CHEMICAL MODIFICATION AND IMMUNOLOGICAL STUDIES ON RECOMBINANT FACTOR VIII

BY

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TO MUM

DECLARATION

I hereby declare that the work presented here is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Fiona Manning

FIONA MANNING

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ABBREVIATIONS

Abs	Absorbance
AC	Alternating Current
AHF	Antihemophilic factor
AIDS	Acquired Immune-Deficiency Syndrome
AMP	2-amino-2-methyl-1-propanol
AP	Alkaline phosphatase
aPC	Activated Protein C
APS	Ammonium persulphate
APTT	Activated Partial Thromboblastin Time
Arg	Arginine
Asp	Aspartic acid
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BiP	Immunoglobulin binding Protein
BMME	Bis-(maleimido)-methyl ether
BMPPD	N-N'-bis (3-maleimido-propionyl)-2-hydroxy-1,3-propanediamine
BSA	Bovine serum albumin
BQ	Benzoquinone
cDNA	Complementry deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CHO	Chinese hamster ovary
CRM^+	Cross-reactive material
Cys	Cysteine
Da	Daltons
DAB	3,3'-diaminobenzidine tetrachloride
DEAE	Diethylaminoethyl
DFP	Diisopropyl fluorophosphate

DMA	Dimethyl adipimidate
DMEM	Dulbecco's modification of Eagle's medium
$DMEMS_{10}$	Dulbecco's modification of Eagle's medium containing 10% (v/v) foetal calf serum
$DMEMS_{10}$/T24	Dulbecco's modification of Eagle's medium containing 10% (v/v) foetal calf serum and 5% (v/v) T24 medium
DMF	Dimethyl formamide
DMP	Dimethyl pimelimidate
DMS	Dimethyl suberimidate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E	Applied potential
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
EIA	Enzyme-immunoassay
ELISA	Enzyme-linked immunosorbent assay
e.r.	Endoplasmic reticulum
FCS	Foetal calf serum
FII	Factor II (thrombin)
FV	Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FVIIIC	Procoagulant activity of Factor VIII
FVIIIC:Ag	Antigenic determinants of FVIII:C
FIX	Factor IX
FIXa	Activated Factor IX
FX	Factor X
FXa	Activated Factor X
GA	Glutaraldehyde
GAR	Goat anti-rabbit antibody

GAR-AP	Goat anti-rabbit antibody linked to alkaline phosphatase
GAR-HRP	Goat anti-rabbit antibody linked to horseradish peroxidase
Glu	Glutamic acid
GMBS	N-(γ-maleimidobutyryloxy) succinimide
GP-Ib	Glycoprotein Ib
HAT medium	Medium containing hypoxanthine, aminopterin and thymidine
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
His	Histidine
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
HQ	Hydroxyquinone
HRP	Horseradish peroxidase
HT	Medium containing hypoxanthine and thymidine
I	Inactive state of a protein
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin of the G class
IgM	Immunoglobulin of the M class
Ile	Isoleucine
IL-6	Interleukin-6
i.p.	Intraperitoneally
IRMA	Immunoradiometric assay
2-IT	2-Iminothiolane
K	Rate constant for protein unfolding
k	Rate constant protein inactivation
kDa	kilodaltons
KLH	Keyhole limpet haemocyanin
Lys	Lysine
M	Molar
MAbs	Monoclonal antibodies

MHV	Mouse hepatitis virus
mM	millimolar
Mr	Molecular weight
mRNA	Messenger ribonucleic acid
N	Native state of a protein
nA	Nanoamp
NRK	Normal rat kidney cells
OPD	o-Phenylenediamine
O-ph	o-Phenanthroline
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-Tween	PBS containing 0.05% (v/v) Tween-20
PEG	Polyethylene glycol
PL	Phospholipids
pNA	p-Nitroaniline
Pristane	2,6,10,14-tetramethylpentadecane
PVC	Polyvinyl chloride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCE	Saturated calomel electrode
Ser	Serine
SDS	Sodium dodecyl sulphate
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate
TEMED	N,N,N′N′-Tetramethylethylenediamine
TK	Thymidine kinase
TMAO	Trimethylamine N-oxide
TMB	3,3′, 5,5′-Tetramethylbenzidine
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
Tyr	Tyrosine

U	Reversibly denatured state of a protein
(v/v)	Volume per volume
Val	Valine
vWF	von Willebrand factor
vWFAg	Antigenic determinants of vWF
(w/v)	Weight per volume
W-P	Wiebel-Palade bodies

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Manning, F., Ó Fágáin, C. and O'Kennedy, R. Factor VIII, Structure, Function and Analysis. *Biotech. Advs.*, 1993; **11**: 79-114.

Manning, F., Ó Fágáin, C., O'Kennedy, R., Deasy, B. and Smyth, M. Development of an antibody-based biosensor for determination of FVIII using horseradish peroxidase-labelled anti-rFVIII antibody. *Anal. Proceedings incl. Anal. Communications*, 1994; **31**: 13-15.

Manning, F., Ó Fágáin, C. and O'Kennedy, R. The effects of chemical modifiers on rFVIII activity. (Submitted for publication)

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POSTER PRESENTATION

Manning, F., Ó Fágáin, C. and O'Kennedy, R. Chemical Modification of rFVIII. Presented at FEBS '92, Trinity College, Dublin. August 1992.

CHEMICAL MODIFICATION AND IMMUNOLOGICAL STUDIES ON RECOMBINANT FACTOR VHI

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Factor VIII (FVIII) is a high molecular weight glycoprotein which is deficient or functionally defective in Haemophilia A. It circulates in normal plasma as part of a complex with von Willebrand factor (vWF). In the absence of vWF, FVIII is highly unstable.

The effects of chemical modifiers on recombinant FVIII (rFVIII) were investigated. A variety of homobifunctional and heterobifunctional chemical cross-linkers and protein-modifying agents with different side chain specifities was employed. The amino-specific reagents, with the exception of 2-Iminothiolane, caused significant loss of FVIII activity. However, reaction with thiol-specific compounds did not lead to any loss of FVIII activity. It would appear therefore, that amino groups are involved in FVIII activity.

Monoclonal and polyclonal antibodies were produced to rFVIII. The effect of binding of these antibodies on FVIII procoagulant activity was investigated. It was found that the binding of the monoclonal antibodies caused loss of activity, whereas, binding of the polyclonal antibodies did not neutralise procoagulant activity.

The antibodies were used in the development of enzyme-linked immunosorbent assays (ELISAs) for the detection and quantitation of rFVIII and anti-rFVIII antibodies using a number of different formats.

The antibodies were also utilised in the development of a biosensor. Using this device, FVIII in normal human plasma and calibration plasma, as well as anti-rFVIII antibodies could be detected. The biosensor also provides a novel method to monitor antibody-antigen interactions. rFVIII was immobilised onto an electrode surface and incubated in a horseradish peroxidase-labelled anti-rFVIII antibody solution. The electrochemical response was based on the enzymic reduction of H_2O_2 in the presence of an electron mediator (hydroquinone). The oxidised quinone produced was reduced at the electrode surface and current measured.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The coagulation mechanism of vertebrate blood was first proposed to be composed of a series of reactions which function as a biological amplifier by Macfarlane in 1964. This basic hypothesis has not been altered, although knowledge of the basic components and reactions, as well as the involvment of other mechanisms besides the coagulation factors (platelet) has greatly increased.

Factor VIII (FVIII) is an essential component of the blood coagulation cascade. A deficiency or abnormality in this protein results in the bleeding disorder known as classic haemophilia.

The low concentration of FVIII in normal plasma (approximately 5-10 µg/ml), in addition to its extreme sensitivity to proteolysis, hampered early attempts to isolate the protein. Isolation and purification were eventually achieved and led to the determination of the complete amino acid sequence (Toole *et al.*, 1984; Vehar *et al.*, 1984).

In early attempts to study Factor VIII a much larger protein was always co-isolated. This protein, von Willebrand factor (vWF), is very closely associated with FVIII. The two proteins circulate in normal plasma as a non-covalently linked complex. vWF comprises the majority of the protein mass of the FVIII/vWF complex (approx. 95%-99%) and, hence, these early studies on "FVIII" actually described properties of vWF, which were generally unaffected by the presence or absence of FVIII, the procoagulant activity of the complex. Thus, to avoid confusion, the International Committee on Thrombosis and Haematosis proposed a nomenclature to describe the properties of the FVIII/vWF complex (Table 1.1). von Willebrand protein functions in the cellular aspects of coagulation. It facilitates platelet-vessel wall interactions (Roth, 1992). FVIII functions as a cofactor in the intrinsic phase of the blood coagulation cascade (Hoyer, 1981). It forms a complex, the "tenase complex", in combination with calcium ions, phospholipids and activated Factor IX (IXa), which activates Factor X. (Figure 1.1).

The two proteins FVIII and vWF are under the control of different genes. FVIII is a product of the X chromosome, and, thus, classic haemophilia is inherited as a recessive, sex-linked disorder. The vWF gene is located on the short arm of chromosome 12 (Ginsberg *et al.*, 1985). von Willebrand disease, a condition resulting from a deficiency or abnormality in vWF, is inherited as an autosomal disorder.

Abbreviation	Corresponding property of the FVIII/vWF complex
FVIII/vWF	The FVIII/vWF complex
FVIII:C	The procoagulant activity of FVIII, measured by clotting assay.
FVIIIC:Ag	The antigenic determinants of FVIII:C, measured by immunological methods.
FVIII:R	FVIII-related protein, von Willebrand factor.
FVIII:RAg	Antigenic determinants on FVIII:R, determined by immunological techniques employing heterologous antisera against the FVIII/vWf complex.
FVIIIR:R	Ristocetin cofactor activity, the property of vWF which supports ristocetin-induced agglutination of normal platelets.

Table 1.1 Nomenclature of the FVIII/vWF complex.

