 after DDAVP (40), the somewhat cumbersome term, prostacyclin metabolite, has been used in preference.

In summary, DDAVP infusion, venous occlusion and exercise produce a specific increase in plasma PA and factor VIII, but the extent of release and the antigen:activity ratio for each protein differs for each stimulus. Different subjects also gave low responses to each stimulus. Such differences must be explained by any hypothesis advanced to explain the mechanism of release by different stimuli. Despite numerous studies such mechanisms remain unclear, although it has been suggested that DDAVP acts via a cerebral receptor (5, 43) and that the factor VIII, but not PA, release following exercise is inhibited by beta-adrenergic blocking agents, whereas such drugs have no effect on the response to venous occlusion (44, 45). Such results imply that, if factor VIII and PA release occurs from a common tissue store, different stimuli act through different pathways and this might account for some of the results presented here.

Acknowledgements

The assistance of Mr. G. McKay, Mr. I. Abbott and Mrs. B. Griffin in performing factor VIII:RCF, antithrombin III and VIII:CAg assays respectively is gratefully acknowledged. Dr. D. Collen (Department of Medical Research, Catholic University of Leuven, Belgium) kindly supplied specific antiserum and melanoma activator used in setting up the radioimmunoassay for t-PA. A. Farrugia was supported by a British Council Commonwealth Scholarship (MLT/1/81).

References

1 Cash J D, Gader A M A, Da Costa J. The Release of Plasminogen Activator and Factor VIII by LVP, AVP, DDAVP, ATIII and OT in Man. Br J Haematol 1974; 27: 363–364.
2 Mannucci P M, Aberg M, Nilsson I M, Robertson B. Mechanism of Plasminogen Activator and Factor VIII Increase After Vasoactive Drugs. Br J Haematol 1975; 30: 81–93.
3 Nilsson I M, Vilhardt H, Holmberg L, Astedt B. Association between Factor VIII Related Antigen and Plasminogen Activator. Acta Med Scand 1982; 211: 105–112.
4 Prowse C V, Sas G, Gader A M A, Cort J H, Cash J D. Specificity in the Factor VIII Response to Vasopressin Infusion in Man. Br J Haematol 1979; 41: 437–447.
5 Cash J D, Gader A M A, Mulder J L, Cort J H. Structure-Activity Relations of the Fibrinolytic Response to Vasopressin in Man. Clin Sci Mol Med 1978; 54: 403–409.
6 Brozovic M. Physiological Mechanisms in Coagulation and Fibrinolysis. Br Med Bull 1977; 33: 231–238.
7 Mannucci P M, Ruggeri Z M, Pareti F I, Capitanio A. DDAVP: A New Pharmacological Approach to the Management of Haemophilia and von Willebrand's Disease. Lancet 1977; 1: 689–872.
8 Kobayashi I. Treatment of Haemophilia A and von Willebrand's Disease Patients with an Intranasal Dripping of DDAVP. Thromb Res 1979; 16: 775–780.
9 Brommer E J P, Barrett-Bergshoeff M M, Allen R A, Schicht I, Bertina R M, Schalekamp M A D H. The Use of Desmopressin Acetate as a Test of Fibrinolytic Capacity of Patients – Analysis of Responders and Non-Responders. Thromb Haemostas 1982; 48: 156–161.
10 Rijken D C, Wijngaards G, Welbergen J. Relationship between Tissue Plasminogen Activator and the Activators in Blood and Vascular Wall. Thromb Res 1980; 18: 815–830.
11 Kluft C. Studies on the Fibrinolytic System in Human Plasma: Quantitative Determination of Plasminogen Activators and Pro-activators. Thromb Haemostas 1979; 41: 365–383.
12 MacGregor I, Pepper D. A Direct Functional Assay and an Immunoassay for Plasminogen Activators. Haemostasis 1982; 11 (suppl. 1): Abstract 117.
13 Peake I R. In: The Haemophilias. Bloom A (Ed). Churchill Livingstone 1982; pp. 92–105.
14 Belch J J F, Small M, McKenzie F, Hill P A, Lowe G D O, McIntyre D E, Forbes C D, Prentice C R M. DDAVP Stimulates Prostacyclin Release. Thromb Haemostas 1982; 47: 122–123.
15 Greaves M, Preston F. Plasma 6-Keto-Prostaglandin F_1: Fact or Fiction. Thromb Res 1982; 26: 145–157.
16 Prowse C V, Douglas J G, Forrest J A H, Forsling M L. Haemostatic Effects of Lysine Vasopressin and Triglycyl Lysine Vasopressin Infusion in Patients with Cirrhosis. Eur J Clin Invest 1980; 10: 49–54.
17 Macfarlane D E, Stibbe J, Kirby E P, Zucker M B, Grant R A, McPherson J. A Method for Assaying von Willebrand Factor (ristocetin cofactor). Thrombos Diathes Haemorrh 1975; 34: 306–308.
18 Rotblat F, Tuddenham E G D. Immunological Studies of Factor VIII Coagulant Activity (VIII:C):1:Assays Based on a Haemophilic and an Acquired Antibody to VIII:C. Thromb Res 1981; 21: 431–445.
19 Laurell C B. Electroimmunoassay. Scand J Clin Lab Invest 1972; 29 (suppl. 124): 21–37.
20 Clauss A. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol 1957; 17: 237–246.
21 Gornall A G, Bardawill C J, David M M. Determination of Serum Proteins by Means of the Biuret Reaction. J Biol Chem 1949; 177: 751–766.
22 MacGregor I R, Prowse C V. Tissue Plasminogen Activator in Human Plasma Measured by Radioimmunoassay. Thromb Res 1983; 31: 461–474.
23 Prowse C V, Pepper D S, Dawes J. Prevention of the Platelet Alpha-Granule Release Reaction by Membrane Active Drugs. Thromb Res 1982; 25: 219–227.
24 Dawes J, Clemetson K J, Gogstad G O, McGregor J, Clezardin O, Prowse C V, Pepper D S. A Radioimmunoassay for Thrombospondin Used in a Comparative Study of Thrombospondin, B-Thromboglobulin and Platelet Factor 4 in Healthy Volunteers. Thromb Res 1983; 29: 569–581.
25 Robertson B A, Pandolfi M, Nilsson I M. Fibrinolytic Capacity in Healthy Volunteers as Estimated from the Effect of Venous Occlusion of Arms. Acta Chir Scand 1972; 138: 429–440.
26 Rijken D C, Collen D. Purification and Characterisation of the Plasminogen Activator Secreted by Human Melanoma Cells in Culture. J Biol Chem 1981; 256: 7035–7041.
27 Rijken D C, Juhan-Vague I, De Cock F, Collen D. Measurement of Human Tissue-Type Extrinsic Plasminogen Activator by a Two-Site Immunoradiometric Assay. J Lab Clin Med 1983; 101: 274–284, 285–294.
28 Marsh N, Gaffney P. Some Observations on the Release of Extrinsic and Intrinsic Plasminogen Activators During Exercise in Man. Haemostasis 1980; 9: 238–247.
29 Holmberg L, Nilsson I M, Astedt B. Immunoreactive Tissue Activator and Urokinase in Plasma after Various Stimuli. Haemostasis 1982; 11 (suppl. 1): abstr. 55.
30 Davis G L. Haemostatic and Physiological Changes Induced by Various Lengths of Venous Occlusion (abstr.). Fed Proc 1982; 41: 704.
31 Ruggeri Z M, Mannucci P M, Lombardi R, Federici A B, Zimmerman T S. Multimeric Composition of Factor VIII – von Willebrand Factor Following Administration of DDAVP – Implications for Patho-

