STUDIES ON THE PROCUREMENT OF BLOOD COAGULATION FACTOR VIII

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Ph.D.

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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 GIE 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.

The fibrinopeptide A assays which were done by Dr. Hagop Bessos at the MRC/SNBTS Blood Components Assay Group.

ALBERT FARRUGIA
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Finally, I thank my wife, Josephine, for her constant love and encouragement for the whole period of our stay in Edinburgh.
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(A) INTRODUCTION

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(C) PROCESSING OF PLASMA TO BLOOD BANK FACTOR
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
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 quo Bulloch and Fildes 1911). 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 Thromboplastin 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 byautosomal 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>
<thead>
<tr>
<th>Name of Component (Abbreviation)</th>
<th>Characteristics and Physiological Function</th>
<th>Disease Associated With Deficiency</th>
<th>Methods of Detection / Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII Coagulant Antigen (VIII:Ag)</td>
<td>Antigenic expression of VIII:C</td>
<td>Haemophilia A. Mostly found as haemophilia A with total lack of VIII:C, about 10-20% of patients have detectable VIII:C, about 10% have haemophilia A²</td>
<td>a) Immunodensitometric assays using an antibody to VIII:C, liquid (Hanschke and Hoyeraal 1977); and solid phase assays (Peake 1982) can be used. b) Inhibition: neutralisation assay (Hanschke 1983)</td>
</tr>
<tr>
<td>von Willebrand Factor Protein (VWF)</td>
<td>Protein which corrects the bleeding time abnormality in von Willebrand's disease. Multimeric protein M₄ = 8 x 10^{-12} x 10^{-5}, subunit size 220,000 (Hoyer 1981), synthesised</td>
<td>von Willebrand's disease - various types due to deficiency and/or abnormality of VWF (Birnbaum and Rupprecht 1992): Usually autosomal dominant trait. Incidence about the same as</td>
<td>Exploration of the bleeding time. Semi-quantitative methods - platelet adhesion in glass bead column (Salzman 1983); platelet adhesion to perfused subendothelium</td>
</tr>
<tr>
<td>von Willebrand Factor Protein (VWF)</td>
<td>in vascular endothelium (Hill et al 1973), Concentration in plasma 5-10 mg/ml. Mediation of platelet adhesion to subendothelium (Weiss et al 1978)</td>
<td></td>
<td>(Birnbaum et al 1978)</td>
</tr>
<tr>
<td>Ristocetin Co-factor (VIIIIR:RCP)</td>
<td>A property of von Willebrand Factor which promotes agglutination of platelets in the presence of the antibiotic ristocetin. The equivalent property in bovine and porcine VWF agglutinates human platelets without ristocetin</td>
<td>von Willebrand's disease</td>
<td>VIIIIR:RCP assay using fixed platelets and ristocetin (Ristorino et al 1971); does not always correlate with assays for VWF activity (Ristow et al 1983)</td>
</tr>
</tbody>
</table>
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

PGI2 = prostacyclin

GP = glycoprotein

TXA2 = thromboxane A2
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
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 

\[ \text{M} \text{M}_{\text{in}} \approx 8 \times 10^4 - 2 \times 10^5 \]

(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 fibrinogenrich 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 Blomb~ck modification of Cohn Fraction I (Blomb~ck 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>
<thead>
<tr>
<th>Name of Preparation</th>
<th>Method of Production</th>
<th>Purification, Over Plasma (X)</th>
<th>General Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corp Fraction (P-2) (Cola et al. 1958)</td>
<td>Precipitation of plasma proteins with 95 ethanol at -70°C, pH 7-8 main of the fibrinogen and factor VIII are precipitated</td>
<td>3-20</td>
<td>Careful techniques allow factor VIII to be harvested at high yields. However, P-2 is poorly soluble in dilute plasma and can not be sterilized, and so has to be prepared by elaborate sterile techniques.</td>
</tr>
<tr>
<td>Fp-O (Blomback 1955)</td>
<td>Extraction of Fp-O at 0°C with a glycerol-citrate buffer and subsequent precipitation in ammonium sulfate</td>
<td>10-30</td>
<td>Extraction procedure improves greatly the solubility and stability of Fp-O; it will be used extensively in Scandinavia.</td>
</tr>
<tr>
<td>Ether-Trimethyl Phosphatase (P-3) (Berkman &amp; Wolf 1977)</td>
<td>Precipitation of plasma proteins with 0.9% NaCl at 0°C</td>
<td>25</td>
<td>Same characteristics and drawbacks as Corp P-2.</td>
</tr>
<tr>
<td>Fp-Ox (Blomback et al. 1955)</td>
<td>Treatment of P-4 with tannic acid to remove fibrinogen</td>
<td>40-160</td>
<td>Experience is with small batches; attempts to replicate initial findings produced widely varying results, probably due to wide variability in batches of tannic acid.</td>
</tr>
<tr>
<td>Reninil-C (Saunder 1977)</td>
<td>Treatment of P-5 with benzylate to remove fibrinogen</td>
<td>40-160</td>
<td>As with Fp-Ox.</td>
</tr>
<tr>
<td>Sheep Plasma-derived Fraction (Hapke et al. 1964)</td>
<td>Addition of salicylic acid to plasma to a concentration of 2.1 M at 0°C to precipitate factor VIII with some fibrinogen</td>
<td>25-30</td>
<td>Use of salicylic acid concentrations expensive and wasteful as it precipitates the use of the supernatant plasma — the technique still used to prepare high purity concentrate (Table 1-4A).</td>
</tr>
</tbody>
</table>
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 summarized 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>
<thead>
<tr>
<th><strong>TABLE 1-3 VARIABLES INVOLVED IN CRYOPRECIPITATE PRODUCTION AND THEIR EFFECT ON PRODUCT QUALITY</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Anticoagulant</strong></td>
</tr>
<tr>
<td><strong>(B) Plasma pH</strong></td>
</tr>
<tr>
<td><strong>(C) Plasma Freezing</strong></td>
</tr>
<tr>
<td><strong>(D) Plasma Thawing</strong></td>
</tr>
<tr>
<td><strong>(E) Blood-Group</strong></td>
</tr>
<tr>
<td><strong>(F) Ionic Composition of Plasma</strong></td>
</tr>
<tr>
<td><strong>(G) Additives in Plasma</strong></td>
</tr>
<tr>
<td><strong>(H) Conditions of Storage of Frozen Plasma</strong></td>
</tr>
<tr>
<td><strong>(I) Storage of Blood Prior to Processing</strong></td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Name of Preparation</th>
<th>Method of Preparation</th>
<th>Improvements Over Conventional Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power-driven Cryoprecipitate (Milligan et al 1981)</td>
<td>Small numbers of individual cryos are pooled into stabilising buffer, aliquoted and lyophilised</td>
<td>Product can be stored at 4°C and can be subjected to more rigorous quality control</td>
</tr>
<tr>
<td>'Thaw-siphon' Cryoprecipitate (Russon 1978)</td>
<td>Cryoprecipitate produced by fast-thaw in 4°C water bath with continuous removal of thawed plasma supernatant by siphoning</td>
<td>Marked improvements in yield and purity (Russon and McGill 1979); however only two centres (Edinburgh and Brisbane) produce it routinely - careful attention to detail is required to produce good results</td>
</tr>
<tr>
<td>'Cold-Insoluble Globulin' Cryoprecipitate (Sute-Sibbons et al 1981)</td>
<td>Cryoprecipitate harvested from plasma derived from blood collected in heparin cryo, then subjected to a cold precipitation at 0°C, solubilised in buffer and lyophilised</td>
<td>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 (Sute-Sibbons et al 1981).</td>
</tr>
<tr>
<td>Group</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Concentrates of low purity - &lt; 0.2 i.u./mg protein soluble at ≤ 5 i.u./ml, contain 50-80% fibrinogen, obtained at a yield &gt; 300 i.u./litre fresh frozen plasma</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>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</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>Concentrates of high purity - &gt; 0.5 i.u./mg protein soluble at &gt; 20 i.u./ml, contain &lt; 50% fibrinogen, obtained at a yield of less than 200 i.u./litre fresh frozen plasma</td>
<td></td>
</tr>
</tbody>
</table>
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.
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