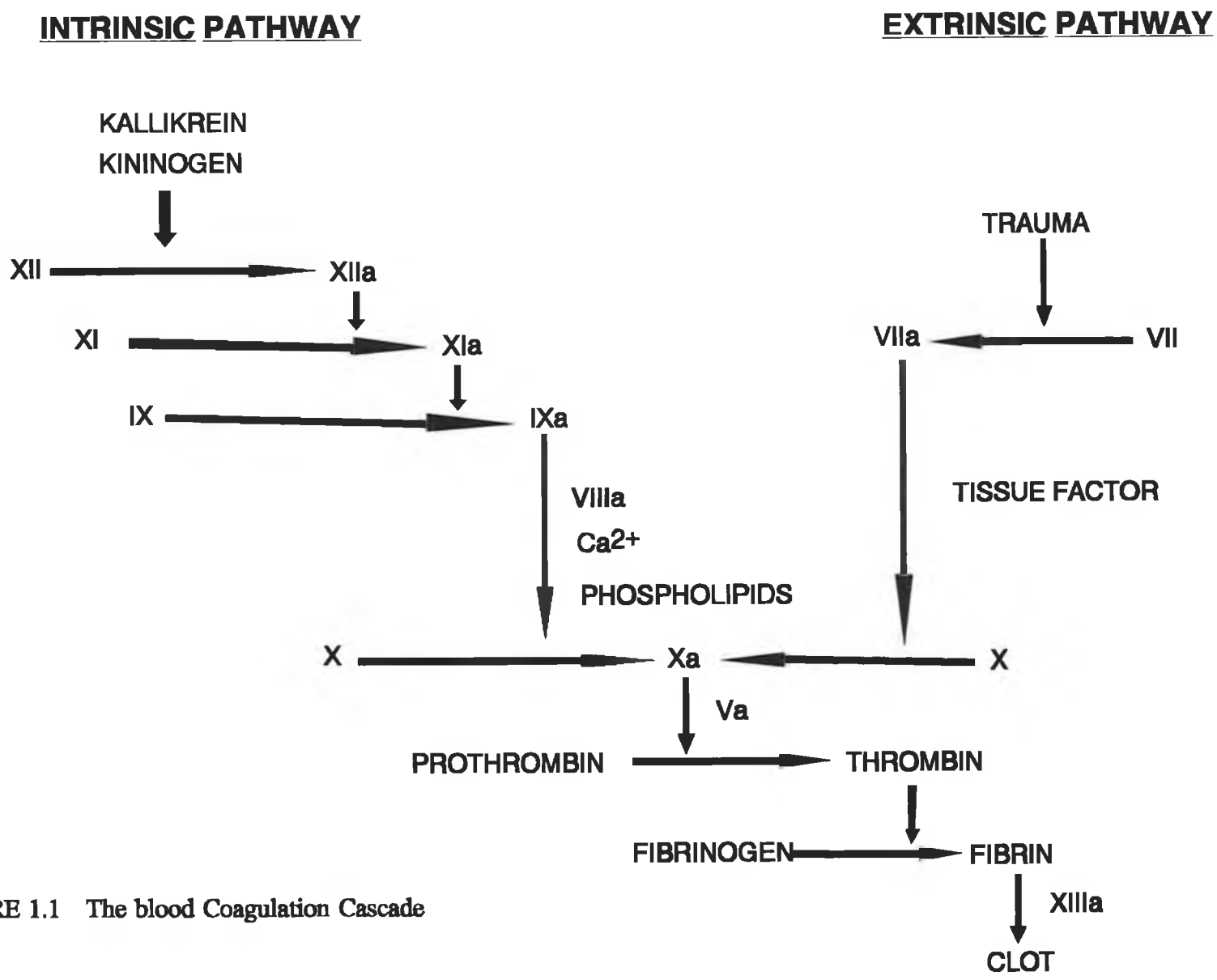


FIGURE 1.1 The blood Coagulation Cascade

FVIII is inherently unstable in the absence of vWF. Its optimal pH lies between 6.9 and 7.2, with marked decrease in stability below pH 6 and above pH 8 (Wolf, 1959). Calcium ions (Ca^{2+}) are also necessary for FVIII stability, but other divalent cations have a comparable effect (Mikaelsson, 1983). The optimal calcium concentration lies between 0.3 and 1.0 mM. The presence of protease inhibitors (DFP or heparin) increases the stability (Rock *et al.*, 1983). Removal of Ca^{2+} reduces initial activity and leads to increased instability (Rock *et al.*, 1983). Activity and stability can be recovered upon recalcification (Ganz *et al.*, 1987). Sakuragawa *et al.* (1988) modified FVIII using polyethylene glycol and found that the modified protein was stable against inactivation by activated Protein C and plasmin, but not against thrombin.

1.2 Purification

Structural and other studies on factor VIII require the isolation of the pure protein. Despite FVIII's low concentration in normal plasma and its susceptibility to proteolytic attack, bovine, porcine and human FVIII have now been successfully isolated.

Vehar and Davie (1980) isolated bovine FVIII and estimated its molecular weight (Mr) to be in the region of 300 kiloDaltons (kDa). After reducing the protein and subjecting it to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), they observed three bands corresponding to Mr of 85, 88 and 90 kDa. Knutson (1982) and Fass (1982) both isolated porcine FVIII which showed similar properties to the bovine FVIII reported by Vehar and Davie, but their protein gave a different series of bands when run on SDS-PAGE. Bands were observed at Mr 166, 130 and 76, kDa. The 130 kDa band was found to be derived from the 166 kDa peptide but the Mr 76 kDa was a distinct protein bound by calcium ions to the other chain.

Human FVIII was much more difficult to isolate. Both bovine and porcine FVIII had been isolated from large volumes of fresh plasma. Use of the same volume of human plasma was not feasible, so alternative methods for the isolation had to be used. Fulcher and Zimmerman (1982) employed a heterologous antibody to isolate the protein. When this FVIII was run on SDS-PAGE, two main bands at Mr 70 kDa and 80 kDa were evident, with others up to Mr 188 kDa. Weinstein *et al.* (1983) confirmed Fulcher and Zimmerman's earlier results that all these bands were derived from the FVIII protein.

These findings suggest that some degree of proteolysis must have occurred during purification. Rotblat (1985) purified FVIII from human plasma to which protease inhibitors had been added. A band was identified which corresponded to a molecular weight (Mr) of 365 kDa which was proposed to be the molecular weight of native FVIII. Minor bands of lower Mr were said to result from limited proteolysis.

1.3 The Structure of the FVIII molecule

The purification of bovine, porcine and human FVIII, free of von Willebrand's factor, the cloning of the factor VIII gene and the isolation of complementary cDNA encoding Factor VIII represents the major steps in the understanding of Factor VIII. The molecular cloning and characterization of the FVIII coding region (Gitschier *et al.*,1984; Wood *et al.*,1984) allowed the nucleotide sequence to be determined and from this the 2351 amino acid sequence of the FVIII molecule was deduced. The first 19 amino acid residues constitute a signal sequence similar to the leader sequence for most secretory proteins and are lost during post-translational modifications. Thus, the mature FVIII polypeptide consists of 2332 amino acids with a calculated Mr of 264,763 Da. Glycosylation, mainly in the central B domain, accounts for the anomaly between the calculated Mr and that observed following purification (Mr 360 kDa).

Analysis of the sequences revealed that the protein is made up of three distinct structural domains (Figure 1.3). The triplicated A domains each contain approximately 330 amino acids and are found at positions 1-329, 380-711 and 1649-2019 of the mature polypeptide. A unique B domain containing approximately 980 amino acids, is located between the second and third A domains. The duplicated C domains, each containing about 150 amino acids, are located at the COOH-terminal of the molecule. The central B domain contains 19 of the 25 potential asparagine-linked glycosylation sites. The majority of the cysteine residues, meanwhile, are situated in the A and C domains, suggesting internal disulphide bond formation.

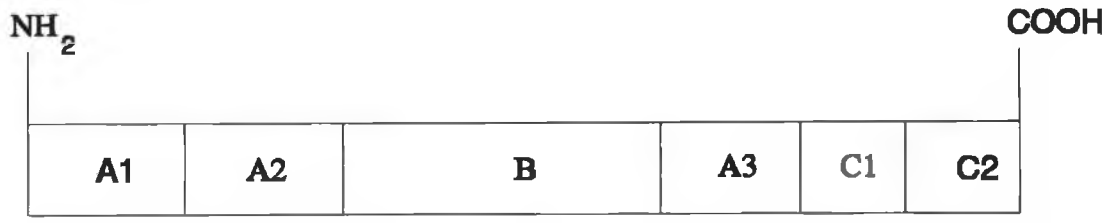


Figure 1.3 The domain structure of FVIII. The 2332 amino acid sequence of the FVIII molecule is made up of repeated domains. Two regions of a triplicated A domain are separated from the third region by a unique B domain containing approximately 980 amino acid residues. The duplicated C domains are located beside the third A domain at the carboxyl terminal end of the molecule. After thrombin activation of FVIII, the central B domain is excised, leaving the A and C domains to form the functional molecule.

Although FVIII is synthesised as a single polypeptide chain (Toole *et al.*, 1984; Wood *et al.*, 1984), it is isolated from plasma as a series of heterodimers, ranging in size from approximately 80 to 210 kDa, linked by divalent ions (Fass *et al.*, 1982). These are formed as a result of proteolysis between the B-A3 domains, and within the B domain (Vehar *et al.*, 1984). The A1 and A2 domains represent the majority of the FVIII heavy chain, but some or all of the B domain may also be present. The heavy chain thus exhibits a large degree of size heterogeneity. The light chain is composed of the A3-C1-C2 domains with an invariant size of 684 residues of Mr 80 kDa. (Figure 1.3). The binding sites for von Willebrand factor (Foster *et al.*, 1988) and phospholipids (Bloom *et al.*, 1987) have been localised in the light chain.

The A domains show considerable internal homology (about 30%) and share approximately 30% homology with ceruloplasmin, the copper-binding protein present in human plasma (Vehar *et al.*, 1984). This suggests some metal binding capabilities for FVIII. The C domains exhibit internal homology (approximately 40%) and show about 20% homology with discoidin I, a phospholipid-binding lectin from the slime-mold *Dictyostelium discoidium* (Kane and Davie, 1988). They also show homology to a mammary epithelial cell surface protein (Stubbs *et al.*, 1990).

1.4 Homology between FVIII and Factor V

The similarities between FVIII, Factor V (FV) and ceruloplasmin were noted by Fass *et al.*, (1983). Factors V and VIII both function as cofactors in the blood coagulation cascade. FVIII is involved in the activation of factor IX in the intrinsic phase, and FV in the activation of factor X during the common phase of the coagulation cascade (Figure 1.1). Both cofactors are also activated by thrombin.

Analysis of their amino acid sequences revealed a homology of approximately 40%, with the greatest similarities occurring at the amino- and carboxyl-terminals (Kane and Davie, 1988). These regions represent the active form of the protein after thrombin activation.

Both proteins are composed of repeated domains - a triplicated A domain, a duplicated C domain and a unique B domain situated between the second and third A domains (Vehar *et al.*, 1984; Toole *et al.*, 1984).

The amino-terminal A domains show similarities with the copper-binding plasma protein, ceruloplasmin, suggesting that these three proteins constitute a family of structurally related proteins (Church *et al.*, 1984). The central B domains which are released during thrombin activation of both FVIII and FV, contain the majority of possible glycosylation sites, but apart from this similarity, they share only about 14% homology.

1.5 Activation and inactivation cleavages of FVIII by thrombin

FVIII has no detectable activity prior to proteolytic activation. Thrombin and FV are the only enzymes capable of activating FVIII. The activation of FVIII is initiated by cleavage between Arg 740-Ser 741 in the heavy chain, producing a uniform 90 kDa fragment. This is followed by further cleavages at Arg 372, between the A1 and A2 domains of the heavy chain, and Arg 1689 in the light chain (Figure 1.5.1). The 50 and 43 kDa heavy chain-derived peptides, and the 73 kDa light chain, are thought to constitute the active form of FVIII.

The structure of activated FVIII (FVIIIa) has been difficult to discern due to its lability. Lollar *et al.* (1989) isolated thrombin-activated porcine FVIII, consisting of a heterotrimer of the A1, A2 and A3-C1-C2 peptides. They proposed that loss of procoagulant activity was due to dissociation of the A2 subunit (Lollar *et al.*, 1991). Fay *et al.* (1991b) suggested that human FVIIIa was also a heterotrimer. Dissociation of the A2 subunit resulted in loss of procoagulant activity, but this could be restored by reconstitution of the inactive A1/A2-C1-C2 dimer and the A2 subunit. An acidic region at the COOH terminus of the A1 subunit is thought to be involved in the association of the A2 subunit with the A1/A3-C1-C2 dimer (Fay *et al.*, 1993). Addition of the A2 subunit to the A1/A3-C1-C2 dimer of recombinant human FVIII increased the procoagulant activity by 22 fold (Pittman *et al.*, 1992). Lamphear *et al.* (1992), showed that both the A1 and A1/A3-C1-C2 subunits were required for association with FIXa, suggesting that assembly of the "tenase complex" stabilises FVIIIa by preventing subunit dissociation.

Using site-directed mutagenesis, Pittman *et al.* (1988) selectively mutated the arginine residues at 740, 372 and 1689 to isoleucine and studied the effect of these mutations on FVIII activity.