physiology and Therapy of von Willebrand's Disease Subtype. Blood 1982; 59: 1272–1278.
32 Todd A S. The Histological Localisation of Fibrinolysin Activator. J Pathol 1959; 281–283.
33 Jaffe E A, Hoyer L W, Nachman R L. Synthesis of Antihaemophilic Factor Antigen by Cultured Human Endothelial Cells. J Clin Invest 1973; 52: 2757–2764.
34 Jaffe E A, Mosher D F. Synthesis of Fibronectin by Cultured Human Endothelial Cells. J Exp Med 1978; 147: 1779–1791.
35 Mosher D F, Doyle M J, Jaffe E A. Synthesis and Secretion of Thrombospondin by Cultured Human Endothelial Cells. J Cell Biol 1982; 93: 343–348.
36 Chan T K, Chan V. Antithrombin III, The Major Modulator of Intravascular Coagulation, is Synthesised by Human Endothelial Cells. Thromb Haemostas 1981; 46: 504–506.
37 Busch C, Dawes J, Pepper D S, Wasteson A. Binding of Platelet Factor 4 to Cultured Human Endothelial Cells. Thromb Res 1980; 19: 129–137.
38 D'Angelo A, Capitanio A, Smith J B, Valsecchi C, Mannucci P M. Effects of Des-Amino-D-Arginine Vasopressin (DDAVP) on Plasma Levels of Platelet and Endothelial Cell Release Products. Thromb Haemostas 1983; 49: 64.
39 Belch J J F, McLaren M, Lowe G D O, McIntyre D E, Forbes C D, Prentice C R M. Effect of Des-Amino-D-Arginine Vasopressin (DDAVP) on Plasma Levels of Platelet and Endothelial Cell Release Products – Reply. Thromb Haemostas 1983; 49: 65.
40 Barrow S E, Heavey D J, Jacqz E, Blair I A, Dollery C T. Des-Amino-D-Arginine Vasopressin does not Increase Circulating Levels of Prostacyclin or Thromboxane A_2 during Infusion in Man. Thromb Haemostas 1983; 50: 622.
41 Viinka L, Ylikorkala O. Different Results of Plasma 6-Keto Prostaglandin F_1 Measurements Utilising Two Antisera with Apparently Similar Specificity. Prost Leuk Med 1982; 9: 1–7.
42 Rosenkranz B, Fisher C, Frohlich J C. Prostacyclin Metabolites in Human Plasma. Clin Pharm Thera 1981; 29: 420.
43 Prowse C V, Dow R C, Sheward W J, Fink G, Boulton F E. The Plasminogen Activator Response to Drug Infusion and to Direct Hypothalamic Stimulation in Dogs and Rats. Progress in Fibrinolysis. J F Davidson (Ed). Churchill Livingstone 1983; 6: 104–106.
44 Ponari O, Civardi E, Megha A, Pini M, Poti R, Dettori A G. Effect of Alpha and Beta-Blocking Drugs on the Clotting and Fibrinolytic Response to Venous Stasis in Man. Br J Haematol 1973; 24: 463–470.
45 Cohen R J, Epstein S E, Cohen L S, Dennis L H. Alterations of Fibrinolysis and Blood Coagulation Induced by Exercise and the Role of Beta-Adrenergic Receptor Stimulation. Lancet 1968; 2: 1264–1266.