<table>
<thead>
<tr>
<th>Starting Material</th>
<th>Fractionation Procedure</th>
<th>Product Characteristics and Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethylene glycol (R.E.G.) (Newman et al 1971) - first precipitation at about 4% P.E.G. 8000 pre-cipitates fibrinogen which is removed. Factor VIII is precipitated and concentrated by increasing P.E.G. concentration to 11%</td>
<td>Separating on means of starting materials (plasma or storage etc.) product is 125-300 fold purified over plasma. Yield is usually 10%</td>
<td></td>
</tr>
<tr>
<td>Solubilized cryoprecipitate</td>
<td>Fractional precipitation with P.E.G. to remove fibrinogen, followed by glycine precipitation at the cold (Kleinschmidt and Baron 1971) to precipitate Factor VIII. Homotrim in processing buffers to increase yield and purity (Fukatsi et al 1974)</td>
<td>Product is 110-330 fold purified over plasma, yield ≥ 1%</td>
</tr>
<tr>
<td>Polyethylene glycol (R.E.G.) (Newman et al 1971) - second precipitation</td>
<td>Addition of NaCl to final 1.4M or pH 5.5 and 1/50 heparin precipitates about 90% of the fibrinogen and fibrinogen and leaves factor VIII in the supernatant (Paster et al 1984)</td>
<td>Product has roughly twice the specific activity of normal concentrate and is obtained at a yield of &gt; 90% from cryoprecipitate. Product can be subjected to heat treatment to inactivate contaminating viruses (Meissner et al 1980)</td>
</tr>
<tr>
<td>Solubilized cryoprecipitate</td>
<td>Precipitation with glycine at 10°C precipitates fibrinogen but leaves factor VIII in solution, from which it can be subsequently concentrated by precipitation with P.E.G. or NaCl (Blazevic and Thoren 1982)</td>
<td>Final product is obtained at a yield ≥ 60% from cryoprecipitate (Dow M &amp; Millar 1980). Specific activity is between 7 and 7.5 mg/kg depending on the final precipitating conditions (Thoren et al 1983). Product corrects the bleeding time defect in V.A.E. (Thoren et al 1983)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Starting Material</th>
<th>Fractionation Procedure</th>
<th>Product Characteristics and Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasite</td>
<td>Ion exchange chromatography on 'polyelectrolyte' resin - VIIIC is adsorbed batchwise and eluted with high Ionic strength buffer containing NaCl. VIIIC is then concentrated by P.E.G.</td>
<td>'Polyelectrolyte' VIIIC is ≥ 50 fold purified over plasma and is almost free of von Willebrand factor. The process yield over cryoprecipitate is ≥ 45%. The concentrate produced from porcine plasma has been used successfully to treat patients with VIIIiC inhibitors (Kernoff et al 1981). The human product gave good clinical results in hemophilia A but poor results in V.A.E. (Roodhoon et al 1982)</td>
</tr>
<tr>
<td>Intermediate purity concentrate</td>
<td>Filtration on Controlled Porc Glass (C.P.G.) column (Mepolive and Rhoades 1981). Fibrinogen is retained and subsequently adsorbed by the gel, while factor VIII is collected in the void volume</td>
<td>Concentrate is ≥ 90 fold purified over plasma and is obtained at a yield of 70% yield over the intermediate purity material. Clinical results are good</td>
</tr>
<tr>
<td>Intermediate purity concentrate</td>
<td>Column chromatography over amionexyl Sepharose (Austen and Smith 1982) is claimed to remove much of the fibrinogen with possible reduction of hepatitis B virus and blood group neoheamoglutinins</td>
<td>Final concentrate is ≥ 90 fold purified over plasma at a yield of ≥ 40% of the starting material. No clinical use reported - still in small scale research stage</td>
</tr>
<tr>
<td>Solubilized cryoprecipitate</td>
<td>Adsorption to and elution from Poly-Acryl matrix. Factor VIII is eluted at high ionic strength after low salt wash to remove fibrinogen (Lewis et al 1980)</td>
<td>Final factor VIII fraction has a specific activity of ≥ 5 mg/kg and the yield over the initial cryop ≥ 70%. No clinical use reported - still in small scale research stage</td>
</tr>
</tbody>
</table>
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.
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 VIII:Ag than factor VIII:C (Yang and Duffy 1978, Nilsson et al 1980) suggesting a selective loss of clotting activity during fractionation. Furthermore, the VIII:Ag/VIII:C ratio was highest for the more purified products (Yang and Duffy 1978). Other studies confirmed these results, although an increase in VIII:Ag/ VIII:C ratios for more purified material was not always found (Allain et al 1980).

Use of the VIII:C:Ag assay increased the scope of such investigations. The ratio VIII:C:Ag/VIII:C was found to be much closer to unity than VIII: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:C:Ag/ VIII:C and VIII:Ag/VIII:C:Ag ratios (Prowse et al 1981).

The development of more sophisticated assays for VIII:Ag led to greater understanding. Using immunoradiometric assays (I.R.M.A's) the levels of VIII: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 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 VIIIIR: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 Redner 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.

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-HBsAg) (Enck et al 1979). The prevalence of the antigen (HBsAg) 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 0 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 HBsAg 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 V III : 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 HBsAg 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
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 'isoagglutininfree' 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

<table>
<thead>
<tr>
<th>Countries Reporting</th>
<th>Cryo-precipitate</th>
<th>Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufficient Products Available</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>Insufficient Products Available</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Not Used</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Total Number of Countries Reporting</td>
<td>43</td>
<td>43</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic</td>
<td>6</td>
</tr>
<tr>
<td>Imported</td>
<td>3</td>
</tr>
<tr>
<td>Domestic and imported</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
</tr>
</tbody>
</table>

Both tables taken from Britten (1983)
high concentrations of anti-A in the final concentrate,
whereas processing of single donations to cryoprecipitate
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
38
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 statefunded 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, SmitSibinga 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, i.e. 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 (IEe) 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 x 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.
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 Diagnostic® 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 endpoint (fibrin formation) were done using an 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 dispensed manually

+0.1 ml activator (Silica / Cephalin) dispensed automatically

  Incubated 5 minutes

+0.1 ml 33

FIBRIN CLOT detected optically
FIGURE 2-1
ESTIMATION OF VIII:C CONTENT IN COAGULATION ASSAY

Standard and test lines are shown, with extrapolation from test to standard to obtain test VIII:C content as a % of standard.
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 semilog 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 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 test 2 112% of standard 0.78 u/ml 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 CaCl₂. 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 O°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 (NH₄)₂SO₄ per 100 ml supernatant). Mixing was carried out for 30 minutes at O°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 O°C. The supernatant was brought to 55% saturation with ammonium sulphate (i.e., 11.8 g (NH₄)₂SO₄ per 100 ml 35% supernatant), mixed for 45 minutes at O°C and centrifuged at 13,000 r.p.m. for 15 minutes at O°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 0.5 M trisodium citrate and
Plasma samples were assayed against a known standard using both types of substrate. Also shown is the statistical analysis of the data obtained.
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

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, 0.5M 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
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.
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 ml of a stock solution of 1 mg/ml protamine sulphate were added to 12 such tubes, so that tubes with 2, 4, 6 .... 24 ml 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 VII:C IN PLASMA CONTAINING HEPARIN AND HEPARIN/CALCIUM

<table>
<thead>
<tr>
<th>Sample</th>
<th>VIII:C (u/ml) No Heparin</th>
<th>VIII:C (u/ml) + 5 u/ml Heparin + Protamine Titration</th>
<th>VIII:C (u/ml) + 2 u/ml Heparin + 10 mM Ca²⁺ + Protamine Titration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Pool 1550</td>
<td>0.88</td>
<td>0.84</td>
<td>-</td>
</tr>
<tr>
<td>Plasma Pool 11</td>
<td>0.95</td>
<td>0.98</td>
<td>-</td>
</tr>
<tr>
<td>Plasma Pool 12</td>
<td>0.92</td>
<td>0.91</td>
<td>-</td>
</tr>
<tr>
<td>Plasma Pool 13</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1st Scottish Plasma Standard</td>
<td>0.69</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>11th British Plasma Standard</td>
<td>0.73</td>
<td>0.72</td>
<td>0.74</td>
</tr>
</tbody>
</table>
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 μM 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 parallelism with respect to the standard curve were also unaffected. It should be noted that 1 μM 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.