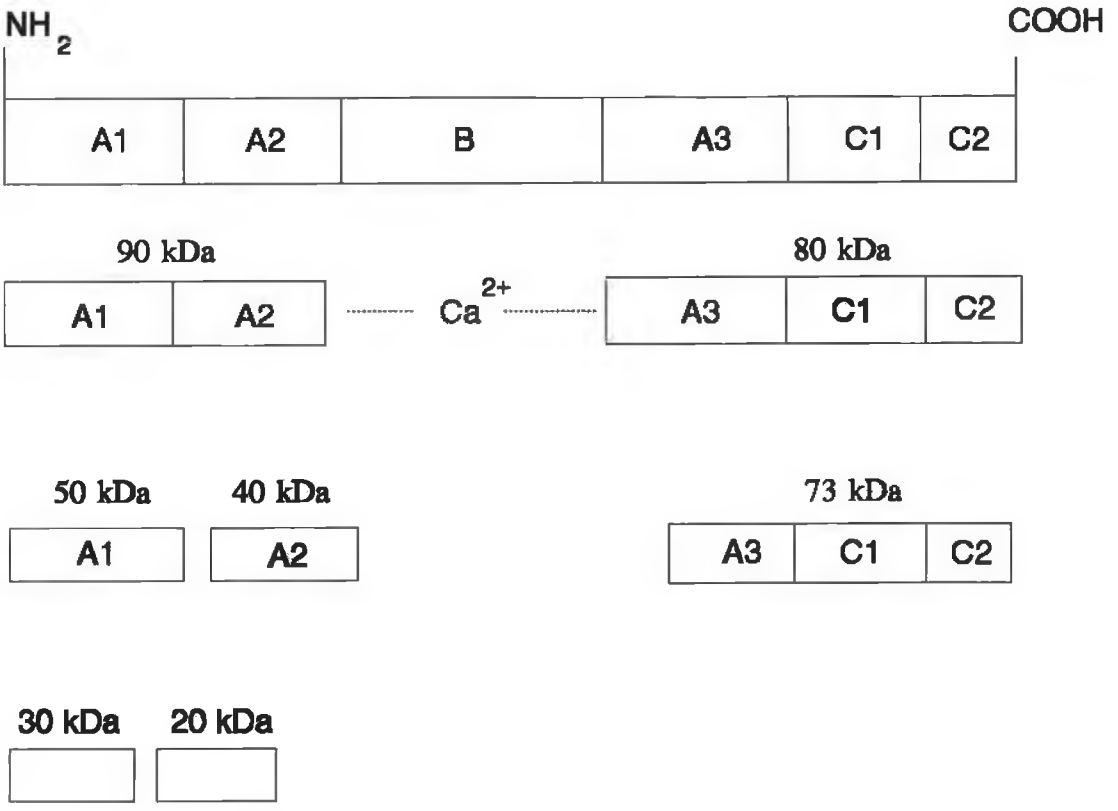


Figure 1.5.1 Thrombin activation of FVIII. The initial cleavage by thrombin occurs after residue 740. Further cleavages at 372 and 1689 release peptides of molecular weight 50, 40 and 73 kDa, respectively, which are thought to constitute the active form of FVIII. The peptides are thought to be linked by calcium bridges. Cleavage at residue 336 (by thrombin, activated Protein C and factor Xa) inactivates the protein.

Mutation at 740 inhibited thrombin cleavage, but had minimum effect on FVIII procoagulant activity. In contrast, while both Arg 372 -→Ile and Arg 1689 -→ Ile mutants exhibited low levels of intrinsic procoagulant activity, they were both resistant to thrombin cleavage and thrombin activation.

Resistance to thrombin cleavage at one site did not affect susceptibility at the other site. These results seem to suggest that while cleavage at 740 is not essential for thrombin activation, cleavage at the remaining sites (372 and 1689) is, but the order in which they occur is irrelevant.

The importance of the 372 and 1689 cleavage sites has been highlighted by Gitschier *et al.* (1988). They identified haemophilia A patients with missense mutations at residues 372 and 1689, resulting in normal levels of circulating FVIII:Ag, but no detectable FVIII coagulant activity.

Longer exposure to thrombin causes inactivation. Further cleavage of the heavy chain occurs at residue 336, resulting in the formation of a 45 kDa fragment. Activated Protein C and Factor Xa also cleave at this position, inactivating FVIII (Fulcher *et al.*, 1984; Eaton *et al.*, 1986: Eaton *et al.*, 1987). The presence of protease inhibitors does not prevent inactivation (Rick and Hoyer, 1977; Hultin and Jesty, 1981) so other inactivation processes must be involved. The dissociation of subunits has been implicated as a possibility, as chelation of metal ions causes loss of activity (Eaton *et al.*, 1987). Fay *et al.* (1987) cross-linked the heavy and light chains, stabilising FVIIIa and preventing dissociation.

In plasma FVIII circulates as part of a complex with von Willebrand factor. In order to participate in coagulation, the FVIII must be released from this complex (Figure 1.5.2). Cleavage after residue 1689 serves to release the 70 kDa light chain fragment from vWF (Hamer *et al.*, 1987b; Lollar *et al.*, 1988). FXa is also capable of activating FVIII, with cleavage also occurring at residue 1689 (Eaton *et al.*, 1986).

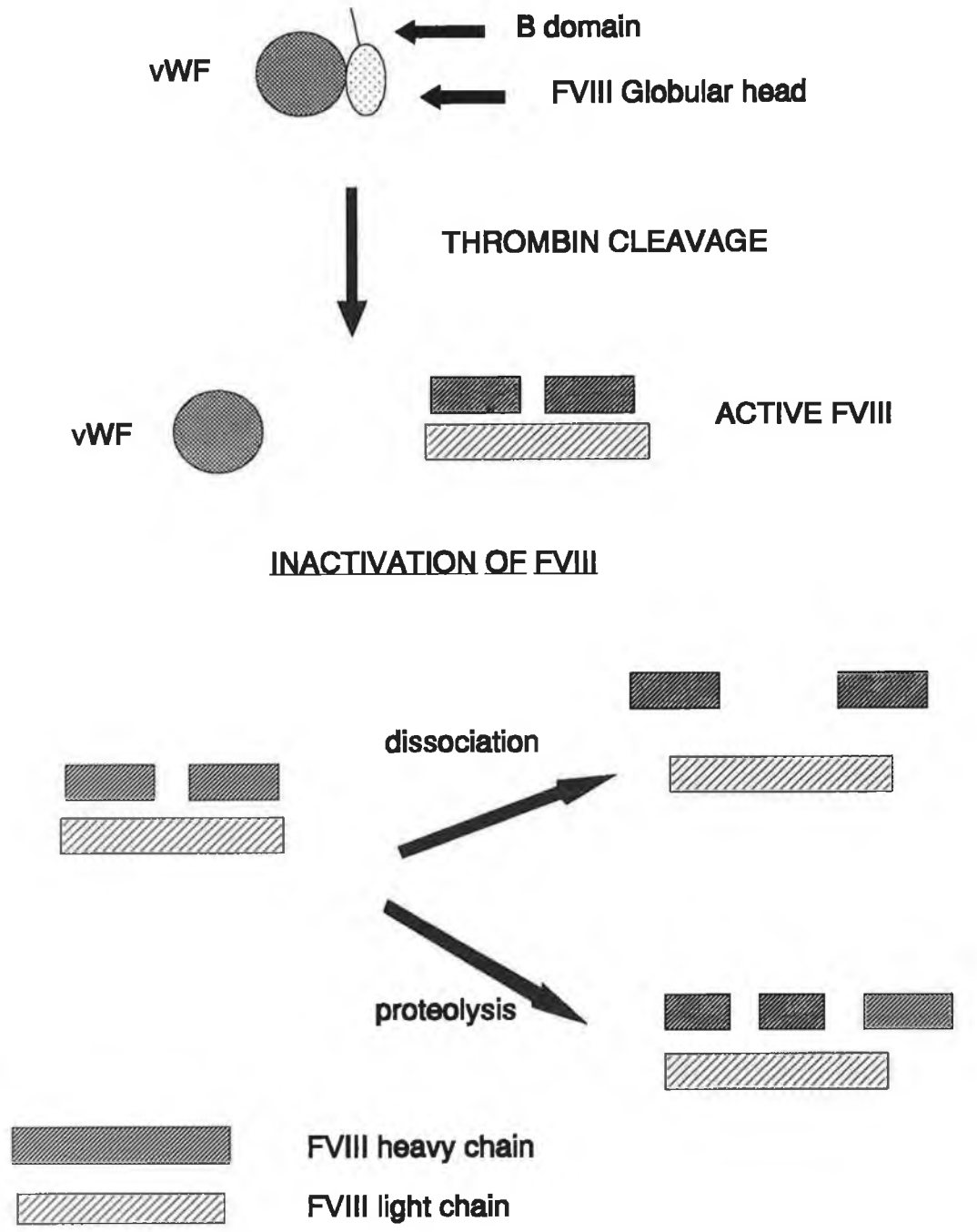


Figure 1.5.2 The activation of FVIII *in vivo*. FVIII circulates in normal plasma complexed with von Willebrand factor (vWF). Thrombin cleaves the light chain, releasing active FVIII. Inactivation occurs as a result of further proteolytic processing by thrombin, activated Protein C and factor X, or by dissociation of the subunits. The light chain is composed of the A3-C1-C2 domains, while the A1-A2 domains make up the heavy chain.

1.6 Inactivation of FVIII by activated Protein C

Protein C, a potent circulating anti-coagulant, is a zymogen of a vitamin K-dependent serine protease (Valcarce *et al.*, 1993). Activation of protein C occurs on the surface of the endothelium and is catalysed by a complex of thrombin bound reversibly to a cell surface receptor, thrombomodulin (Esmon, 1989). Complex formation accelerates the activation of protein C, while inhibiting the ability of thrombin to promote clotting (Esmon *et al.*, 1993). Thrombin cleaves an internal peptide bond (Arg-Ile) in the heavy chain of protein C, in the presence of Ca^{2+} and phospholipids. Activated Protein C (aPC) then inactivates FVIII by cleavage of the light chain. Walker *et al.* (1990) located regions of the light chain A domain (the A3 domain) that served as a binding site for aPC (residues 2009-2019). Koedam *et al.* (1988) and Rick *et al.* (1990) reported that association of FVIII with von Willebrand factor protects the cofactor from aPC. Fay *et al.* (1991a) suggested that the protection conferred on FVIII by vWF results from a reduced affinity of the complex for the phospholipid membrane. aPC is inactivated by an unknown inhibitor present in normal plasma (Marlar *et al.*, 1982).

1.7 The FVIII/von Willebrand factor complex

FVIII circulates in normal plasma as part of a complex, in which it is non-covalently bound to von Willebrand factor (vWF). The larger vWF constitutes approximately 95%-99% of the protein mass. The two proteins differ functionally, immunologically and are under different genetic control. FVIII functions as a cofactor in the blood coagulation cascade and possesses distinct antigenic determinants (FVIII:Ag). It is deficient or abnormal in classic haemophilia.

vWF plays a role in platelet-blood vessel wall interactions, and has its own distinct antigenic determinants (vWFAg or VIIIR:Ag). Abnormality or deficiency in vWF results in the bleeding disorder called von Willebrand disease.

Evidence that FVIII and vWF exist in normal plasma as a complex can be gained from studies in which FVIII and vWF were both isolated together. Using gel filtration and sucrose-density gradient ultracentrifugation, under conditions of high ionic strength (1M NaCl or 0.24M $CaCl_2$), several groups found that the two proteins could be separated

(Owen *et al.*, 1972; Rick and Hoyer, 1973; Weiss *et al.*, 1973). vWF behaved as a high molecular weight protein, eluting in the void volume. FVIII, being the smaller of the two components, remained in the column and was released on addition of 0.24M $CaC1_2$. The dissociation is not irreversible, the two proteins reassociate when the ionic strength is returned to physiologic levels (Cooper *et al.*, 1973; Zucker *et al.*, 1983).

Immunological evidence also suggested that the complex was composed of two distinct molecules. Antibodies raised against vWF, which were cross-linked to agarose beads, isolated both vWF and FVIII but the FVIII could be released upon equilibration with 0.24M $CaC1_2$ (Tuddenham *et al.*, 1979; Fulcher *et al.*, 1982). Fulcher *et al.* (1982) produced antibodies against human FVIII:C which precipitated FVIII:C in normal plasma but were unreactive against haemophilic plasma, containing functional vWF. The association between FVIII and vWF can be disrupted by exposure to thrombin (Davies *et al.*, 1981).