Received June 20, 1983 Resubmitted December 7, 1983
Accepted December 9, 1983

Thromb Haemostas (Stuttgart) 51 (3) ■–■ (1984)

Studies on the Procurement of Coagulation Factor VIII: Selective Precipitation of Factor VIII with Hydrophilic Polymers

A. Farrugia*, B. Griffin**, D. Pepper** and C. Prowse*

From the Edinburgh & S-E Scotland Blood Transfusion Service*, Royal Infirmary, and Scottish National Blood Transfusion Service Headquarters Unit Laboratory, Edinburgh, Scotland

Key words

Factor VIII – Fibrinogen – Polymer – Protein-precipitation

Summary

Early work on the purification of factor VIII using polyethylene glycol (PEG) indicated that other polymers might also be used to precipitate factor VIII leaving fibrinogen in solution. Recently polyvinylpyrrolidone (PVP) has also been advocated for this purpose.

In this study, different concentrations and molecular weights of hydroxyethyl starch, dextran, PEG, PVP, Ficoll, Percoll and albumin were examined for their ability to precipitate the factor VIII complex from *cooled* (not frozen) fresh CPD plasma. At optimal concentrations near quantitative recovery of VIIIR:Ag and VIII:C:Ag was achieved in 2 hr with minimal precipitation of fibrinogen or total protein. The best separations were observed with hydroxyethyl starch, albumin or Ficoll. PVP and PEG gave inferior purifications. Results for cryoprecipitate and intermediate-purity factor VIII concentrate were inferior to those obtained with plasma. Simple pre-concentration of plasma prior to chilling is an attractive alternative for large scale continuous production.

Introduction

Since its introduction, by Pool and Shannon in 1965 (1), cryoprecipitation has become the major method for production of factor VIII concentrates from plasma in blood banks. Bulk cryoprecipitation (2) is also the standard initial stage in production of intermediate and high purity factor VIII concentrates for clinical use (3).

Improvements in the yield and purification of factor VIII from plasma by cryoprecipitation may be achieved by modification of the thawing technique (4, 5) or by collection of blood in heparin (6). The early observations of Polson et al. (7) suggested that polymer precipitation might provide a method of plasma protein fractionation, but in the case of factor VIII, polymers such as polyethylene glycol (3, 8) have only been used in the later steps of purification. Recently, polyvinylpyrrolidone has been proposed as a selective precipitant for factor VIII from plasma and cryoprecipitate (9).

Despite the large differences in molecular weight between factor VIII complex (ca. 10×10^6) and fibrinogen (0.34×10^6) no high-yielding large scale separation has been based on this fact. Small scale separation (by gel filtration) is very successful but cannot be applied on a large scale. When one looks at the specific requirements of a purified factor VIII concentrate, it is clear that removal of fibrinogen is the major unsolved problem. If fibrinogen can be largely eliminated from the product then (a) a smaller reconstitution volume is possible, (b) a more rapid resolution time is possible, (c) the reconstituted material is less likely to clot or clog filters etc., and (d) the material is more easily sterilised by filtration, radiation or heat. It was the purpose of this work to produce a simple, reliable process for the concentration of factor VIII complex with retention of biological activitie(s) in high yield and with the elimination of as much fibrinogen as possible.