1. VIII:CAg in sample
2. 125I anti-VIII:CAg Fab
3. Incubated 18 hours at 37°C
4. FREE Fab
5. VIII:CAg with bound 125I Ag Fab
6. Precipitated with 38% S.A.S.
7. Precipitated bound 125I CAg Fab
8. Washed with 38% SAS to remove unbound Fab
9. Washed precipitate
10. Count radioactivity
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 absorbance at 280 nm was measured. Fractions containing unbound protein were pooled, and the protein content was estimated from absorbance 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 : CA\textsubscript{g} Fab FROM INHIBITOR PLASMA

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Inhibitor Serum</td>
<td>2.9</td>
<td>112</td>
</tr>
<tr>
<td>2. DEAE Unbound Fraction</td>
<td>27.5</td>
<td>24.8</td>
</tr>
<tr>
<td>3. Concentrate of (2)</td>
<td>6.7</td>
<td>21.0</td>
</tr>
<tr>
<td>4. Pepsin Digest</td>
<td>4.0</td>
<td>13.0</td>
</tr>
<tr>
<td>5. Protein A Unbound Fraction</td>
<td>3.4</td>
<td>8.8</td>
</tr>
<tr>
<td>6. (5) Post Dialysis</td>
<td>12.6</td>
<td>8.8</td>
</tr>
</tbody>
</table>
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 125I 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
- 1 g in 10 ml of 50 mM phosphate pH 7.4
- 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 125I (2 mCi)
10 μl Chloramine-T
mixed for 10 seconds
add 0.85 ml Na2S205
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.
swollen in 500 ml of 1 M 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 NaHCO3. 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 baseline, elution was continued with 50 ml of 0.1 M glycine-HCl pH 2.5. 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 K2 HPO4 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 ml/minute was registered in an NE 1600 gammacounter.

Assay of factor VIII:CAg: The assay was performed as
FIGURE 2-6

STANDARD CURVE OF THE VIII:Cag IMMUNORADIOMETRIC ASSAY
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$_4$)$_2$SO$_4$ added

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

precipitates

washed with 1 ml 38% saturated (NH$_4$)$_2$SO$_4$

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 - VIIIIR:Ag

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

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

* Except where otherwise specified, in this thesis total centrifugal force is expressed as g min., being the centrifugal force 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.
Gel filtration was performed on Sepharose CL-4B as described in the text. Fractions containing VIIIIR: Ag without detectable fibrinogen were pooled and used for immunisation.
### TABLE 2-4 PURIFICATION OF VIIIR :Ag

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Volume ml</th>
<th>VIII:C u/ml (Yield)</th>
<th>VIIIR:Ag u/ml (Yield)</th>
<th>Fibrinogen mg/ml (Yield)</th>
<th>Fibronectin mg/ml (Yield)</th>
<th>Protein mg/ml (Yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled Plasma</td>
<td>1755</td>
<td>0.95 (100)</td>
<td>0.84 (100)</td>
<td>2.5 (100)</td>
<td>0.36 (100)</td>
<td>52.3 (100)</td>
</tr>
<tr>
<td>4% Picoll Supernatant</td>
<td>1742</td>
<td>0.18 (20.3)</td>
<td>0</td>
<td>1.6 (65)</td>
<td>0.05 (13)</td>
<td>44.7 (92)</td>
</tr>
<tr>
<td>4% Picoll Precipitate</td>
<td>45</td>
<td>28.9  (78)</td>
<td>31 (95)</td>
<td>25.3 (26)</td>
<td>11.2 (80)</td>
<td>45.4  (2.2)</td>
</tr>
<tr>
<td>V₀ Peak From Gel Filtration</td>
<td>46</td>
<td>N.D.</td>
<td>3.5 (11)</td>
<td>&lt; 0.02</td>
<td>&lt; 0.004</td>
<td>0.04</td>
</tr>
<tr>
<td>Final Preparation</td>
<td>15</td>
<td>N.D.</td>
<td>7.5 (7.6)</td>
<td>&lt; 0.02</td>
<td>&lt; 0.004</td>
<td>0.09</td>
</tr>
</tbody>
</table>
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 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. Fibronectin
5. Pharmacia high molecular weight marker proteins
preparations of VIIIR:Ag were made utilising cryoprecipitate
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 O°C for 2 hours (Chapter V). The resulting
precipitate was harvested by centrifugation for 30,000 g min. at O°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. 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.
was by subcutaneous 4-site injections consisting of 150 \(\mu\)g of antigen in Freund's complete adjuvant. Boosts were given 2 months and 3 months after the primary injection and consisted of 65 \(\mu\)g of antigen in Freund's incomplete adjuvant. The rabbits were bled at intervals to test the sera for reactivity with VIIIIR: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 VIIIIR: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-VIIIIR: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 \(56^\circ\)C for 30 minutes, aliquoted and stored frozen at \(-40^\circ\)C for use. Assay for VIIIIR:Ag - Laurell assay: Commercial and later on locally produced antisera to VIIIIR:Ag were used to set up an electro immunoassay 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 VIIIIR:Ag.*
FIGURE 2-10

STANDARD CURVE OF THE VIIIIR :Ag LAURELL ASSAY
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 VIIIIR: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 VIIIIR:Ag concentrations of between 100% and 25% of normal pooled plasma (Figure 2-10). Samples for assay were prediluted 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: 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 lateral 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 VIIIIR:Ag had been incorporated. The antiserum 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 VIIIIR:Ag (DAKO antibodies) was used to develop an IRMA for VIIIIR:Ag using the same principle as for the VIII:CAg IRMA. Production of immuno-purified 125I anti-VIIIIR:Ag Fab fragments was exactly as for the production of the VIII:CAg reagent. The assay was also similar, except that 125I anti-VIIIIR:Ag
FIGURE 2-11

STANDARD CURVE OF THE VIIIIR :Ag

IMMUNORADIOMETRIC ASSAY
Fab was used in the incubation mixture. The assay was sensitive to levels of VIIIIR: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 VIIIIR:Ag in samples which produced faint immunoprecipitates notably cryosupernatants.

(E) ASSAY OF FACTOR VIII-RELATED RISTOCETIN COFACTOR (VIIIIR: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 VIIIIR: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 VIIIIR : RCF ASSAY
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 J.Ll of calcium-thrombin reagent (0.56 M CaCl2 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
absorbance of the test mixture at 470 nm was read using
a CECIL spectrophotometer. The control mixture was used
as blank. The absorbance 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 VIIIIR:Ag. Antiserum
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 VIII: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 125I an ti- 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. 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 150mMNaCl, 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 (NH4)2SO4 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.
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 Sepharose4B. 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 Ug/ml) indicating its specificity .
FIGURE 2-14

STANDARD CURVE OF THE BIURET PROTEIN ASSAY
(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 measurement 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 (CuSO4 5H2O), 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 absorbance of one was equivalent to a protein concentration of 1 mg/ml unless the extinction coefficient of the protein involved was known.
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 `loved` 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 gelcasting 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 ml of 0.5 M NaOH and 12 ml H3PO4 up to one litre with distilled water).

(b) 22.2% acrylamide (22.4 g + 0.6 g bis in 100 mls H2O).

(c) 10% SDS.

(d) 10% ammonium per sulphate (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

Marker proteins (Pharmacia) were electrophoresed and Rf values were measured:

\[ R_f = \frac{\text{DISTANCE MIGRATED BY PROTEIN BAND}}{\text{DISTANCE MIGRATED BY DYE FRONT}} \]
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 cellfree (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, 106
Carlebj5rk 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 VIIIIR:Ag have been reported to be stable in plasma at room temperature (Rock et al 1983a).