The role of calcium in the FVIII/vWF complex was investigated by Mikaelsson *et al.* (1983). They proposed that in normal plasma, FVIII circulates as a calcium-linked complex (with vWF). Upon exposure to EDTA (a chelating agent), the calcium is removed, the complex dissociates and coagulant activity is lost. It would, therefore, appear that Ca^{2+} ions are essential for FVIII coagulant function. They may also act as a stabiliser in the quaternary structure of the complex. The two proteins may also be linked by Ca^{2+} bridges. Using monoclonal antibodies, Foster *et al.* (1987) located a major binding site for FVIII in the first 272 residues of the amino terminal of each vWF subunit. Each monomer is capable of binding one FVIII molecule, but *in vivo* not all binding sites are occupied. Lollar *et al.* (1987) calculated a stoichiometry of 1.2 vWF monomers per FVIII molecule for porcine FVIII. Hamer *et al.* (1987a) found that one FVIII molecule was bound per four vWF, using a human *in vitro* system under saturating conditions with excess FVIII. Zucker *et al.* (1983), meanwhile, reported a ratio of one FVIII to eight vWF monomers.

The binding site for vWF has been localised in the light chain of FVIII between Val 1670-Glu 1684, using antibodies which inhibit the binding of FVIII to vWF (Foster *et al.*, 1988). Thrombin cleavage at position 1689 in the light chain releases a 41-residue amino-terminal peptide, abolishing binding to vWF (Lollar *et al.*, 1988). Thus, the binding site must be contained within this fragment, preceeding residue 1689.

This region, containing a high proportion of acidic residues, also contains a sulphated tyrosine residue at position 1680. Mutation of this tyrosine to phenylalanine, with subsequent inhibition of sulphonation, reduces the affinity for vWF (Pittman *et al.*, 1987). It can be concluded, therefore, that sulphonation of this Tyr residue is critical for a successful interaction between FVIII and vWF.

The reason for the complexation of FVIII to vWF in normal plasma seems to be the stabilisation of the coagulant protein (Weiss, 1977). The life-time of FVIII in the circulation is increased by binding to vWF (Tuddenham *et al.*, 1982). The FVIII:C is highly unstable in the absence of vWF, and is prone to proteolytic attack. Weiss (1977) suggested that the vWF protects the FVIII:C from proteolysis. vWF also functions as a carrier protein for FVIII. It localises FVIII:C at the site of vascular damage, by binding to the sub-endothelial cells (Sakariassen *et al.*, 1979) and to the thrombin-stimulated platelets.

1.8 The functions of FVIII and vWF

FVIII and vWF are both involved in haemostasis. FVIII is a component of the blood coagulation cascade and vWF is involved in platelet-sub-endothelial cell interactions. The importance of each of these proteins is evident from the bleeding disorders which result from deficiency or abnormality in either of them (classic haemophilia in the case of FVIII and von Willebrand disease in the case of vWF).

The function of FVIII is to act as a cofactor in the activation of Factor X (FX) during the intrinsic pathway of the blood coagulation cascade. It forms a complex along with Ca^{2+}, phospholipids and activated Factor IX (FIXa) to achieve this activation. 1987). McGee *et al.* (1991) showed that blood monocytes can promote fast activation of FX by FIXa/FVIII. FVIII does not possess any intrinsic ability to activate FX, rather it is the enzymatic activity of FIXa which is responsible for the activation of FX. FIXa can slowly activate FX in the presence of Ca^{2+} and phospholipids without FVIII but the rate of activation is greatly increased (by as much as 10^4 fold) in the presence of FVIII (van Dieijen *et al.*, 1981).

FX exists in normal plasma as a zymogen consisting of two polypeptide chains held together by one or more disulphide bridges.

The heavy chain contains the active site, while the light chain contains the characteristic γ-carboxyglutamic acid residues of the vitamin K-dependent clotting factors (Factors II, X and VII). These residues are responsible for the Ca^{2+}-mediated binding of FX to phospholipids. Activation of FX involves cleavage of a specific peptide bond in the heavy chain, causing a peptide to be released from the amino terminal.

FVIII must also be activated from its precursor form. The single chain polypeptide is activated by proteolytic cleavage at specific sites. This modification can be achieved by thrombin and by activated factor X (FXa) (Vehar and Davie, 1980). FIXa has also been shown to activate FVIII (Rick, 1982). Although this activation is not as effective as that achieved by thrombin or FXa, it is important in producing initial levels of FVIIIa.

Phospholipids play an important role in the FX activating complex. Both FX and FIXa bind to the phospholipid surface of the platelet by means of calcium bridges. It is thought that FVIII aligns FX and FIXa in such a way as to provide optimal conditions for the two to interact. The interaction of FVIII with phospholipid appears to cause the FVIII to become dissociated from vWF. Griffin *et al.* (1982) showed that thrombin-activated FVIII was unstable, but its stability was increased when bound to phospholipid.

vWF fulfils dual roles in haemostasis. It forms a non-covalently linked complex with FVIII, stabilising and protecting the cofactor. It also plays a role in platelet plug formation. Here it acts as a ligand between the sub-endothelium at the site of vascular damage, and platelets in the arterial circulatory system (Roth, 1992). This function is dependent on the high shear rates characteristic of this system (Weiss *et al.* 1991). Under these conditions, vWF binds to a receptor on the platelet surface, GP-Ib (Roth, 1990) and to components of the sub-endothelium, possibly type VI collagen (Rand *et al.*, 1991). vWF thus acts as a linker between the platelet and the damaged arterial wall. In venous circulation the plasma coagulation system is predominant as the high shear forces necessary for the interaction between vWF and GP-Ib are not present.

vWF contains a number of binding sites associated with its functions in normal haemostasis. The FVIII binding site has been localised (Foster *et al.*, 1987), as have those for GP-Ib (Mohri *et al.*, 1989) and collagen (Pareti *et al.*, 1987).

1.9.1 The Deficiency disease of FVIII

Classic haemophilia is a hereditary disorder of blood coagulation caused by deficient or abnormal FVIII:C. It is inherited as a recessive sex-linked disorder and occurs in approximately 1 in 10,000 males.

Haemophilia B (also known as Christmas Disease, after the first patient diagnosed with the condition) is caused by a deficiency in FIXa. It is also a sex-linked disorder, unlike deficiency disorders of the other vitamin K-dependent factors (FII, FX and FVII) which are inherited as autosomal traits. The incidence of haemophilia B is about 1 in 40,000 males.

Classic haemophilia (sometimes known as haemophilia A) and haemophilia B are clinically indistinguishable. The clinical features of classic haemophilia include abnormally prolonged or excessive bleeding, producing characteristic haematomas, extensive bruising, bleeding into joints and other forms of deep tissue bleeding. Patients with the disorder are prone to intra- and/or post-operative bleeding.

The severity of the disease (classic haemophilia) is dependent on the degree of deficiency of FVIII:C, the defect being constant throughout the life of the patient. Severe haemophiliacs are classed as having less than 5% of the normal level of FVIII:C. Moderate cases have between 5% and 10% of the normal level and mild sufferers have between 10% and 15% of the normal FVIII:C concentration.

Using human anti-FVIII:C antibodies, FVIII:CAg has been detected in approximately 10% of patients with severe haemophilia. This FVIII:C was non-functional but was antigenically cross-reactive. These plasmas were designated cross reacting material positive (CRM^+) and were later found to have normal antigen levels, while the coagulant activity was very low (2%-10% of normal) (Lazarchick *et al.*, 1978).

Studies using heterologous antisera, showed that all haemophiliac plasma contained normal levels of vWFAg. Thus, in haemophiliac patients, only the FVIII:C activity is impaired, while the vWF function remains intact.

Lazarchick *et al.* (1978), using anti-human FVIII:C antibodies, found a number of different patterns involving FVIII:C and FVIII:CAg. In severe haemophilia, there is no detectable FVIII coagulant activity but there are variable levels of FVIII:CAg. Most sufferers have no FVIII:CAg but in approximately one-quarter of severe haemophiliacs there are low levels of the antigen (1%-10% of normal).

Almost all patients with mild or moderate haemophilia have detectable levels of FVIII:CAg, most having more FVII:CAg than FVIII:C. Some patients even have normal levels of FVIII:CAg while the coagulant activity (FVIII:C) is low. These are patients with CRM^+ haemophilia. An X chromosome mutation which modifies the FVIII:C structure, rendering it inactive, is responsible for this condition.

Patients can now be screened by recombinant DNA analysis to find the genetic defects that cause haemophilia. Point mutations, deletions and insertions have all been identified (Tuddenham, 1989). Point mutations involving single nucleotides can cause premature termination of FVIII translation, resulting in a shorter, non-functional FVIII. Deletions in the FVIII gene result in severe haemophilia.

Female carriers of haemophilia have normal FVIII:CAg levels but FVIII:C is reduced (only half of the X chromosome directs synthesis). Even though the levels of FVIII:C and FVIII:CAg can change (e.g. during pregnancy), the ratio between the two remains constant (Hoyer *et al.*, 1982).

With the advent of reliable methods of estimating FVIII:CAg (Peake *et al.*, 1978; Lazarchick *et al.*, 1978), the prenatal diagnosis of haemophilia has become possible. Foetal blood samples obtained by foetoscopy can be assayed for FVIII:C or FVIII:CAg (Firshein *et al.*, 1979).

1.9.2 Treatment of Classic Haemophilia

Factor VIII concentrates derived from pooled plasma were originally used in the treatment of haemophilia. Certain complications arose over the use of such products as the risks of blood-borne diseases, hepatitis and AIDS (from HIV) became apparent. Screening of donors and heat-inactivation of blood products reduced, but did not remove, the risks posed by these viruses. Peertinck and Vermylen (1993) reported cases of hepatitis A infection in haemophilic patients treated with a solvent/detergent-treated FVIII concentrate. The use of recombinant FVIII (rFVIII) in the treatment of previously treated haemophilic patients was reported by White *et al.* (1989). They found that the recombinant protein was effective in the control of haemostasis and had several practical advantages over plasma-derived FVIII.

Schwartz *et al.* (1990) also used rFVIII and found that the low frequency of inhibitors was consistent with studies using plasma-derived FVIII concentrates. Lusher *et al.* (1993) administered rFVIII to previously untreated patients and found that it was well tolerated and that patients responded well to treatment. There was, however, a relatively high incidence of low titre inhibitor antibodies. Despite this, the authors concluded that the benefits of a recombinant FVIII outweighed the risks.

Cases have been reported where patients have developed inhibitors to monoclonal antibody-purified FVIII (Bell *et al.* 1990; Kessler *et al.*, 1990; Montoro *et al.*, 1991). These cases were unusual in that the inhibitors developed under conditions not normally associated with inhibitor production. These may be related to the use of highly purified FVIII, where new immunogenic regions have been recognised.

The presence of inhibitors creates problems in the treatment of haemophilia. To overcome such problems, patients were treated with activated prothrombin complex concentrate (aPCC), containing porcine FVIII or activated factor IX. Hedner *et al.* (1988) showed that activated factor VII (FVIIa) was capable of inducing haemostasis in haemophilia, and he successfully used recombinant FVIIa (rFVIIa) during synovectomy on a haemophilic patient. Ingerslav *et al.* (1991) also reported the successful use of rFVIIa during oral surgery.

1.9.3 Inhibitors to FVIII

Inhibitors to FVIII are pathologic circulating IgG antibodies which specifically neutralise the procoagulant activity of FVIII (Kessler *et al.*, 1990). They develop in 5-15% of patients who receive factor VIII replacement therapy (Shapiro, 1984), with two-thirds of these cases developing inhibitors in the first 20 years of life (McMillan *et al.*, 1988). Algiman *et al.* (1992) found, however, that 17% of normal sera taken from healthy blood donors contained FVIII-neutralising IgG antibodies.

The risk of severe haemophiliacs, i.e., those with FVIII activity less than 5% of normal, developing inhibitors in the first 25 years of life is 2% (Schwarzinger *et al.*, 1987).

Children under five are four times more likely to develop inhibitors than adults, as are patients with FVIII:C levels less than 0.03 U/ml (McMillan *et al.*, 1988; Ehrenforth *et al.*, 1992). McMillan *et al.* (1988) also found that high titre inhibitors appeared less than 75 days after exposure to FVIII, with all inhibitors appearing within 250 days.