The precipitation of plasma proteins by a variety of hydrophilic polymers was examined to determine if they would allow purification of factor VIII with a high yield and purification. The results indicate that this can be achieved with hydroxyethyl starch, Ficoll or albumin, or simply concentration of plasma, without the need for freezing.

Correspondence to: Dr. C. Prowse, S-E Scotland Blood Transfusion Service, Royal Infirmary, Edinburgh, Scotland

Materials and Methods

Human plasma was obtained from normal blood bank donations taken into CPD anticoagulant.

Cryoprecipitate was prepared by the thaw-siphon technique (10) from single donations. In some experiments, cryoprecipitate was prepared from a two litre plasma pool and extracted into 0.03 plasma volumes of tris buffer (3).

Intermediate purity factor VIII concentrate was obtained from the Protein Fractionation Centre (Edinburgh) and was a freeze-dried preparation, prepared by the method of Newman et al. (3). For these studies, this material was dissolved in one half of the recommended volume.

Citrate-saline (C-S) buffer 0.015 M citrate, 0.15 M NaCl, pH 7.2.

Glycine-citrate-glucose (GCG) buffer 0.06 M NaCl, 0.019 M sodium citrate, 0.007 M glucose, 0.013 M Glycine, pH 6.5.

Polymer stock solutions were made by dissolving 40% w/w of polymer in isotonic saline. The following polymers were used: polyethylene glycol (PEG) Mr = 6,000 (Union Carbide); polyvinylpyrrolidone (PVP 10, PVP 40) Mr = 10,000 and Mr = 40,000 (Sigma); dextran T 70, dextran T 150, Percoll and Ficoll 70 (Pharmacia Fine Chemicals); hydroxyethyl starch Mr = 40,000 (HES 40), Mr = 350,000 (HES 350) and Mr = 450,000 (HES 450) were generously supplied by the Army Blood Supply Depot, Aldershot. A 40% stock solution of human albumin was made by concentrating a 20% solution (Immuno) by ultrafiltration on an Amicon PM-10 Membrane.

Assays

Factor VIII:C was assayed by a one-stage technique based on the activated partial thromboplastin time, using an automated clot detector (Coag-a-pet), and reagents supplied by General Diagnostics Inc. Factor VIIIR:Ag was assayed by a one-site fluid phase immuoradiometric assay (11), factor VIIIR:Ag, fibrinogen and fibronectin were assayed by electroimmunoassay (12) using commercially obtained antisera (Behringwerke).

Total protein was assayed by the Biuret technique (13). Dextran was found to cause interference in this assay. Dextran-containing solutions were, therefore, assayed by absorbance at 280 nm following suitable dilution.

Polymer-Induced Precipitation

Dilutions of stock polymer solutions were added to the protein mixtures under investigation until the desired final concentration was reached. The mixtures were then held under the conditions described in Results, after which the precipitates formed were separated by centrifugation for 13,000 g min at the temperature used for precipitation. Precipitates were dissolved in G-C-G buffer, in the experiments with plasma, or in C-S buffer in the experiments with cryoprecipitate and concentrates. The precipitates were dissolved in 0.1–0.03 of the original volume for plasma, 0.5 volume for cryoprecipitate and 1 volume for concentrate.

Plasma Pre-Concentration and Cold Precipitation

Plasma was concentrated by ultrafiltration on an Amicon PM-10 Membrane or by dialysis against 50% w/w PEG 6000 in tris-buffered saline pH 7.2 (14). The concentrated plasma was then held at 0° C for 30 min, spun, and the resultant precipitate dissolved in 0.1 of the original volume of G-C-G buffer.

Results

Polymer Precipitation of Chilled Plasma

Preliminary studies indicated that low temperatures were necessary to effect precipitation with certain of the polymers. All experiments were therefore performed by adding polymer solution to the appropriate concentration and chilling the mixtures for 2 hr on melting ice.

Fig. 1 shows the dose-response results for the various polymers when tested on 20 ml amounts of plasma. All polymers tested produced selective precipitation of factor VIII:C and VIIIR:Ag, but some resulted in a better purification than others.