VIIIIR: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 VIIIIR:Ag over one week at 4°C (Nilsson et al 1983). VIIIIR: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 107
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 (TBrn and Myllyl~ 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 cryoprecipitate 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 Tutason 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 trisodium citrate. 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 kept 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 VIIIIR: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).
STABILITY OF FACTOR VIII IN CPD DONATIONS Oil

A, B, C, D show data for VIII :C 0-7

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

Results show the mean and standard deviation for
ten individual donations.
### TABLE 3-1 STABILITY OF FACTOR VIII AND FIBRONECTIN IN CPD PLASMA AT 0°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh Plasma Levels</th>
<th>Levels after 18 hours at 0°C</th>
<th>Centrifuged at 0°C</th>
<th>Centrifuged at 20°C after 30 min at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Supernatant</td>
<td>Precipitate</td>
<td></td>
</tr>
<tr>
<td>VIII:C u/ml</td>
<td>0.09</td>
<td>0.20 ± 0.09 (32)*</td>
<td>1.28 ± 0.18 (37)</td>
<td>0.54 ± 0.12</td>
</tr>
<tr>
<td>ViliIR:Ag u/ml</td>
<td>0.68</td>
<td>0.51 ± 0.04 (64)</td>
<td>2.12 ± 0.37 (41)</td>
<td>0.80 ± 0.13</td>
</tr>
<tr>
<td>Fibronectin antigen u/ml</td>
<td>0.63</td>
<td>0.1 (32)</td>
<td>3.3 ± 1.08 (69)</td>
<td>0.79 ± 0.14</td>
</tr>
</tbody>
</table>

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: O°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 O°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 O°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 O°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 O°C as occurred for
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.
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 VIIR : 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.
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 121
TABLE 3-2 IONISED CALCIUM IN PLASMA COLLECTED INTO DIFFERENT ANTICOAGULANTS

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Calcium Ion-Specific Electrode Reading (mV)</th>
<th>Ionised Calcium (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin (3 u/ml)</td>
<td>-90</td>
<td>0.56</td>
</tr>
<tr>
<td>CPD + Heparin (3 u/ml)</td>
<td>-50</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

+ Final heparin concentration in plasma
FIGURE 3-4
TITRATION OF CPD/HEPARIN PLASMA TO IONISED CALCIUM LEVELS FOUND IN HEPARIN PLASMA

55 mM CaCl₂ was added to a 4 ml plasma sample.
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 VIIIIR :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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hours Post-Donation</th>
<th>Factor VIII:C u/ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>6</td>
<td>0.83 ± 0.17</td>
</tr>
<tr>
<td>Blood</td>
<td>Calcium/heparin added 3 hrs, separated 5 hrs</td>
<td>1.03 ± 0.274</td>
</tr>
<tr>
<td>Blood</td>
<td>18</td>
<td>0.71 ± 0.17</td>
</tr>
<tr>
<td>Blood</td>
<td>Calcium/heparin added 18 hrs, separated 20 hrs</td>
<td>0.65 ± 0.148</td>
</tr>
<tr>
<td>Plasma</td>
<td>6</td>
<td>0.84 ± 0.25</td>
</tr>
<tr>
<td>Plasma</td>
<td>Calcium/heparin added 3 hrs, frozen 5 hrs</td>
<td>1.1 ± 0.41</td>
</tr>
<tr>
<td>Plasma</td>
<td>18</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>Plasma</td>
<td>Calcium/heparin added 18 hrs, frozen 20 hrs</td>
<td>0.77 ± 0.255</td>
</tr>
</tbody>
</table>

* 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

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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 nM and 5 u/ml in the plasma. Results show the mean and standard deviation of ten individual donations.
effect of the CPD.

(D) RECALCIFICATION OF CPD DONATIONS

On the basis of the calcium level measurements just outlined, a CaCl2/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

<table>
<thead>
<tr>
<th>Calcium/Heparin Added to Final Concentration mM (u/ml)</th>
<th>VIII:C After Overnight Incubation at 22°C u/ml*</th>
<th>Ionised Calcium After Overnight Incubation at 22°C mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (0)</td>
<td>(starting levels) 1.07 ± 0.09</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>0 (0)</td>
<td>0.67 ± 0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>10 (3)</td>
<td>0.86 ± 0.18*</td>
<td>0.33</td>
</tr>
<tr>
<td>15 (3)</td>
<td>0.78 ± 0.13*</td>
<td>0.44</td>
</tr>
<tr>
<td>20 (3)</td>
<td>0.89 ± 0.16*</td>
<td>0.96</td>
</tr>
<tr>
<td>25 (3)</td>
<td>0.82 ± 0.15*</td>
<td>0.78</td>
</tr>
<tr>
<td>30 (3)</td>
<td>0.78 ± 0.09*</td>
<td></td>
</tr>
</tbody>
</table>

* Mean and standard deviation of 6 individual donations

† Significantly higher than control, p < 0.01

t Difference from 10 mM value not significant

Precipitate formed after overnight incubation
TABLE 3-5 DEPENDENCE OF VllC STABILITY IN HEPARIN PLASMA ON CITRATE CONCENTRATION

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

<table>
<thead>
<tr>
<th>Citrate Added</th>
<th>VllC After Overnight Incubation at 22°C</th>
<th>Ionised Calcium After Overnight Incubation at 22°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Concentration</td>
<td>μM</td>
<td>u/ml*</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.85 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.53 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>0.46 ± 0.16</td>
</tr>
<tr>
<td>0 (+ 10 mM calcium)</td>
<td></td>
<td>0.18 ± 0.02*</td>
</tr>
<tr>
<td>1.03 ± 0.15*</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
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-sodiumcitrate. 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

<table>
<thead>
<tr>
<th>Mixing Procedure</th>
<th>VIII:C (u/ml) Mean and Standard Deviation (range)</th>
<th>Median</th>
<th>Range</th>
<th>% Donation with ≤ 7.0 u/ml</th>
<th>% Donation with ≤ 30 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully Mixed</td>
<td>0.97 ± 0.2 (0.76 - 1.27)</td>
<td>11.9</td>
<td>3.6 - 90</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Weekly Mixed</td>
<td>0.62 ± 0.13 (0.42 - 0.96)</td>
<td>15.5</td>
<td>3.5 - 38.8</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>Standing</td>
<td>0.57 ± 0.31 (0.44 - 1.75)</td>
<td>7.8</td>
<td>2.9 - 26.8</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Mixing Procedure</th>
<th>Group C</th>
<th>Group A + B + AB</th>
<th>All Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmixed</td>
<td>0.74 ± 0.23 (0.41 - 1.12,7)</td>
<td>1.10 ± 0.29 (0.94 - 1.5,3)</td>
<td>0.87 ± 0.31 (0.41 - 1.5,10)</td>
</tr>
<tr>
<td>Partly Mixed</td>
<td>0.80 ± 0.13 (0.62 - 0.97,5)</td>
<td>0.82 ± 0.14 (0.63 - 0.90,5)</td>
<td>0.82 ± 0.13 (0.62 - 0.98,5)</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.64 ± 0.16 (0.74 - 1.1,4)</td>
<td>1.06 ± 0.19 (0.77 - 1.17,6)</td>
<td>0.97 ± 0.21 (0.74 - 1.37,10)</td>
</tr>
</tbody>
</table>
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, Band 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 1983 a) 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 nonchelating 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, VIIIIR:Ag and fibronectin were observed in heparinised blood and plasma stored at $4^\circ$C. These losses are probably due to cryoprecipitation as was observed in CPD blood and plasma at $0^\circ$C. Although the effect of warming to $37^\circ$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 nm 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 nm 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 quo 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 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 quo 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 -80°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 and stored as follows:

The plasma was then frozen

' 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
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 VIIIIR:Ag,
Fibrinogen and Total Protein were assayed as described in Chapter II. Factor VIIIIR :Ag was assayed by electroimmunoassay, except for samples of cryosupernatant plasma, the VIIIIR :Ag in which gives indistinct precipitin lines (Over et al 1978 ). For these samples an immunoradiometric 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 PLAStIC PACK FROZEN IN DIFFERENT MEDIA