Ewing *et al.* (1988) induced immune tolerance to FVIII in haemophiliacs with inhibitors, using 50 U/kg body weight of FVIII. The treatment was particularly effective for patients with low levels of inhibitors. They proposed that the treatment was suitable for patients with low levels of inhibitors who had not been exposed to high levels of FVIII since developing the antibodies. The procedure was, however, expensive.

1.10 vWF Disease

von Willebrand disease is the condition which results from deficiency or abnormality in vWF. It is an autosomal disorder which can appear in a severe, mild or moderate form.

Type I von Willebrand disease is probably the most common form of the disorder. Levels of both FVIII and vWF are decreased, and bleeding time is prolonged. The multimeric composition of the complex is normal and vWF can be detected in endothelial cells.

Type II von Willebrand disease is characterised by the lack of the large vWF multimers in plasma. In Types IIA and IIB, ristocetin cofactor activity is affected. FVIII:C is normal or reduced in both cases. In Type IIC, the repeating triplet in normal vWF is replaced by a repeating doublet.

Type III von Willebrand disease is the most severe form and is relatively uncommon. There is no ristocetin cofactor activity, no detectable vWFAg, and no vWF can be detected in endothelial cells.

1.11.1 Biosynthesis of FVIII

The exact cellular site of synthesis of FVIII is unclear, but the liver has been proposed as the primary site of synthesis. Transplantation studies in haemophilic dogs (Marchioro *et al.*, 1969; Webster *et al.*, 1971) and in patients (Lewis *et al.*, 1985; Bontempo *et al.*, 1987;) have shown that replacement of normal livers increases FVIII coagulant levels. FVIII mRNA has been detected in many tissues, including liver, kidney, spleen and lymph nodes (Wion *et al.*, 1985). FVIII antigen has also been detected immunochemically in hepatocytes using both light (Kelly *et al.*, 1984) and electron microscopy (Zelechowska *et al.*, 1985). No known naturally derived cell lines express FVIII. With the discovery of the FVIII gene, mammalian cells can now be transfected with copies of this gene allowing expression of the protein in culture. Kaufman *et al.* (1988) successfully transfected Chinese hamster ovary (CHO) cells and were able to study the probable biosynthetic pathway of FVIII.

The protein is synthesised as a 2351 amino acid chain, the first 19 of which are lost during translocation into the lumen of the endoplasmic reticulum (e.r.). In the e.r. a considerable proportion of the FVIII binds to a resident protein GRP78 (also known as BiP, immunoglobulin binding protein) (Dorner *et al.*, 1987). It is not known whether this FVIII is subsequently secreted or degraded. High-mannose oligosaccharides are added to asparagine residues in the "free", *i.e.*, unbound FVIII. The protein is released from the e.r. and enters the Golgi apparatus. Here, the majority of the FVIII is cleaved, generating the heterologus heavy chain amino-terminal-derived fragments, and the COOH-terminal-derived light chain. Modifications are also carried out. Carbohydrates are added to serine and threonine residues. The asparagine-linked high mannose oligosaccharides are modified to complex types, and specific tyrosine residues in both the heavy and light chains are sulphonated.

In the presence of vWF in the medium, the heavy and light chains associate upon secretion, forming a stable complex with vWF. In the absence of vWF, the chains do not associate and are degraded. When vWF is co-expressed by the cells, synthesis and secretion of FVIII remains unaffected, but recovery of the protein in the medium is higher (Kaufman *et al.*, 1989).

1.11.2 Biosynthesis of vWF

von Willebrand factor (vWF) circulates as a large glycoprotein consisting of a series of multimers ranging in size from 500 to 2000 kDa. (Chopek *et al.*, 1986). It is synthesised in endothelial cells (Jaffe *et al.*, 1973) and megakaryocytes (Nachman *et al.*, 1977), as a 2813 residue polypeptide of approximately 360 kDa (pro-vWF), including a 741-residue, 95 kDa propeptide. This propeptide functions in the multimerisation process and is cleaved during post-translational processing in the Golgi apparatus (Wagner *et al.* 1990).

The pro-vWF subunits dimerise, through the formation of disulphide bridges at the carboxyl terminal, to form the protomer of the multimeric series (Sadler, 1991). These protomers undergo a certain amount of processing including cleavage of the pro-peptide (Wagner *et al.*, 1990), glycosylation, sulphonation (Browning *et al.*, 1983) and further disulphide formation. These bonds, involving cysteine residues at the amino-terminals, link the protomers to form the biologically functional multimers.

The multimerisation process is believed to take place in the Golgi apparatus (Wagner *et al.*, 1986) and is dependent on the presence of the pro-peptide, an acidic pH - less than 6.2 with an optimum of 5.8 - and the presence of free sulphydryl groups on the vWF protomer (Mayadas *et al.*, 1989). These multimers are then either secreted constitutively (Sporn *et al.*, 1986) or stored in specialised storage organelles called Wiebel-Palade (W-P) bodies (Wagner *et al.*, 1982). The two pools of vWF (*i.e.*, those secreted and those stored in the W-P bodies) are structurally different. The secreted vWF is largely dimeric, with pro-vWF and mature subunits also present (Sporn *et al.*, 1986). In constrast, the stored vWF in the W-P bodies consist only of large multimers (the biologically active form of vWF). Free propeptide is also present in a stoichiometry of 1:1 with the multimers.

vWF multimers also occur in the α-granules of platelets (Cramer *et al.*, 1985). The release of stored vWF can be triggered by α-thrombin (Sporn *et al.*, 1986), plasmin, adrenalin, bradykinin and interleukin-1 (Booth *et al.*, 1987).

1.12 Analysis of FVIII

The diagnosis of bleeding disorders, including haemophilia A, depends on the ability to analyse the blood coagulation system in conjunction with the patient's bleeding history. It has proved extremely difficult to reproduce the entire coagulation cascade *in vitro* because of the complexity of the coagulation process. Therefore, only partial components of the process can be analysed at one time. An example of one such assay is the Activated Partial Thromboplastin Time (APTT). This test is used to screen components of the intrinsic phase of blood coagulation (Figure 1.1). APTT reagent contains phospholipids and a contact phase activator (a negatively charged compound). Patient plasma is incubated with the APTT reagent and $CaCl_2$ is then added. The time taken for the formation of a clot (the Activated Partial Thromboplastin Time) is measured in seconds. Normal clotting times are between 20 and 30 seconds, with an increase of 10 seconds or more in this time indicating a defect in the clotting system. Prolonged APTT may be due to a deficiency or abnormality in one or several of the factors in the intrinsic phase of the coagulation process, the presence of coagulant inhibitors or heparin. APTT reagents may differ in their contact activator (the most common being allagic acid, glass, charcoal, celite or kaolin) and the origin of the phospholipids (brain extracts, placenta and plant phospholipids being the most widely used).

The need to quantitate levels of anti-haemophilic factor (FVIII) in the diagnosis of haemophilia led to the development of biological assays for the measurement of FVIII coagulant activity in the 1950's. The one-stage assay developed by Langdell *et al.* (1953) was the first of these. Biggs *et al.* (1955) developed an assay based on the thrombin generation test, called the two-stage assay. This assay formed the basis of most of the assays carried out today, although some modifications have been made. Denson (1976) simplified the method, allowing for the possibility of automation, and Rosen *et al.* (1984) used a chromogenic substrate for the detection of FXa.

Lazarchick *et al.* (1978) and Peake and Bloom (1978) developed immunoradiometric assays for the detection of FVIII:CAg, using inhibitor antibodies from haemophilic patients. This opened a whole new field in the detection of FVIII - the use of immunological techniques. Dinesen (1983) developed the first ELISA using peroxidase-labelled inhibitor antibodies. Recent modifications to this method include the use of $F(ab')_2$ fragments and microtitre plates (Nordfang *et al.*, 1983) and the use of monoclonal antibodies (Jourquera, 1989). The avidin-biotin complex has also been used to increase the sensitivity of the assay (Tackaberry *et al.*, 1987). Recent advances in biosensor technology have led to an increasing number of novel techniques. Included in these are sensors which can monitor the coagulation process and detect factor VIII (Muramatsu *et al.*, 1991).

1.12.1 Estimation of FVIII:C

The one-stage clotting assay: This assay, developed by Langdell *et al.* (1953), is based on the ability of FVIII to correct the prolonged clotting time of FVIII-deficient plasma to a degree determined by the amount of FVIII present. Phospholipids are added to the plasma (as a substitute for platelets) along with an activator (e.g., kaolin) to provide a negatively charged surface to initiate the cascade. Ca^{2+} ions are also added to start the FVIII-dependent part of the coagulant cascade.

The assay may be influenced by contamination with coagulation factors that act subsequently, particularly when the samples being measured are blood-derived. Clotting times are also shortened by traces of thrombin and tissue factor. Thus, the assay suffers from a lack of specificity. However, the assay does have the advantage of being rapid and simple.

The two stage assay: This was developed by Biggs *et al.* (1955) and is based on the formation of activating complexes (Figure 1.12). In the first stage, FVIII is added to a source of FIXa, Ca^{2+} and phospholipids (PL). These form a complex which activates FX (FXa). FXa in turn forms prothrombinase along with FV, Ca^{2+} and PL. In the second stage, prothrombin and fibrinogen are added, resulting in the formation of a clot.

This assay provides a more direct measurement of FVIII and is less susceptible to the influence of other factors. However, it can give artificially high levels of FVIII in FVIII concentrates (Kirkwood, 1978) but it detects relatively more activity in the more highly purified preparations than does the one-stage assay (Kirkwood, 1977).

Chromogenic Assay: Seghatchian *et al.* (1978) modified the two-stage assay by using a highly selective FXa substrate, S-2222, which released p-nitroaniline, (pNA), when hydrolysed by FXa. The free pNA could be measured by a spectrophotometer at 405nm. The assay was somewhat insensitive for detecting low levels of FVIII, and so Rosen *et al.* (1984) developed a chromogenic assay, using bovine coagulation factors, which proved to be highly sensitive, accurate and had a high degree of precision. A thrombin inhibitor, I-2581, was included to prevent hydrolysis of the substrate by thrombin. van Dieijen *et al.* (1987) developed a similar assay for the detection of human FVIII in plasma and Wagenvoord *et al.* (1989) modified this assay for clinical use, using lyophilised reagents and minimising the number of pipetting steps. The assay was highly sensitive, detecting less than 1% FVIII:C. In addition, the lyophilised reagents could be stored for several months. Mazurier *et al.* (1990) compared the different FVIII coagulant assays, and the effect of pre-diluent, using high purity FVIII concentrates. They found that shorter clotting times were always obtained when using the one-stage assay, when the concentrate was diluted in FVIII-deficient plasma. However, the prediluent had less influence on the chromogenic assay than on the one-stage assay. Therefore, they concluded that the use of the chromogenic assay, with FVIII-deficient plasma as a diluent, provided the best results.

STAGE 1

(a) Formation of Complex 1

FVIII + FIXa, Ca^{2+}, phospholipids ----------→ Complex 1

(b) Activation of FX and the formation of Complex 2

Complex 1 + FX ----------→ FXa

+

Ca^{2+}, phospholipids, FV ----------→ Complex 2

STAGE 2

Complex 2

Prothrombin ----------→ Thrombin

|

|

Fibrinogen ----------→ Fibrin

|

|

Clot

Figure 1.12.1 The two-stage clotting assay. FVIII forms a complex with activated factor IX (FIXa), Ca^{2+} and phospholipids which activates factor X (FX). This in turn forms a complex with factor V (FV), Ca^{2+} and phospholipids which, in the second stage of the assay, leads to the formation of an insoluble clot.

1.12.2 Estimation of FVIIICAg

The immunological properties of FVIII:C have been studied, using antibodies from multi-transfused haemophilic patients who have developed inhibitors, or from patients with autoantibodies. Quantitative immunoradiometric assays for the determination of FVIII:CAg, utilising radiolabelled human antibodies, were developed. These assays, consisting of either a two-site solid phase immunoradiometric assays (Lazarchick and Hoyer, 1978; Peake and Bloom, 1978) or a one-site fluid phase immunoradiometric assays (Rotblat and Tuddenham, 1981) showed a good correlation between FVIII:C and FVIII:CAg in normal plasma.