There is some evidence that polymers at their highest concentrations demonstrated inhibition of the factor VIII:C bioassay. This was indicated by the discrepancy between bioassay and factor VIIIR:Ag immunoassay, an effect that was particularly noticeable with HES 450, dextran T 150 and PVP, although at low polymer concentrations there was concordance between the yields of factor VIII:C and factor VIIIR:Ag. An inhibition of factor VIII:C bioassay by PVP has been demonstrated by Zuber and Morgenthaler (15). Such an inhibition might explain the drop in factor VIII:C, but not factor VIIIR:Ag at high polymer concentrations. Specific inactivation of factor VIII:C at these concentrations cannot be discounted and it was noticeable that PVP produced clotted precipitates with low factor VIII:C yields in our hands. The virtually complete recovery of both factor VIII:C and factor VIIIR:Ag in precipitation obtained with, for example, HES 40 and Ficoll 70 indicate that, at the concentrations tested, these compounds did not degrade factor VIII:C or affect the bioassay.

The various hydroxyethyl starches and Ficoll 70 gave quantitative recoveries of FVIII:C and FVIIIR:Ag at concentrations which produced minimal precipitation of fibrinogen and total protein. At higher concentrations, increased fibrinogen precipitation was obtained and, in the case of HES 350 and HES 450, some degree of insolubility. At concentrations of between 2 and 4%, maximal recovery and purification of factor VIII was obtained.

Other polymers were found to be less selective. Except at the lowest concentrations tested, PEG precipitated FVIII and fibrinogen together and formed very insoluble precipitates. Precipitates obtained by dextran addition were sparingly soluble in the buffer used. Dextran solutions tended to be very viscous and polymer was retained in the precipitate. Since no attempt was made to wash these precipitates, the insolubility could be due to retention of dextran. Precipitates obtained with PVP were unstable as some clotting was observed upon freezing and thawing samples.

Precipitation of factor VIII from plasma by albumin addition is shown in Fig. 2. At final concentrations between 5 and 6% w/w added albumin, virtually complete precipitation of factor VIII was obtained, with little fibrinogen or other proteins being precipitated.

Table 1 summarises the optimal conditions for FVIII precipitation from plasma at 0° C, using the various polymers on a small scale (20 ml). These optimal conditions were then applied to plasma precipitation on a medium scale (<1 litre). The effect of an overnight incubation as well as the standard 2 hr one was also investigated. The results are summarised in Table 2. As can be seen, precipitations on a medium-scale for 2 hr resulted in concentrates containing the bulk of the factor VIII with little contaminating proteins. In some cases the yield of factor VIII:C was confirmed by assay of VIIIR:Ag, this assay being only available for the latter part of this study. Between 60 and 90 percent of the precipitated protein was fibrinogen and fibronectin. Overnight incubation, however, resulted in decreased yields of factor VIII and increased precipitation of other proteins. In overnight incubation experiments, plasma was subjected to a

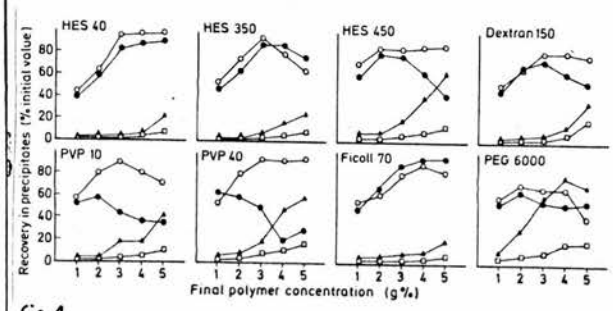


Fig. 1

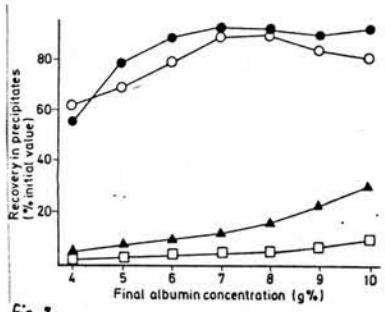


Fig. 2

Fig. 1 Precipitating effect of various polymers on human plasma at 0° C. Polymer stock solutions were added to 20 ml aliquots until the indicated final concentrations (weight/weight) were achieved. The mixtures were held in melting ice for 2 hr, centrifuged and activities in the precipitates determined. (–●– FVIII:C; –○– FVIIIR:Ag; –▲– fibrinogen; –□– total protein). Mean percent precipitation is shown for 3 to 6 experiments for each polymer.

Fig. 2 Precipitation effect of human albumin on human plasma at 0° C. Conditions were as in Fig. 1.

Table 1 Small scale (20 ml) polymer precipitation of fresh plasma. Conditions for optimal factor VIII purification.