-70°C LIQUID NITROGEN/ETHANOL
-40°C CABINET FREEZER

PACK CORE TEMPERATURE (°C)

TIME (minutes)

TIME (hours)
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 2 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 2 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: 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

<table>
<thead>
<tr>
<th>Temperature of Storage</th>
<th>Period of Storage</th>
<th>Total VIII:C Recovered (Cryo + Sugar) u/kg Plasma</th>
<th>VIII:C Cryo Yield u/kg Plasma</th>
<th>Fibrinogen Cryo Yield mg/kg Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td>16 hours</td>
<td>693 ± 105</td>
<td>427 ± 84</td>
<td>695 ± 172</td>
</tr>
<tr>
<td>-20°C</td>
<td>3 months</td>
<td>699 ± 123</td>
<td>500 ± 130</td>
<td>699 ± 107</td>
</tr>
<tr>
<td>-20°C</td>
<td>6 months</td>
<td>690 ± 82</td>
<td>416 ± 67</td>
<td>538 ± 115</td>
</tr>
<tr>
<td>-40°C</td>
<td>16 hours</td>
<td>698 ± 125</td>
<td>493 ± 116</td>
<td>697 ± 99</td>
</tr>
<tr>
<td>-40°C</td>
<td>3 months</td>
<td>718 ± 102</td>
<td>522 ± 77</td>
<td>573 ± 51</td>
</tr>
<tr>
<td>-40°C</td>
<td>6 months</td>
<td>730 ± 103</td>
<td>449 ± 47</td>
<td>542 ± 47</td>
</tr>
</tbody>
</table>
TABLE 4-1B EFFECT OF STORAGE PERIOD ON PLASMA FROZEN IN -40°C DEEP FREEZE. RESULTS SHOW MEAN ± S.D. FOR SIX DIFFERENT

<table>
<thead>
<tr>
<th>Period of Storage</th>
<th>Total VIII:C Recovered (Cryo + Super) W/kg Plasma</th>
<th>VIII:C Cryo Yield W/kg Plasma</th>
<th>Fibrinogen Cryo Yield ng/kg Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 hours</td>
<td>610 ± 161</td>
<td>310 ± 111</td>
<td>22 ± 77</td>
</tr>
<tr>
<td>5 months</td>
<td>611 ± 163</td>
<td>306 ± 62</td>
<td>20 ± 79</td>
</tr>
</tbody>
</table>
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

- Control: frozen in liquid nitrogen/ethanol to -180°C and held at -180°C for 2 days
- Insulated 10°C warmer to -8°C after 2 days, then replaced in -80°C storage
- Insulated twice as above and subjected to same cycle after 5 days

PLASMA CORE TEMPERATURE (°C)

DAYS POST-COLLECTION
FIGURE 4-5

EFFECT OF TEMPERATURE INOCULATION AND REPRIPE ON CYPHERFICATE

A) VLDL OF VIE C OR PLASMA FRACTIONS
B) VLDL OF VIE C OR CYPHERFICATE
C) SPECIFIC ACTIVITY OF CYPHERFICATE VIE 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 (1983 a)
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 al1971). 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 saltingout 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 fastfreezing 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 cryoprecipitate 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) tubestoppers 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⁻¹ 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, VIIIIR: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 VIIIIR: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 gelatinagarose (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 (RES 40, RES 350, RES 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

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>VIII:C Yield</th>
<th>VIIIIR:Ag Yield</th>
<th>Fibrinogen Yield</th>
<th>Specific Activity VIII:C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>u/kg plasma</td>
<td>u/kg plasma</td>
<td>mg/kg plasma</td>
<td>u/mg protein</td>
</tr>
<tr>
<td>No heparin added</td>
<td>3</td>
<td>366 ± 53.5</td>
<td>529 ± 55</td>
<td>678 ± 73</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Heparin added to plasma, 1 u/ml</td>
<td>3</td>
<td>347 ± 74</td>
<td>519 ± 50</td>
<td>676 ± 69</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Heparin added to plasma, 5 u/ml</td>
<td>1</td>
<td>435</td>
<td>680</td>
<td>690</td>
<td>0.11</td>
</tr>
<tr>
<td>Heparin added to blood bag pre-donation, 2 u/ml in plasma*</td>
<td>2</td>
<td>475</td>
<td>600</td>
<td>140</td>
<td>0.18</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>VIII:C Yield</th>
<th>VIIIIR:Ag Yield</th>
<th>Fibrinogen Yield</th>
<th>Specific Activity VIII:C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>u/kg plasma</td>
<td>u/kg plasma</td>
<td>mg/kg plasma</td>
<td>u/mg protein</td>
</tr>
<tr>
<td>No heparin added</td>
<td>3</td>
<td>627 ± 150</td>
<td>690 ± 165</td>
<td>505 ± 84</td>
<td>0.74 ± 0.19</td>
</tr>
<tr>
<td>1 u/ml heparin added 1 hour post donation</td>
<td>3</td>
<td>625 ± 126</td>
<td>699 ± 142</td>
<td>512 ± 123</td>
<td>0.70 ± 0.1</td>
</tr>
<tr>
<td>1 u/ml heparin added 3 hours post donation</td>
<td>3</td>
<td>637 ± 163</td>
<td>672 ± 128</td>
<td>537 ± 83</td>
<td>0.71 ± 0.14</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Anti-coagulant</th>
<th>n</th>
<th>VIII:C Yield</th>
<th>VIIIIR:Ag Yield</th>
<th>Fibrinogen Yield</th>
<th>Specific Activity VIII:C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>u/kg plasma</td>
<td>u/kg plasma</td>
<td>mg/kg plasma</td>
<td>u/mg protein</td>
</tr>
<tr>
<td>CPD</td>
<td>6</td>
<td>366 ± 120</td>
<td>588 ± 135</td>
<td>690 ± 136</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Heparin*</td>
<td>6</td>
<td>523 ± 156</td>
<td>641 ± 159</td>
<td>735 ± 187</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>VITE-C Yield μg/kg plasma</th>
<th>FIIb-K2 Yield μg/kg plasma</th>
<th>Fibrinopeptide Yield ng/kg plasma</th>
<th>Specific Activity VITE-C μg/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PEG added</td>
<td>5</td>
<td>591 ± 153</td>
<td>740 ± 284</td>
<td>495 ± 711</td>
<td>0.4 ± 0.15</td>
</tr>
<tr>
<td>PEG added to 1% w/v in plasma</td>
<td>6</td>
<td>563 ± 133</td>
<td>607 ± 172</td>
<td>906 ± 218*</td>
<td>0.32 ± 0.04*</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Number of Experiments</th>
<th>Plasma Volume</th>
<th>VIII:C Yield u/kg plasma</th>
<th>VIIIIR:Ag Yield u/kg plasma</th>
<th>Fibrinogen Yield mg/kg plasma</th>
<th>Specific Activity Protein VIII:C u/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>253 ± 75</td>
<td>376 ± 65</td>
<td>812 ± 23</td>
<td>2350 ± 424</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable Investigated</th>
<th>VIII:C u/ml</th>
<th>VIIIIR:Ag u/ml</th>
<th>Fibrinogen mg/ml</th>
<th>Fibromectin mg/ml*</th>
<th>Total Protein mg/ml</th>
<th>Conductivity mMho</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Ionic Strength:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control*</td>
<td>9.96 ± 0.18</td>
<td>1.6 ± 0.25</td>
<td>2.3 ± 0.8</td>
<td>0.36 ± 0.10</td>
<td>56 ± 9</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>Low</td>
<td>1.1 ± 0.22</td>
<td>1.1 ± 0.28</td>
<td>2.3 ± 0.8</td>
<td>0.38 ± 0.15</td>
<td>54 ± 7</td>
<td>1.27 ± 0.05</td>
</tr>
<tr>
<td>B) Protein Content:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.73</td>
<td>1.04</td>
<td>2.05</td>
<td>0.24</td>
<td>47</td>
<td>7.5</td>
</tr>
<tr>
<td>Low</td>
<td>&lt; 0.05</td>
<td>0.72</td>
<td>1.27</td>
<td>0.16</td>
<td>14</td>
<td>1.7</td>
</tr>
<tr>
<td>C) Fibrinogen Content:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.23 ± 0.3</td>
<td>1.24 ± 0.2</td>
<td>3.1 ± 0.15</td>
<td>0.69 ± 0.21</td>
<td>61 ± 30</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Low</td>
<td>1.23 ± 0.14</td>
<td>1.1 ± 0.21</td>
<td>1.4 ± 0.4x10⁻²</td>
<td>0.33 ± 0.11</td>
<td>59 ± 6</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>D) Fibromectin Content:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.98 ± 0.14</td>
<td>1.07 ± 0.14</td>
<td>2.33 ± 0.35</td>
<td>0.25 ± 0.03</td>
<td>63 ± 12</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>Low</td>
<td>1.1 ± 0.2</td>
<td>1.21 ± 0.14</td>
<td>3.24 ± 1.29</td>
<td>6.6 ± 0.5x10⁻¹</td>
<td>63 ± 17</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