These assays made use of polyclonal antibodies which differed in their specificities and affinities. With the advent of monoclonal antibody technology, anti-FVIII:C antibodies with defined specificities and affinities were produced. Muller *et al.* (1981) described a monoclonal antibody against FVIII:C which neutralised FVIII:C. They used this antibody in an immunoradiometric assay (IRMA) for FVIII:CAg. Similarly, Brown *et al.* (1983) described a one-step two site IRMA for FVIII:CAg in which I^{125}-labelled monoclonal IgG was used to detect FVIII:CAg.

One of the first enzyme-linked immunosorbent assays (ELISA) for estimation of FVIII:Ag was developed by Dinesen *et al.* (1983). They used protein A-purified IgG from haemophilic patients with inhibitors. The ELISA was carried out using polystyrene cuvettes as the solid phase. The inhibitor antibody was bound to this, test samples were added, and bound antigen was detected using horseradish peroxidase (HRP)-labelled inhibitor IgG.

Nordfang *et al.* (1985) and Ingerslev *et al.* (1986) both describe a micro ELISA using inhibitor antibodies from haemophilic patients. Polystyrene microtitre plates were used as the solid phase and bound FVIII:C was detected using HRP-labelled $F(ab_2')$ fragments of the inhibitor antibody. Use of these fragments resulted in decreased non-specific binding (caused by the Fc portion of the antibody molecule).

Jorquera *et al.* (1989) developed an ELISA in which the test antigen was sandwiched between a polyclonal human anti-FVIII antibody bound to the solid phase and a commercial anti-FVIII monoclonal antibody. Any bound monoclonal antibody was detected using a polyclonal goat anti-mouse IgG labelled with HRP.

Hawes *et al.* (1982) developed a procedure for the assay of antibodies in sera based on the application of the antigen as a spot on nitrocellulose paper. This system requires only microgram quantities of antigen, but it is prone to a greater degree of non-specific binding. Tackaberry *et al.* (1987) used the avidin-biotin amplification system to detect picogram quantities of FVIII:CAg. The sensitivity of the assay can be attributed to the capacity of nitrocellulose paper to bind proteins, the high titre of monoclonal antibodies for FVIII, and the amplification of the second antibody signal by the avidin-biotin complex.

Muramatsu *et al* (1991) have developed a biosensor which they used to detect factors VIII and IX. They employed a quartz crystal viscosity sensor with a monitoring system made up of 16 oscillating circuits, a channel selector, a frequency counter, a temperature controller and a microcomputer. The resonant frequency of quartz crystal is known to vary with changes in the viscosity of a liquid and, thus, can be used as a viscosity monitoring sensor. The authors used this device to monitor the coagulation procedure. The FVIII (or FIXa) was incubated along with an activated partial thromboplastin time reagent with pre-incubated FVIII- (or FIXa-) deficient plasma. $CaCl_2$ was also added. The mixture was poured over the quartz crystal sensor and the resonance frequency monitored.

The use of factor VIII in replacement therapy in the future will very much depend on the availability of essentially pure contaminant-free supplies of the protein. The availability of pure, contaminant free FVIII has meant that giant steps forward have been possible in the understanding of FVIII and its role in haemostasis (Kaufman, 1991). This in turn could lead to novel techniques for the treatment of, and perhaps ultimately a cure for, haemophilia.

1.13 Monoclonal Antibody Production

1.13.1 Introduction

By definition, all of the antibody produced by a single clone, will have the same specifity and affinity for the target antigen. It is not possible to isolate individual B-lymphocytes and grow them in culture, as they have a finite life-span. Köhler and Milstein (1975) described the technique of cell hybridization, fusing an immunized splenocyte with a myeloma cell (Figure 1.13.1). Tumor cells have an infinite life-span, and, thus, the hybrid cells inherit the ability to survive in culture from the myeloma, and the ability to secrete antibody from the lymphocyte. The importance of monoclonal antibodies has increased enormously in recent years. Today, the applications of monoclonal antibodies (MAbs) are wide-spread, from clinical use in the diagnosis and treatment of disease, to preparative uses as well as uses in basic research (Campbell, 1984; Peters and Baron, 1993). New technologies including human MAbs, bispecific, catalytic and recombinant antibodies (Peters, 1993b) are constantly being developed.

1.13.2 Antigen

The production of monoclonal antibodies is dependent on a number of factors. The antigen should be capable of eliciting a good immune response. Proteins and large molecules are good immunogens, eliciting a good immune response. Some antigens, *i.e.*, those with low molecular weights, are too small to be immunogenic and must be conjugated to a protein carrier such as bovine serum albumin (BSA), ovalbumin, thyroglobulin or keyhole limpet haemocyanin (KLH). Immunisation with these conjugates, however, results in the production of antisera against determinants on the carrier protein as well as against the antigen (Tijssen, 1985).

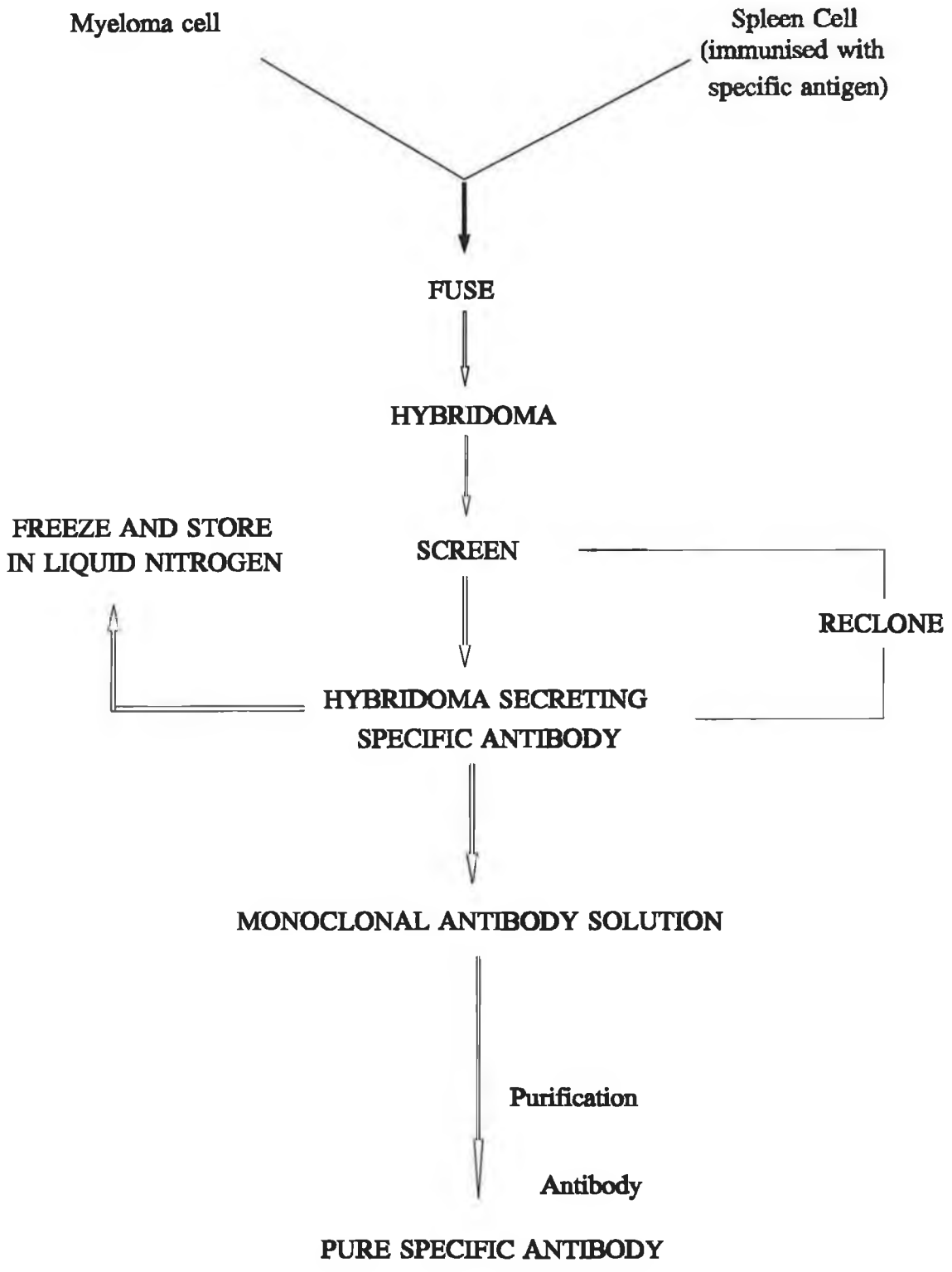


Figure 1.13.1 The Production of Monoclonal Antibodies.

1.13.3 Adjuvant

Adjuvants are used to enhance the immune response. They stimulate non-specific production of antibody and cause a polyclonal activation of the immune system. (Baumgarten, 1993). The most commonly used adjuvants are Freund's complete (CFA) and incomplete (IFA) adjuvant, but aluminum hydroxide can also be used. Freund's adjuvant consists of water-in-oil emulsions, with heat-inactivated *Mycobacterium tuberculosis* also present in CFA. The antigen is injected emulsified in CFA or IFA and is slowly released from the emulsion, prolonging exposure to the immune system. CFA is used only for the first immunisation, with boosting being given with IFA.

Aluminum hydroxide can also be used as an adjuvant, and is commercially available in ready-to-use forms (Tijssen, 1985). It is also possible to immunise with antigens bound to a solid matrix such as Sepharose, nitrocellulose paper or acrylamide gel, even after staining with Coomassie Blue. The incorporation of antigen into polymers which then release them slowly, without causing damage to the animal, has also been described (Schröder and Stahl, 1984; Gurvick and Korukova, 1986; Kohn *et al.*, 1986). Antigens can also be incorporated into liposomes. These consist of phospholipid double layered membranes, separated by an aqueous phase and can be prepared from nonimmunogenic, biologically degradable materials (Baumgarten, 1993). No adjuvant is required when cells are used as immunogens.

1.13.4 Immunisation Protocol

Rats and mice are generally used for the generation of MAbs. Larger animals, such as rabbits, sheep, goats and donkeys are used for the production of polyclonal antibodies. The site of immunisation can influence the immune response (Baumgarten and Schulze, 1993). Antigens can be injected at a number of different sites: intradermally, intraperitoneally, intravenously, intraorally, intrasplenically, intramusculary and subcutaneously. The difference in injection sites affects the speed with which the antigen is presented to the immune system. Injections may be given at single or at multiple-sites. The amount of antigen required to elicit an immune response is small. An animal is typically immunised with between 0.1mg/ml and 1mg/ml of a soluble antigen (*e.g.*, a protein) in adjuvant and between 10^6 and 10^7 cells without adjuvant (Campbell, 1984).

Injections can be given at 2-4 week intervals. Animals are bled after 2 weeks and the antibody levels measured.

Immunisation can also be carried out *in vitro*. This is particularly relevant to the production of human MAbs as *in vivo* immunisation is not feasible. There are a number of advantages over the *in vivo* system: the concentration of antigen required is smaller (μg rather than mg quantities), the system is less complex and allows responses against self-antigens, it is a simple and rapid method, with immunisations complete in 3 to 5 days as opposed to several weeks. There are also disadvantages to the system. The responses are mainly primary producing predominantly IgM antibodies. However, a secondary response may be obtained if the animal used as a source of lymphocytes is given an *in vivo* immunisation 28 days or more prior to *in vitro* immunisation (De Boer *et al.*, 1988).

A rich source of B-lymphocytes as well as a source of T-cell derived growth factors (supplied by T-cell conditioned medium) are essential for this system.