Polymer	Final concentration (% w/w)	n	VIII:C u (% yield)	VIIIR:Ag u (% yield)	Fibrinogen mg (% yield)	Total protein mg (% yield)	SP. ACT. u/mg
HES 40	4	6	14.2 (89)*	19.4 (108)	5.4 (9)	24 (2)	0.59
HES 350	3	6	14.9 (93)	17.1 (95)	5.4 (9)	12 (1)	1.24
HES 450	2	3	13.6 (85)	15.5 (86)	5.4 (9)	48 (4)	0.28
Dextran T-70	2	4	12.6 (79)	13 (72)	4.2 (7)	36 (3)	0.35
Dextran T-150	3	4	11.7 (73)	14.4 (80)	4.2 (7)	36 (3)	0.33
PVP 10	3	5	6.7 (42)	15.8 (88)	10.8 (18)	48 (4)	0.14
PVP 40	2	4	9.8 (61)	14.4 (80)	4.8 (8)	24 (2)	0.41
Ficoll 70	4	5	14.6 (91)	15.5 (86)	4.8 (8)	48 (4)	0.30
PEG 6000	2	3	9.8 (61)	12.8 (71)	18.6 (31)	96 (8)	0.10
Percoll	3.5	3	4.3 (27)	9 (50)	0.7 (1.1)	12 (1)	0.36
Human Albumin	6	7	14.2 (89)	15.8 (88)	4.8 (8)	24 (2)	0.59

Results presented as the mean amounts of the respective activities recovered in the precipitates, with the respective yields as % of starting plasma in brackets.

* To allow clarity standard deviations have not been shown above but were typically ± 15 percent from the mean values shown.

Table 2 Medium scale polymer precipitation of fresh plasma.

Process	n	VIII:C (% yield)	VIIIR:Ag (% yield)	VIIIR:Ag (% yield)	Fibrinogen (% yield)	Fibronectin (% yield)	Total protein (% yield)	SP. ACT. u/mg
A. Two hours incubation								
4% HES 40	8	67 ± 17	64 ± 12	99 ± 25	16 ± 3	51 ± 28	1.2 ± 0.3	0.93 ± 0.21
3–4% HES 350	2	75 ± 11	n.d.	106 ± 18	25 ± 8.4	n.d.	2.6 ± 0.7	0.51 ± 0.21
4% Ficoll 70	6	66 ± 16	58 ± 19	75 ± 23	15 ± 4	n.d.	1.4 ± 0.5	0.84 ± 0.23
Concentration ×2 (Dialysis vs. 50% PEG)	1	46	n.d.	82	8	83	0.6	1.27
B. Overnight incubation								
4% HES 40	2	61 ± 16	n.d.	96 ± 13	22 ± 8	n.d.	4.2 ± 1.3	0.24 ± 0.06
3% Ficoll 70	2	59 ± 11	53 ± 13	83 ± 17	16 ± 4	n.d.	1.9 ± 0.4	0.52 ± 0.22
4% Ficoll 70	6	49 ± 19	60 ± 23	98 ± 17	30 ± 12	n.d.	3.5 ± 1.7	0.21 ± 0.15
Concentration ×2 (Amicon membrane)	4	83 ± 17	n.d.	89 ± 18	4 ± 1.3	n.d.	0.9 ± 0.3	1.53 ± 0.41

Results presented as percentage yields of starting plasma. Plasma volumes ranged from 400–1800 mls.
n.d. = not determined.

second spin prior to precipitation in order to minimise cellular contamination. This was found to be necessary to ensure the precipitates would dissolve adequately.

Plasma Concentration and Cold Precipitation

Since albumin addition resulted in factor VIII precipitation, simple concentration of plasma was also tried as a means of precipitating factor VIII (14). In one experiment using dialysis against 50% PEG for 2 hr, precipitation of VIIIR:Ag with minimal precipitation of proteins was accomplished, but only half of the VIII:C was recovered in the precipitate (Table 2). Ultrafiltration gave better results, but at least with our equipment, took approximately 8 hr when processing 400 mls plasma.

Polymer Precipitation of Plasma Fractions

A. *Cryoprecipitate.* The two types of cryoprecipitate used differed in their composition: cryoprecipitate prepared by the modified thaw-siphon technique having a lower protein, factor VIII and fibrinogen content than bulk cryoprecipitate extracted into tris buffer.