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 normal pooled plasma 0.33 mg/ml (Mosseson and Amrani 1980).
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.

### TABLE 5-7 EFFECT OF PLASMA COMPOSITION ON CRYOPRECIPITATE

<table>
<thead>
<tr>
<th>Plasma Type</th>
<th>VIII:C Yield in Cryo % plasma</th>
<th>VIIIIB:Ag Yield in Cryo % plasma</th>
<th>Fibrinogen Yield in Cryo % plasma</th>
<th>Fibronectin Yield in Cryo % plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiologic Ionic Strength</td>
<td>47 ± 4</td>
<td>64 ± 12</td>
<td>34 ± 17</td>
<td>56 ± 22</td>
</tr>
<tr>
<td>Low Ionic Strength</td>
<td>60 ± 13</td>
<td>51 ± 6</td>
<td>42 ± 14</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>High Ionic Strength</td>
<td>5 ± 2</td>
<td>32 ± 19</td>
<td>19 ± 7</td>
<td>89 ± 47</td>
</tr>
<tr>
<td>Physiologic Protein Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Protein Content</td>
<td>53</td>
<td>84</td>
<td>50</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Physiologic Fibrinogen Level</td>
<td>47 ± 8</td>
<td>73 ± 24</td>
<td>55 ± 28</td>
<td>68 ± 26</td>
</tr>
<tr>
<td>Low Fibrinogen</td>
<td>11 ± 6</td>
<td>11 ± 6</td>
<td>92 ± 30</td>
<td>13 ± 26</td>
</tr>
<tr>
<td>Physiologic Fibronectin Level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Fibronectin</td>
<td>42 ± 4</td>
<td>40 ± 20</td>
<td>32 ± 6</td>
<td>00 ± 22</td>
</tr>
<tr>
<td></td>
<td>3 ± 1</td>
<td>5 ± 3</td>
<td>2 ± 1</td>
<td>25</td>
</tr>
</tbody>
</table>
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 ml 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 VIII:Ag, fibrinogen and fibronectin are shown. Fractions containing the bulk of these proteins were pooled as shown and concentrated as described in Methods.

![Graph showing elution profile of VIII:Ag, fibrinogen, and fibronectin](image-url)
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

• • VIII:Ag

Fibrinogen

Total protein
4. Fibronectin depleted plasma:

Adsorption 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-SMALL SCALE PRECIPITATION OF CPD PLASMA WITH HUMAN ALBUMIN

Conditions for precipitation and recovery of precipitates were as in Figure 5-2.
### TABLE 5- 8 SMALL SCALE (20 ml) POLYMER PRECIPITATION OF FRESH PLASMA . CONDITIONS FOR OPTIMAL FACTOR VIII PURIFICATION

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>
<thead>
<tr>
<th>Polymer</th>
<th>Final Concentration (% w/w)</th>
<th>n</th>
<th>VIII:C</th>
<th>VIII:Ag</th>
<th>Fibrinogen</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES 40</td>
<td></td>
<td>4</td>
<td>6</td>
<td>14.2 (89)*</td>
<td>19.4 (108)</td>
<td>5.4 (9)</td>
<td>24 (2)</td>
<td>0.59</td>
</tr>
<tr>
<td>HES 350</td>
<td></td>
<td>3</td>
<td>6</td>
<td>14.9 (93)</td>
<td>17.1 (95)</td>
<td>5.4 (9)</td>
<td>12 (1)</td>
<td>1.24</td>
</tr>
<tr>
<td>HES 450</td>
<td></td>
<td>2</td>
<td>3</td>
<td>13.6 (85)</td>
<td>15.5 (86)</td>
<td>5.4 (9)</td>
<td>48 (9)</td>
<td>0.28</td>
</tr>
<tr>
<td>Dextran 50-70</td>
<td></td>
<td>2</td>
<td>4</td>
<td>12.6 (78)</td>
<td>13 (72)</td>
<td>4.2 (7)</td>
<td>36 (3)</td>
<td>0.35</td>
</tr>
<tr>
<td>Dextran 5-150</td>
<td></td>
<td>3</td>
<td>4</td>
<td>11.7 (73)</td>
<td>14.4 (80)</td>
<td>4.2 (7)</td>
<td>36 (3)</td>
<td>0.33</td>
</tr>
<tr>
<td>PVP 10</td>
<td></td>
<td>3</td>
<td>5</td>
<td>6.7 (42)</td>
<td>15.8 (88)</td>
<td>6.8 (18)</td>
<td>48 (4)</td>
<td>0.14</td>
</tr>
<tr>
<td>PVP 40</td>
<td></td>
<td>2</td>
<td>4</td>
<td>9.8 (63)</td>
<td>14.4 (80)</td>
<td>4.8 (8)</td>
<td>24 (2)</td>
<td>0.41</td>
</tr>
<tr>
<td>Ficoll 70</td>
<td></td>
<td>4</td>
<td>5</td>
<td>14.6 (94)</td>
<td>15.5 (86)</td>
<td>4.8 (40)</td>
<td>40 (4)</td>
<td>0.20</td>
</tr>
<tr>
<td>PEG 6000</td>
<td></td>
<td>2</td>
<td>3</td>
<td>9.0 (63)</td>
<td>12.0 (71)</td>
<td>18.6 (31)</td>
<td>96 (6)</td>
<td>0.10</td>
</tr>
<tr>
<td>Percoll</td>
<td></td>
<td>3</td>
<td>3</td>
<td>4.3 (27)</td>
<td>9 (50)</td>
<td>6.7 (11)</td>
<td>52 (2)</td>
<td>0.09</td>
</tr>
<tr>
<td>Human Albumin</td>
<td></td>
<td>6</td>
<td>7</td>
<td>14.2 (89)</td>
<td>15.8 (88)</td>
<td>4.8 (8)</td>
<td>24 (2)</td>
<td>0.59</td>
</tr>
</tbody>
</table>
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 O°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 O°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
Six donations of each anticoagulant were processed. Precipitation with 4% Ficoll was carried out with plasma in polythene bottles at DoC 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.
Adsorption 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>VIII:C u/mL</th>
<th>VIII:C % yield</th>
<th>VIII:C:Ag mg/mL</th>
<th>Protein mg/mL</th>
<th>Specific Activity VIII:C u/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.92</td>
<td>100</td>
<td>0.98</td>
<td>2.44</td>
<td>56</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.3</td>
<td>34.4</td>
<td>0.39</td>
<td>2.32</td>
<td>54</td>
</tr>
<tr>
<td>Dissolved precipitate</td>
<td>18.5</td>
<td>60.3</td>
<td>15.6</td>
<td>8.8</td>
<td>20.2</td>
</tr>
</tbody>
</table>
| 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 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