1.13.5 Feeder Cells and Conditioned Medium

Feeder cells or conditioned medium are used during the production of MAbs and are essential during the cloning of hybridomas. Newly formed hybridomas do not grow well at low densities. They require the presence of a monolayer of non-growing cells. Feeder cells should have a limited lifetime and should be able to provide metabolites which nourish the hybridomas during initial stages of growth (Peters, 1993a). Macrophages are commonly used as feeder cells. They are actively phagocytic and provide an excellent post-fusion clean-up mechanism, removing dead myeloma and parent lymphocytes (Campbell, 1984).

Alternatively, conditioned media provide a convenient source of soluble growth factors to cells without the problems of feeder cells. Growth factors like interleukin-6 (IL-6) are used to promote hybridoma growth and have proved to be highly successful (Debus *et al.*, 1993). Problems can arise with the use of such additives, including variation between batches, increase in protein concentration and an increase in undefined substances (Debus *et al.*, 1993). Some of these problems can be overcome with the use of a defined substance like a recombinant protein, *e.g.* recombinant IL-6.

1.13.6 Myeloma Cell Lines

In order to perform a successful fusion, the myeloma cell line must satisfy a number of criteria. It must not secrete antibody itself, it must have an enzymatic defect which allows for the selection of hybridomas, and it must show good fusion properties. The cells should originate from the same system as the immunised splenocytes (the murine system is generally used). Balb/c mice are the usual strain of mouse chosen for immunisation as they are compatible with all murine myeloma cell lines. Hybridomas can subsequently be grown as an ascitic tumor in Balb/c mice. In the human system, no cell lines exist so far with anything like the favourable properties of the mouse myeloma cell lines. As a result, human myeloma cells, lymphoblastoid cell lines or heteromyelomas are used (Baron, 1993a). Epstein-Barr Virus (EBV) is used in the transformation of human B-lymphocytes, producing immortalised B-cells which are still capable of synthesising specific antibody. This enables the generation of monoclonal antibodies without involving cell fusion (Baron, 1993a).

1.13.7 Fusion Procedures

Early fusions used inactivated viruses, such as Sendai virus to fuse cells but chemical fusogens have replaced these. Polyethylene glycol (PEG) is the major chemical used. The exact mechanism by which it acts has not been elucidated, although it is known to be a complex one, involving cell agglutination, membrane fusion and cell swelling (Campbell, 1984). The molecular weight of PEG is variable, molecular weights between 1000 and 6000 have been successfully used (Fazekas de St. Groth *et al.*, 1980).
The temperature at which the fusion takes place is critical. PEG is held at 37°C until it is added to the fusion mixture. Too low a temperature hinders the production of clones after a fusion step (Campbell, 1984).

Other methods for fusion are also available. The use of viruses was the original method used by Kohler and Milstein in 1975. Herpes-, myxo- and paramyxoviruses contain proteins which are capable of mediating fusion. The frequency of hybridization is lower however, than that achieved by PEG (Baron, 1993b).

Cells can also be fused by electrofusion. Using this method, the frequency of hybridisation can be increased 500-fold as compared with chemically mediated fusion. (Baron, 1993b). The process consists of two stages:

(i) Dielectrophoresis - leading to cell-cell contact. Here, an AC current is applied and the cells migrate to form a chain between the electrodes. This is called pearl chain formation.

(ii) Pulsing. A high voltage is applied which induces pore formation, allowing mixing of adjacent cell contents. The membrane reforms around the two adjacent cells forming a hybrid cell.

1.13.8 Selection of Hybridomas

The fusion procedures do not have a hybridisation frequency of 100%. Therefore, after the fusion, there will be unfused cells present, as well as the fused hybridomas. Lymphocytes will die off naturally with time, while macrophages or other cells used as feeder cells will grow slowly, or not at all. The myeloma cells are capable of survival in culture, and within a short period of time, will outgrow the hybridomas. A selection method must, therefore, be used which allows for growth of the hybridomas but not of the myeloma cells. The myeloma cell line should contain a selective drug marker, which allows for post-fusion selection of the hybridomas.

Nucleic acids can be synthesised by two pathways - the de novo main metabolic pathway and the salvage pathway (Figure 1.13.8). If there is a defect in the main pathways, the cell is still capable of survival, provided there are exogenous supplies of nucleosides.

Aminopterin blocks the main metabolic pathway for the synthesis of purines and pyrimidines, by inhibition of the enzyme dihydrofolate reductase. The cell can switch to the salvage pathways provided exogenous nucleosides are provided. Hypoxanthine is utilized in the synthesis of purines, by the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Thymidine is utilized in the synthesis of pyrimidines, by the enzyme thymidine kinase (TK). Myeloma cell lines may be deficient in one of the enzymes (HGPRT or TK) and so, if the main nucleic acid metabolic pathway is blocked, the cells are unable to synthesise either pyrimidines or purines and, thus, are unable to survive.

Figure 1.13.8 The synthesis of DNA precursors. There are two pathways leading to the production of DNA, the de novo pathway and the salvage pathway. Cells normally utilise the de novo pathway, but in the presence of aminopterin, this route is blocked. The cell can switch to the salvage pathway provided exogenous nucleosides (hypoxanthine and thymidine) are supplied. Myeloma cells contain a genetic defect in that they do not have the enzymes (thymidine kinase (TK), or hypoxanthine guanine phosphoribosyl transferase (HGPRT)) to utilise these nucleosides and they subsequently die. Hybridoma cells survive because of their ability to utilise the salvage pathway inherited from the lymphocyte.

Cells can be made deficient in one of these enzymes by growing them in bromodeoxyuridine (which selects for TK-negative cells) or 8-azaguanine or 6-thioguanine (which selects for HGPRT-negative cells).

The immunised B-lymphocytes used in the fusion contain no enzymic defect. The hybridomas formed after fusion will not have the genetic defect either, because of genetic complementation, *i.e.*, the B-lymphocyte will compensate for the defect in the myeloma. The hybridoma will therefore be insensitive to the aminopterin, as it can use hypoxanthine and thymidine. The myeloma cells cannot and they die.

1.13.9 Large Scale Production of Antibodies

Large amounts of MAbs can be produced *in vivo* or *in vitro*. The method chosen is dependent on the amount of antibody required and also in the facilities available. Antibodies can be produced *in vivo* as ascitic fluid (Baumgarten and Peters, 1993). This is grown in the peritoneal cavity of a primed mouse. Priming can be carried out with pristane (2,6,10,14-tetramethylpentadecane) or IFA (Baumgarten and Peters, 1993). Ascitic fluid contains about 1-10mg/ml of antibody and each mouse can yield up to 10ml of fluid (Campbell, 1984). The ascites is, however, contaminated with mouse immunoglobulins.

Production *in vitro* involves the growth of hybridomas in large culture vessels (bioreactors). The concentration of antibody in these systems is less than that of ascites (about 100µg/ml) (Campbell, 1984) but the end product has a higher degree of purity. The supernatants may contain contaminating foetal calf serum (FCS) which is used in the growth medium. To remove this, cells can be grown in serum-free medium prior to harvesting. A wide range of methods are available for the production of MAbs *in vitro*: from culture flasks, spinner flasks and roller bottles for laboratory use, to large scale fermenters and hollow-fibre systems for industrial production (Franze and Baumgarten, 1993).

1.14 Protein Stabilisation

1.14.1 Introduction

The generation of stable or stabilised proteins is one of the main priorities of modern biotechnology. The requirement for protein use in biotechnological industries such as therapeutics, diagnostics, bioreactors, fine chemicals and biosensors requires molecules be highly stable, have a long shelf-life and be able to withstand the harsh conditions experienced in these applications.

There are many ways of analysing protein stability. In studying thermodynamic stability, a number of physical parameters are monitored, to detect changes in the protein structure caused by the unfolding and denaturation of the native structure. The reversible change in the proportions of these states make it possible to calculate the thermodynamic parameters of denaturation - ΔG_D, the free energy value of denaturation, ΔH_D, the enthalpy of denaturation, ΔS_D, the change in entropy, which is characteristic of the degree of protein unfolding, and Δc_p, the change in heat capacity on denaturation (Mozhaev, 1993).

Long-term stability is the ability of a protein to withstand irreversible changes of structure under denaturing conditions. To measure this, protein is heated to a high temperature, samples removed at intervals and assayed for activity. The rate of disappearance of the native form of the protein (V_{in}), is considered a quantitative measure of long-term stability.

Using these parameters, it is possible to study and compare the loss of activity of a native protein and mutant or modified protein.

1.14.2 Protein Denaturation

It is important to distinguish between the terms "denaturation" and "inactivation" . Denaturation refers to the conformational changes (usually unfolding) that result in the loss of molecular function; these changes may or may not be reversible. Inactivation, on the other hand, is caused by changes in the degree of association or aggregation of the molecule, along with possible primary structural changes, such as peptide bond hydrolysis or destruction of amino acid side chains (Ó Fágáin and O'Kennedy, 1991). Such changes are irreversible.

Inactivation is considered to be a two-step process:

$$N \overset{K}{\longleftrightarrow} U \overset{k}{\dashrightarrow} I$$

while denaturation is an "all-or-nothing" transition between the two states:

$$N \overset{K}{\longleftrightarrow} U$$

where N is the native form, U is the reversibly unfolded form and I is the irreversibly inactivated form of the protein. K is the equilibrium constant for the transition from the native state (N) to the unfolded state (U) and k is the rate constant for the transition from the reversibly denatured state (U) to the irreversibly inactivated state (I).

Inactivation mechanisms involve specific changes in protein structure. The relative importance of a particular mechanism is dependent on the experimental conditions. Ahern and Klibanov (1985) showed that at 90°C, the primary cause of inactivation of ribonuclease A at pH 4 was hydrolysis of peptide bonds while, at pH 8, reactions involving disulphide bonds were the major inactivation mechanism.

The inactivation rate depends on both K and k. Therefore, strategies to increase protein stability will alter these rates by preventing unfolding (decreasing K) or decelerating the inactivation process (k).

1.14.3 Stabilisation of proteins

There are a number of approaches to the stabilisation of proteins. The most commonly used ones are as follows:

(i) Use of additives

(ii) Suspension of proteins in organic solvents

(iii) Immobilisation

(iv) Protein engineering

(v) Chemical modification

(vi) Screening for stable proteins

1.14.3.1 Use of Additives

A variety of compounds can be used to increase the stability of proteins in solution or when undergoing processes like freeze-drying. These include sugars (sucrose), salts (ammonium sulphate), polyols (glycerol), and other proteins (Ó Fágáin and O'Kennedy, 1991).

1.14.3.1.1 Osmolytic stabilisers

Osmolytic stabilisers are compounds which are not strongly charged and have little effect on the protein activity up to concentrations of at least 1M (Yancey, 1973). Their major effects are on the viscosity and surface tension of water and hence on solvent ordering. Osmolytes can be polyols, sugars, polysaccharides, neutral polymers, amino acids and their derivatives, and large dipolar molecules like trimethylamine N-oxide (TMAO) (Schein, 1990). Glycerol is the most commonly used osmolyte. Its stabilisation effect on proteins seems to be due to its ability to enter into and strengthen the lattice structure of water (Schein, 1990). Xylitol is a good alternative to glycerol (which is an excellent substrate for bacteria).

1.14.3.1.2 Ionic stabilisers

Ionic compounds and salts can stabilise protein structure by shielding surface charges. A particular salt is stabilising or destabilising depending on its position in the Hofmeister lyotropic series:

$$(CH_3)_4N^+ > NH_4^+ > K^+, Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$

$$SO_4^{2+} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

Both cations and anions are ranked in order of their stabilising effects. Therefore, $(NH_4)_2SO_4$ should be a strong stabiliser whereas NaSCN should destabilise proteins. The stabilising ions "salt out" hydrophobic residues in the protein causing the adoption of a more compact structure.

1.14.3.1.3 Denaturants, chaotrophs, cryoprotectants and other additives

It is possible to solubilise almost any protein (usually at the expense of its activity) by chemical denaturation with perturbing ions. Urea stabilises the unfolded states of proteins because essentially all protein parts are more soluble in 6M urea than water (Schein, 1990).