Fig. 3 shows the results obtained for thaw-siphon cryoprecipitate with HES 450 and albumin. Precipitation of factor VIII and fibrinogen occurred concurrently, although some discrimination from total protein was observed. Separate experiments with

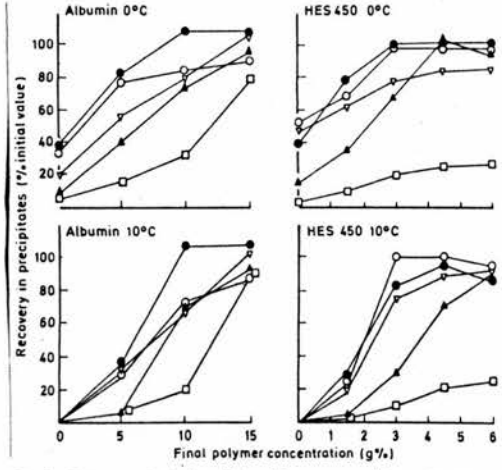


Fig. 3 Polymer precipitation of thaw-siphon cryoprecipitate. Conditions were as in Fig. 1 for incubation at 0° C; incubation at 10° C were carried out in a waterbath. (-▽- FVIII:CAg).

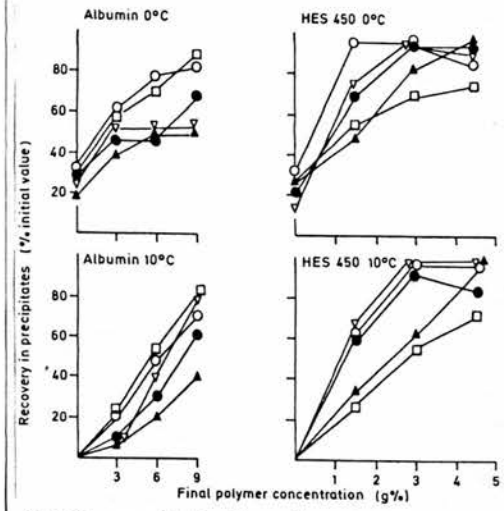


Fig. 4 Polymer precipitation of intermediate purity FVIII concentrate. Conditions were as in Figs. 1 and 3.

precipitation carried out at 10° and 20° did not improve the discrimination between factor VIII and fibrinogen. Results obtained with tris extract of bulk cryoprecipitate were similar (results not shown).

B. *Intermediate purity concentrate.* Albumin and HES 450 were again used as precipitation agents. As can be seen in Fig. 4, no useful discrimination between factor VIII, fibrinogen and total protein was observed under the conditions studied.

Discussion

This study demonstrates that precipitation of plasma with hydrophilic polymers allows selective harvesting of factor VIII. Precipitation of chilled citrated plasma with albumin, hydroxyethyl starches or Ficoll 70 was particularly effective and allowed a single step purification of factor VIII at a specific activity of 0.5 to 1.0 units/mg protein in greater than 80% yield, in 2 hr. In larger scale experiments, the yield of factor VIII:C reduced to about 70%. Precipitation with PVP in our hands, did not produce as good results as those described by Casillas and Simonetti (9), but it was found that these precipitates tended to clot, possibly due to the presence of impurities in the polymer (15). The other polymers tested in this study, dextran, polyethylene glycol and Percoll, while resulting in selective precipitation of factor VIII, gave inferior purification to those described above.

Previous studies by Alexander et al. (16, 17) with low concentrations of polymers added to chilled plasma indicated that prolonged incubation resulted in precipitation of fibrinogen and factor VIII with dextran. Although no data was given, these papers also indicated hydroxyethyl starch can precipitate factor VIII from plasma while albumin was found to be ineffective. Such differences presumably arise from the different experimental conditions. It seems likely that the mechanism of precipitation by the polymers described in this study is a molecular exclusion process similar to that described for dextran (18) and PEG (19), although this does not fully explain the differences between the effect of the various polymers tested.

It may be significant that the discrimination between factor VIII:C/VIIIR:Ag and fibrinogen/total protein was worst with a linear flexible polymer – PEG – while compact polymers such as albumin gave much better discrimination. Another factor influencing discrimination between factor VIII complex and fibrinogen is the time of holding at 0° C, 2 hr gives significantly better discrimination than 20 hr, i.e. it appears that FVIII complex is rapidly and completely precipitated within 2 hr whereas fibrinogen is slowly (and incompletely) precipitated over 20 hr.

With cryoprecipitate as starting material, discrimination between FVIII complex and fibrinogen was better at 10° C than 0° C, though it was never as good as with plasma as raw material (Fig. 3). However, a significant degree of concentration (ca. 2fold) could be achieved based on a similar final concentration of fibrinogen (3% HES 450, 10° C). Zuber and Morgenthaler (15) have presented similar results for PVP. Discrimination in intermediate purity FVIII concentrates as raw material was even poorer than that with cryoprecipitate, however the situation is complicated because depolymerisation of some of the FVIIIR:Ag/C complex to less than 1×10^6 MW has occurred during processing and this is likely to reduce the potential discrimination between fibrinogen and FVIII.