<table>
<thead>
<tr>
<th>Component</th>
<th>VIII:C u/mL</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Dissolved precipitate</td>
<td>10.5 ± 3.8</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>2) Dried precipitate dissolved in dispensed volume</td>
<td>10.1 ± 4.5</td>
<td>7.2 ± 0.06</td>
</tr>
<tr>
<td>3) Dissolved precipitate adsorbed with Al(OH)₃</td>
<td>9.6 ± 2.6</td>
<td>6.8 ± 0.08</td>
</tr>
<tr>
<td>4) Dried adsorbed precipitate dissolved in dispersed volume</td>
<td>10.2 ± 3.5</td>
<td>7.3 ± 0.14</td>
</tr>
<tr>
<td>5) (1) after 24 hours at 22°C</td>
<td>8.2 ± 4.0</td>
<td>6.9 ± 0.17</td>
</tr>
<tr>
<td>6) (2) after 24 hours at 22°C</td>
<td>5.7 ± 2.6</td>
<td>7.4 ± 0.12</td>
</tr>
<tr>
<td>7) (3) after 24 hours at 22°C</td>
<td>8.3 ± 1.9</td>
<td>7.0 ± 0.03</td>
</tr>
<tr>
<td>8) (4) after 24 hours at 22°C</td>
<td>5.9 ± 3.6</td>
<td>7.5 ± 0.19</td>
</tr>
</tbody>
</table>

Six donations of CPD plasma were each precipitated with 4% HES 40. The precipitates were each dissolved in 16 mi. 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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIIIcC at Reconstitution</td>
<td>9.6 ± 2.7 u/ml</td>
</tr>
<tr>
<td>After 6 hours</td>
<td>9.2 ± 1.9 u/ml</td>
</tr>
<tr>
<td>After 24 hours</td>
<td>4.5 ± 1.4 u/ml</td>
</tr>
<tr>
<td>Recovery from Plasma</td>
<td>694 u/mg</td>
</tr>
<tr>
<td>Specific Activity</td>
<td>0.67 ± 0.22 u/mg</td>
</tr>
<tr>
<td>Factor VIIIIR:Ag</td>
<td>12 ± 4.6 u/ml</td>
</tr>
<tr>
<td>Factor VIIIIR:Ag</td>
<td>14.3 ± 5.7 u/ml</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>87 ± 1.2 mg/ml</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>32 ± 1.1 mg/ml</td>
</tr>
<tr>
<td>Total Protein</td>
<td>14.4 ± 1.5 mg/ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.6 ± 0.03</td>
</tr>
<tr>
<td>Conductivity</td>
<td>5 ± 0.7 mho</td>
</tr>
</tbody>
</table>

(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 8.5, 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 DoC. 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 reconstitution remaining after 24 hours. Adsorption 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%.
TABLE 5-14 PURIFICATION OF FACTOR VIII BY CONCENTRATION OF PLASMA PROTEIN AND SUBSEQUENT COOLING

<table>
<thead>
<tr>
<th>Method of concentration</th>
<th>N</th>
<th>VIII:C u/kg</th>
<th>VIII:Ag u/kg</th>
<th>Fibrinogen mg/kg</th>
<th>Fibrinocin mg/kg</th>
<th>Specific Activity u/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis vs 50% PEG</td>
<td>3</td>
<td>614</td>
<td>728</td>
<td>216</td>
<td>274</td>
<td>0.85</td>
</tr>
<tr>
<td>Ultrafiltration on PM-10 Membrane</td>
<td>4</td>
<td>772 ± 148</td>
<td>801 ± 162</td>
<td>116</td>
<td>ND</td>
<td>1.53 ± 0.41</td>
</tr>
</tbody>
</table>

* 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 O°C for 30 min. and centrifuged for 30,000 g min at O°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, VIII: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 immediately 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 fastthawing method, cryoprecipitate remains suspended in supernatant plasma throughout thawing, allowing redissolving.

The conditions for the final separation of cryoprecipitate are however also of importance. Thus, when centrifuging bags of thawed plasma after fastthawing, 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 fastthawed plasmatopolycarbonate 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 O°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 slowermigrating 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 adsorption (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 RES 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 ionexchange chromatographic techniques (Curling 1980, Suomela 1980) are likely to be much more suitable for production on a blood-bank scale. The presence of an unchanged 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.

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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 saltingout 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 VII, 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

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Yield in Component % of Starting Plasma</th>
<th>Specific Activity VIII:C u/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VIII:C</td>
<td>VIIIR:Ag</td>
</tr>
<tr>
<td>Plasma</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cryosupernatant</td>
<td>15 ± 7</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Tris Extract of Cryo</td>
<td>56 ± 12</td>
<td>72 ± 18</td>
</tr>
<tr>
<td>Residue from Extraction</td>
<td>&lt; 1</td>
<td>6.8 ± 2.5</td>
</tr>
</tbody>
</table>

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 glycinedextrose 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.

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FIGURE 6-1

DIAGRAMATIC REPRESENTATION OF A PRECIPITIN ARC IN A TWO-DIMENSIONAL IMMUNOELECTROPHORESIS, TO ILLUSTRATE THE MIGRATION DISTANCES MEASURED
Analysis of factor VIII:Ag in two dimensional immunoelectrophoresis (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. d₁ and d₂ were then measured and recorded in mm.

An increase in distance d₁ is interpreted to be the result of a decrease in the amount of antigen with slower electrophoretic mobility. An increase in d₂ 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

<table>
<thead>
<tr>
<th>Yield of Protein*</th>
<th>Cryoprecipitate Produced by Fast-Thaw, Dissolved in 10 ml of Residual Plasma, and Held for 2 Hours at 0°C</th>
<th>Cryoprecipitate Produced by Thaw-Siphon/Centrifugation with Complete Predonage of Plasma, Dissolved in 10 ml of Citrate-Saline, and Held for 2 Hours at 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anticoagulant (a)</td>
<td>Anticoagulant (m)</td>
</tr>
<tr>
<td></td>
<td>Cryo (g)</td>
<td>Cryo/Repepia (m)</td>
</tr>
<tr>
<td>VIII:C Yields</td>
<td>1</td>
<td>63 ± 17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>74 ± 11</td>
</tr>
<tr>
<td>VIII:Kag Yields</td>
<td>1</td>
<td>63 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65.5 ± 17</td>
</tr>
<tr>
<td>Fibrinogen Yields</td>
<td>1</td>
<td>32.5 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>57.3 ± 12</td>
</tr>
<tr>
<td>Fibrinectin Yields</td>
<td>1</td>
<td>31 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>55.6 ± 38</td>
</tr>
<tr>
<td>Specific Activity Cryo</td>
<td>0.12 ± 0.04</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Specific Activity</td>
<td>0.32 ± 0.11</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>Final Precipitate</td>
<td>0.38 ± 0.32</td>
<td>0.38 ± 0.32</td>
</tr>
</tbody>
</table>

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

t 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).
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

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.
FIGURE 6-4
PRECIPITATION OF CRYOEXTRACT AT DIFFERENT pH VALUES
Cryo extract was prepared from FFP and precipitated at O°C with Ficoll-70. pH was varied by addition of 0.1 N NaOH.
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
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

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Yield of VIII:C % of Glycine Supernatant</th>
<th>Specific Activity VIII:C μg/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M Glycine Supernatant</td>
<td>100</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>6.5 % PEG Precipitate</td>
<td>92.5 ± 9.3</td>
<td>1.4 ± 0.45</td>
</tr>
</tbody>
</table>

Cryos were subjected to glycine precipitation and the precipitate removed by centrifugation. The supernatants were brought to 6.5% PEG 6000 at O°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 M 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 M solution led to near quantitative
TABLE 6-5A PURIFICATION OF CRYOPRECIPITATE USING ZINC

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Yield in Fraction, % of Cryo Extract</th>
<th>Specific Activity VIII:C U/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryo Extract</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 mM Zinc Acetate Supernatant</td>
<td>97 ± 6</td>
<td>94 ± 12</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Yield in Fraction, % of HES 40 Precipitate</th>
<th>Specific Activity VIII:C U/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES 40 Precipitate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 mM Zinc Acetate Supernatant</td>
<td>99 ± 16</td>
<td>78 ± 12</td>
</tr>
</tbody>
</table>

Results show the mean and standard deviation of 4 experiments. added as in Table 6-SA.
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 densitometry

A. Cryoprecipitate dissolved in buffer
B. 2 M glycine supernatant of cryoprecipitate
C. 4% HES 40 precipitate of plasma
D. 1 roM zinc acetate supernatant of 4% HES precipitate
E. Clinical intermediate-purity concentrate
F. 1 roM 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 B-CHAIN OF FIBRINOGEN
IV γ-CHAIN OF FIBRINOGEN
recovery of VIII:C and VIIIIR: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 zincprecipitation 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 VIII: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.
### TABLE 6-6 ANALYSES OF VIIR-Ag BY TWO-DIMENSIONAL IMMUNOELECTROPHORESIS

<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_1$</th>
<th>$d_2$</th>
<th>$d_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilised Cryo</td>
<td>6</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Cold Precipitate of Cryo</td>
<td>6</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Glycine-Purified Cryo</td>
<td>7</td>
<td>12.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Extracted Cryo</td>
<td>10</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Zinc-Purified Extracted Cryo</td>
<td>9.5</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>4.5% PEG Fraction of Extracted Cryo</td>
<td>6.5</td>
<td>10.5</td>
<td>18</td>
</tr>
<tr>
<td>8% PEG Fraction of Extracted Cryo</td>
<td>11.5</td>
<td>17</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Samples were analysed by 2DIEP as described in Methods.

d1, f, and d2 denote the respective migration 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 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 d1 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 largescale fractionation) is rich in slow and medium moving forms of VIIIR:Ag.
TABLE 6-7 SUMMARY OF CHARACTERISTICS OF HIGHER-PURITY FACTOR VIII CONCENTRATES

<table>
<thead>
<tr>
<th>Product</th>
<th>Specific Activity VIII:C u/mg Protein</th>
<th>Fibrinogen % of Total Protein</th>
<th>Yield of VIII:C % of Intermediate Material</th>
<th>U/kg Starting Plasma</th>
<th>Purification Over Intermediate Material*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold Insoluble Globulin Precipitate of Cryo</td>
<td>0.35</td>
<td>40</td>
<td>75</td>
<td>310</td>
<td>3.0</td>
</tr>
<tr>
<td>PEG Precipitate of Glycine-Purified Cryo</td>
<td>1.4</td>
<td>16</td>
<td>78</td>
<td>420</td>
<td>2.6</td>
</tr>
<tr>
<td>Znco-Purified Cryo Extract</td>
<td>0.9</td>
<td>25</td>
<td>87</td>
<td>370</td>
<td>1.9</td>
</tr>
<tr>
<td>Znco-Purified HES Precipitate</td>
<td>1.3</td>
<td>19</td>
<td>96</td>
<td>530</td>
<td>1.9</td>
</tr>
<tr>
<td>PEG-Purified Cryo Extract</td>
<td>0.9</td>
<td>12</td>
<td>56</td>
<td>210</td>
<td>2.1</td>
</tr>
</tbody>
</table>

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, SmitSibinga 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 70mg/ml 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 coprecipitation 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. 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
VIIIIR: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 VIIIIR: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
VIIIIR: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 VIIIIR:Ag
this might be due to the use of low ionic strength
extraction buffers, as VIIIIR: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 VIIIIR:Ag in
the discarded 4.5% PEG precipitate. The VIIIIR: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 VIIIIR: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 VI I

ADDITIONAL APPROACHES TO

OPTIMAL USE OF DONATED FACTOR VIII

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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 sorbita lglycine (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 sorbitol 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 from a semilogarithmic 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

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

Gels were 5% polyacrylamide. Electrophoresis was at 30 V for 18 hours.
TABLE 7-2 ANALYTICAL PROFILE OF CLINICAL HEAT-TREATED
FACTOR VIII
CONCENTRATE

Data for vials are constituted in 25 ml of distilled water.

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

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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 pursued 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

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

<table>
<thead>
<tr>
<th>Preparation Infused</th>
<th>Recovery (%)</th>
<th>Half-Life (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen Cryoprecipitate*</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>Intermediate Purity Concentrate*</td>
<td>122</td>
<td>10.5</td>
</tr>
<tr>
<td>Heat-Inactivated Concentrate†</td>
<td>92</td>
<td>12.3</td>
</tr>
</tbody>
</table>

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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.
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

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

* Clinical albumin solution

+ Stabilised as in routine production with sodium caprylate
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.

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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.
INTRODUCTION

It has long been known that persons of blood groups A and B have higher factor VIII levels than persons of group 0 (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 0 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 0 plasma (Prowse et al 1982). Oligosaccharide structures characteristic of blood group A, Blo 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 0-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 VIII: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-VIII: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 VIII:Ag by elution with 300 mM CaCl2, 50 mM imidazole / HCl pH 7.2, 0.01% Tween 80, 0.1% turkey serum. VIII: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 VIII:Ag-containing fractions were obtained.
FIGURE 7-4

PRINCIPLE OF IMMUNOADSORPTION OF BLOOD GROUP ASSOCIATED FACTOR VIII

Irrmmobilised 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.
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

 ADSORPTION OF FACTOR VIII BY IMMOBILISED ANTIBODIES

Results show the mean and standard deviations of 3 experiments. The percentage adsorption compared to the initial values was estimated by subtracting the residual activities from the starting activities.
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 0 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 adsorption 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 adsorption of 54% by the anti-A and 22% by the nonimmune IgG, a difference which was significant \( p < 0.005 \).

2. Group 0 pool.

For both incubation periods, the two antibodies adsorbed small amounts of VIIIIR :AgI; 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 haemagglutination 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 adsorption 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 0 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 unassociated 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.

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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 l-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.

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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 VIII:Ag.
FIGURE 7-6

CHANGES IN FACTOR VIII FOLLOWING DDAVP INFUSION

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

Significant (p < 0.05) increases are shown as filled symbols.
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, VIIIIR :RCF 97 and
VIIIIR:Ag 98 u/dl.
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 III

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 (Hassig 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 (Hessig 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 (Höggren 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 VII B) 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 VII C) 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
FIG1.JRE 8 - I PLASTIC BAG SYSTEMS FOR OPTIMAL PRESERVATION OF DONATED FACTOR VIII
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 anticoagulants 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 anticoagulation, 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 anticoagulant. 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 singledonation 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
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.

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

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)
Scheme II - Quadruple bag system

Blood is collected into A, platelet-rich pRisma 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 (i) production of platelet rich plasma does not allow hard centrifugation in
order to remove the maximum volume of plasma in the SAG procedure, (ii) $b$ 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 RES 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 RES 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.
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 reservoir 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 RES 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 (Myllly 1983).
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 - Myllylg (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, Blornback 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

Figure 8-3 describes the various stages for production of a factor VIII concentrate as described in this Thesis (Chapters V, VI and VII). The procedure is shown in a flow diagram below. The following steps are involved:

1. **Whole Blood Donation**
   - Separate Plasma
   - Express into HES 40 Solution
   - Chill and Harvest Precipitate

2. **Factor VIII-Rich Precipitate**
   - Store Frozen
   - Pool 100-200 Precipitates into Buffer

3. **Pooled Concentrate Solution**
   - Filter (0.22 μm)
   - Add Zinc Acetate
   - Dispense
   - Lyophilise

4. **Factor VIII Concentrate**
   - Add Glycine/Sorbitol
   - Adjust pH
   - Heat 70°C, 30 min.

5. **Sterilised Concentrate**
   - Remove Reagents (Precipitation)
   - Filter (0.22 μm)
   - Dispense
   - Lyophilise

6. **Factor VIII Concentrate**

The diagram illustrates that a range of viruses are inactivated using this technique. A heat-sterilised concentrate can therefore reduce significantly the risk of transmission and characterisation of the disease. The introduction of sensitive assays for viral markers and the holding of pooled plasma in quarantine to allow follow-up (Smith 1983a). This last option cannot be applied.
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-Siblinga 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 2~ million units of factor VIII concentrate, which are adequate to cover needs. Processing by thawsiphon 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.
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