It is suggested that polymer precipitation of factor VIII from plasma has a potential application in at least three areas. Namely, in the preparation of purified factor VIII at high yield for research investigations and in the preparation of factor VIII from plasma for clinical use, without the necessity of freezing plasma either in bulk, e.g. from large pools of animal plasma, or on a smaller scale at blood bank level.

Despite the limited number of experiments it is clear that simple pre-concentration of plasma is an attractive alternative to polymer addition. With a concentration factor of ×2 (which could be achieved continuously by ultrafiltration) followed by a 2 hr chilling period at 0° C, it ought to be possible to produce "cryoprecipitate" continuously on an industrial scale in good yield and purity (Table 2). It remains to be shown, however, that this approach works as well with thawed bulk fresh frozen plasma as it does with fresh (non-frozen) plasma.

Acknowledgements

A.F. is the recipient of a grant from the British Council (MLT 01/81) and is on study leave from the Pathology Department, St. Luke's Hospital, Malta. We are grateful for supplies of hydroxyethyl starch from the Army Blood Supplies Depot, Aldershot, and to Colonel Parry for unpublished data on his work with HES.

B.G. is supported by a Scottish Home and Health Department research grant No. K/MRS/50/C387.

References

1 Pool J G, Shannon A E. Production of high-potency concentrates of antihaemophilic globulin in a closed-bag system; assay in vitro and in vivo. New Eng J Med 1965; 273: 1443–7.

2 Pool J G, Hershgold E J, Pappenhagen A R. High-potency antihaemophilic factor concentrate prepared from cryoglobulin precipitate. Nature 1964; 203: 312.

3 Newman J, Johnson A J, Karpatkin M, Puszkin S. Methods for the production of clinically effective intermediate and high purity factor VIII concentrate. Br J Haematol 1971; 21: 1–20.

4 Mason E C. Thaw-siphon technique for production of cryoprecipitate concentrate of factor VIII. Lancet 1978; 2: 15–9.

5 Prowse C V, McGill A. Evaluation of the 'Mason' (continuous-thaw-siphon) method for cryoprecipitate production. Vox Sang 1979; 37: 235–43.

6 Rock G A, Cruikshank W H, Tackaberry E S, Palmer D S. Improved yields of factor VIII from heparinized plasma. Vox Sang 1979; 36: 294–300.

7 Polson A, Potgieter G M, Largier J F, Mears G E F, Joubert F J. The fractionation of protein mixtures by linear polymers of high molecular weight. Biochim Biophys Acta 1964; 82: 463–75.
8 Hao Y C, Ingham K C, Wickerhauser M. Fractional precipitation of proteins with polyethylene glycol. In Methods of Plasma Protein Fractionation. Academic Press 1980. pp. 57–74.
9 Casillas G, Simonetti C. Polyvinylpyrrolidone (PVP) – a new precipitating agent for human and bovine factor VIII and fibrinogen. Br J Haematol 1982; 50: 665–72.
10 Mason E C, Pepper D S, Griffin B. Production of cryoprecipitate of intermediate purity in a closed system thaw-siphon process. Thromb Haemostas 1981; 46: 543–6.
11 Rotblat F, Tuddenham E G D. Immunologic studies of factor VIII coagulant activity (VIII:C). I. Assays based on a haemophilic and an acquired antibody to VIII:C. Thromb Res 1981; 21: 431–45.
12 Laurell C B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 1966; 15: 45–52.
13 Gornall A G, Bardawill C J, David M M. Determination of serum protein by means of the Biuret reaction. J Biol Chem 1949; 177: 751–66.
14 Owen W G, Wagner R H. Antihemophilic factor. A new method for purification. Thromb Res 1972; 1: 71–88.
15 Zuber T, Morgenthaler J J. Polyvinylpyrrolidone as a precipitating agent for factor VIII and fibrinogen. Br J Haematol 1982; 52: 517–8.
16 Alexander B, Odake K, Lawler D, Swanger M. Coagulation, hemostasis and plasma expanders: a quarter century enigma. Fed Proc 1975; 34: 1429–40.
17 Alexander B. Effects of plasma expanders on coagulation and haemostasis: dextran, hydroxyethyl starch and other macromolecules revisited. In Blood Substitutes and Plasma Expanders. A. R. Liss New York 1978. pp. 293–326.
18 Laurent T C. The interaction between polysaccharides and other macromolecules. The solubility of proteins in the presence of dextran. Biochem J 1963; 89: 253–7.
19 Juckes I R M. Fractionation of proteins and viruses with polyethylene glycol. Biochim Biophys Acta 1971; 229: 535–46.

Received March 5, 1984 Accepted March 23, 1984