Another class of denaturants, "chaotrophs" like guanidinium, disrupt hydrogen bond formation and disturb the hydration shell around proteins (Gekko and Timmasheff, 1981). Organic solvents are another class of denaturants. The denaturing activity of hydrophobic solvents is due to a limited detergent effect and the provision of a competing interaction for the intramolecular hydrophobic interactions responsible for a stable tertiary structure.

Some organic solvents such as dimethylsulphoxide (DMSO) and ethylene glycol, are used as cryoprotectants. They maintain the native protein conformation at temperatures below the freezing point of water.

1.14.3.2 Proteins in organic solvents

Enzyme catalysis in organic solvents is being increasingly used for a variety of applications. It is possible to catalyse unusual reactions with enzymes in bi-phasic aqueous-organic systems. However, certain rules govern the activity of enzymes in such systems:

(i) the solvent must be hydrophobic and show little affinity for water. In bi-phasic systems, the enzyme molecule remains in an aqueous milieu (Klibanov, 1989). Sufficient water remains to hydrate the enzyme molecule and maintain its correct, functional conformation. Hydrophilic solvents tend to strip away the aqueous monolayer surrounding the protein molecules.

(ii) enzymes should be lyophilised from solutions with pH values corresponding to the enzyme's optimum. Enzymes retain the ionic state they possessed in the previous aqueous system throughout lyophilisation and redispersal in organic phase (Kilbanov, 1989). The enzyme will, therefore, function best if it retains its pH optimum.

(iii) the enzyme should be agitated vigorously or sonicated to ensure homogeneous dispersion in the organic solvent. Enzymes are suspended and not truly dissolved in an organic solvent. Heterogeneous dispersions are therefore, plagued by problems of

diffusional limitation. These can be prevented by the formation of a homogeneous solution.

1.14.3.3 Protein Engineering

The stability of a protein may be altered by changing amino acids which affect either stabilising or destabilising interactions in the folded or unfolded form of the protein. Protein engineering refers to the use of genetic manipulation to alter proteins in specific ways. It is one of the most powerful and useful techniques recently developed. There are a number of key techniques:

(i) Site-directed mutagenesis is probably the most widespread technique used. Synthetic oligonucleotides are used to direct the required mutations (Ó Fágáin and O'Kennedy, 1991). It is highly specific, allowing for individual bases within a codon to be altered. The synthetic oligonucleotide containing the desired mutation is annealed to a single-stranded gene copy. Extension of the oligonucleotide occurs from the 3' end.

(ii) Cassette mutagenesis involves the insertion of a double-stranded segment of DNA into a suitable DNA vector between two non-identical restriction sites. The resulting recombinant DNA contains no mismatches and results in highly efficient mutagenesis since DNA repair does not occur (Ó Fágáin and O'Kennedy, 1991).

Known sequences from mesophilic and thermophilic organisms should be used to work out the necessary mutations required for increased thermostability, and highly conserved residues within proteins should not be altered (Imanaka *et al.*, 1986).

1.14.3.4 Immobilisation

Proteins can be immobilised in a variety of ways, onto a variety of matrices. Immobilisation can influence the stability of proteins by steric hindrance, partitioning or diffusion restrictions (Volkin and Klibanov, 1989). A wide range of supports have been used, including porous glass and ceramics, charcoal, sand, stainless steel, synthetic polymers and cellulose (Axen *et al.*, 1970). Immobilisation is usually via an amine or carboxyl group on the protein, and involves two stages: activation of the solid support, and attachment of the protein.

Proteins can be entrapped in polymeric gels (Ó Fágáin and O'Kennedy, 1991). The protein is added to a solution of monomers and gel formation initiated, by a change in temperature or adding a gel-inducing chemical. The gels may be covalent (*e.g.*, polyacrylamide cross-linked with N,N'-methylenebisacrylamide) or non-covalent (*e.g.*, calcium alginate).

The encapsulation of proteins involves the envelopment of the protein in a membrane that is permeable only to low molecular weight substrates and products (*e.g.*, liposomes).

1.14.3.5 Chemical Modification of Proteins

Soluble proteins can be chemically modified in a variety of ways, altering properties which can lead to increase in catalytic activity and stability. A wide range of reagents are available with selectivity for specific groups on amino acids (Table 1.14). Bifunctional compounds are widely used.

The mechanisms of chemical stabilisation are categorised into four areas:

(i) Cross-linking by bifunctional reagents

(ii) Strengthening of hydrophobic interactions by non-polar reagents

(iii) Introduction of new polar or charged groups leading to additional ionic or hydrogen bonds.

(iv) Hydrophilisation of the protein structure to reduce unfavourable surface hydrophobic contacts with water.

(i) Bifunctional cross-linking reagents, including homobifunctional and heterobifunctional reagents, have been successfully used to stabilise a wide range of proteins (Wold, 1972). They are also used to conjugate enzymes to antibodies for use in immunological techniques (Tijssen, 1985). They act by forming a "bridge" of variable length either inter- and intra-molecularly. Particular amino acids containing specific functional groups can be targeted using certain bifunctional reagents.

(ii) Chemical modification of proteins by non-polar reagents strengthens hydrophobic interactions. The most efficient mechanism for hydrophobic stabilisation should be the introduction of non-polar molecules inside the hydrophobic nucleus of the protein (Mozhaev and Martinek, 1984).

Residue for Modification	Reagent	Reaction
Amine (Lys)	O-Methylisourea	Guanidination
	Acid Anhydrides	Acylation
	Imidates	Amidation
	Iodoacetic Acid	Alkylation
	Borohydrides and Carbonyl compounds	Reductive Alkylation
Carboxyl (Asp, Glu)	Carbodiimides	Amidation
Guanidino (Arg)	Dicarbonyls	Not fully known
Imidazole (His)	Diethylpyrocarbonate	Addition
Indole (Trp)	N-bromosuccinimide	Oxidation
Thiol (Cys)	Maleimido compounds	Addition
	Iodoacetic Acid	Reduction and S-carboxymethylation
	N-ethylmaleimide	Alkylation

Table 1.14 Side-chain modification reagents used in protein stabilisation.

The protein is unfolded, and changes introduced by

(a) refolding under "non-native" conditions, *e.g.*, in the presence of organic solvents or at elevated temperatures.

(b) refolding in the presence of substances which interact non-covalently with the protein in a multipoint fashion. For example, non-polar compounds can be incorporated into the protein in such a way as to leave the polar or charged fragments of the protein exposed to the solvent (Mozhaev and Martinek, 1984).

(c) chemical modification, followed by refolding.

(iii) Introduction of new polar or charged groups

New polar or charged functional groups can be introduced into the protein molecule by chemical modification, leading to formation of additional salt or hydrogen bonds.

(iv) Hydrophilisation of the protein surface reduced the surface area responsible for thermodynamically unfavourable hydrophobic interactions (Mozhaev and Martinek, 1984). Amine groups on the protein surface, can be substituted by more hydrophilic groups, such as $-HCH_2COO^-$ groups, by chemical modification (Melik-Nubarov *et al.*, 1987; Mozhaev *et al.*, 1988).

1.14.3.6 Screening for stable proteins

There is a wide variety of microorganisms that are capable of survival at elevated temperatures (Ó Fágáin and O'Kennedy, 1991). Proteins derived from such thermophilic organisms have been extensively studied and compared with their mesophilic counterparts in an effort to elucidate the molecular basis of their thermostability. While this approach cannot be considered strictly as a method of stabilisation, it has, however, provided an insight into the mechanisms of stabilisation.

1.15 Biosensors

1.15.1 Introduction

A biosensor can be defined as a probe that encorporates a biological sensing component with a physiochemical transducer. The field of biosensor development is one of the most rapidly expanding areas of both analytical chemistry and biochemistry. Since Clark and Lyons developed the first real biosensor in 1962, the field has expanded to incorporate a wide range of disciplines, ranging from micro-electronics to immunology.

The "model" biosensor should exhibit a number of characteristics. It should be relatively small in size, have a fast response time, be highly selective and show targeted specificity and the electronic processing should allow for easy manipulation of data and integration with other devices (Griffiths and Hall, 1993).

A biosensor is composed of two components: a means of recognition and a method of transduction. The means of recognition is provided by a biological element and a transducer converts the biochemical signal into a electronic signal which can be further processed or outputted as data (Coulet, 1991). The biological component can be either catalytic *e.g.*, enzymes, or based on affinity, *e.g.*, antibodies (Vadgama and Crump, 1992). The biomolecule determines the selectivity of the sensor. This is based on the interaction of molecules with complementary structures, binding to each other (enzyme to substrate; or antibody to antigen). The development of monoclonal antibody technology has been a major step in the field of bioaffinity biosensors.

There are a wide range of transduction techniques currently in use. These include electrochemical, optical, calorimetric, acoustic and piezoelectric crystal transducers. The nature of the interaction of the biological component with the analyte has a major influence on the choice of transducer. Other considerations include the intended use of the biosensor and the cost (Griffiths and Hall, 1993).

Biosensors offer a number of advantages over conventional analytical techniques. They can provide "real time" analysis, are generally highly sensitive and selective, and are simple to use (Taylor *et al.*, 1991). At the current stage of development however, there are a number of problems associated with their development. The biological component often has a relatively short operating life-span and needs frequent calibration.

Problems also arise when the analyte is present in a complex matrix, for example blood, where some degree of sample "clean-up" is necessary.

Despite these problems, biosensors have the potential to revolutionise the fields of analytical chemistry and biology. Some of the main types of of biosensor technologies and configurations are summarised below.

1.15.2 Voltammetry

Electrochemical techniques are the biosensor-transduction method most widely used. The most commom of these, amperometry and potentiometry, involve electrical changes (in current or potential) which can be detected by the transducer and reported. Voltammetry is the study of current-potential relations in an electrolysis cell where the current is determined solely by the rate of diffusion of an electrochemical species. The current is defined as the rate at which charge passes through the electrode-solution interface.

The potential is the driving force of the reaction, causing the electrochemical species to be oxidised or reduced at the surface of the working electrode. The applied potential determines the strength of the oxidation/reduction reaction. At negative potentials, the current is cathodic (positive) causing reduction, while at positive potentials, the current is anodic (negative) causing oxidation.

In thermodynamic systems, the standard potential E°, is dependent on the concentration of the electrochemical species. The Nernst equation describes this dependence:

$$Ox + ne^{-} \rightleftharpoons Red$$

$$E = E^{\circ} - \frac{2.3026RT}{nF} \log \frac{[Red]}{[Ox]}$$

where

Ox = Oxidised state of a species

Red = Reduced state of a species

E = reduction potential at specific concentration.

n = number of electrons transferred in the reaction.

R = gas constant (8.3143 V coul deg^{-1} mol^{-1}).

T = absolute temperature.

F = Faraday constant (96,487 coul eq^{-1}).

Therefore, at 25°C,

$$E = E^{\circ} - \frac{0.059}{n} \log \frac{[Red]}{[Ox]}$$

The faradaic current is the current resulting from a change in the oxidation state of the electroactive species. It is a direct measure of the rate of the redox reaction taking place at the surface of the working electrode. It is dependant on the rate at which a species moves from the bulk solution to the electrode surface, and the rate of electron transfer between the electrode and the species in solution.

1.15.3 Electrochemical Transduction

There are two methods used in electrochemical transducers

(i) Amperometry

(ii) Potentiometry

Amperometry involves the application of a constant potential with respect to a reference electrode, while the current between the working and the counter electrodes is measured. The current is generated by the oxidation or reduction of an electrochemical species at the working electrode, and is proportional to the concentration of the analyte (Czaban, 1985).

Potentiometric transducers measure the difference in potential between the working electrode and a second reference electrode, while the current remains constant. Under these conditions, there is no consumption of the analyte. They are based on the Nernst equation, resulting in a logarithmic relationship between the potential and the activity of the ion in solution. Examples of potentiometric devices include ion-selective electrodes and gas-sensing electrodes (Vadgama and Crump, 1992).

1.15.4 The Electrochemical Cell

Electrochemical reactions take place in an electrochemical cell. The cell contains three electrodes, the working, reference and the counter electrodes, immersed in the sample solution (Figure 1.15.4).

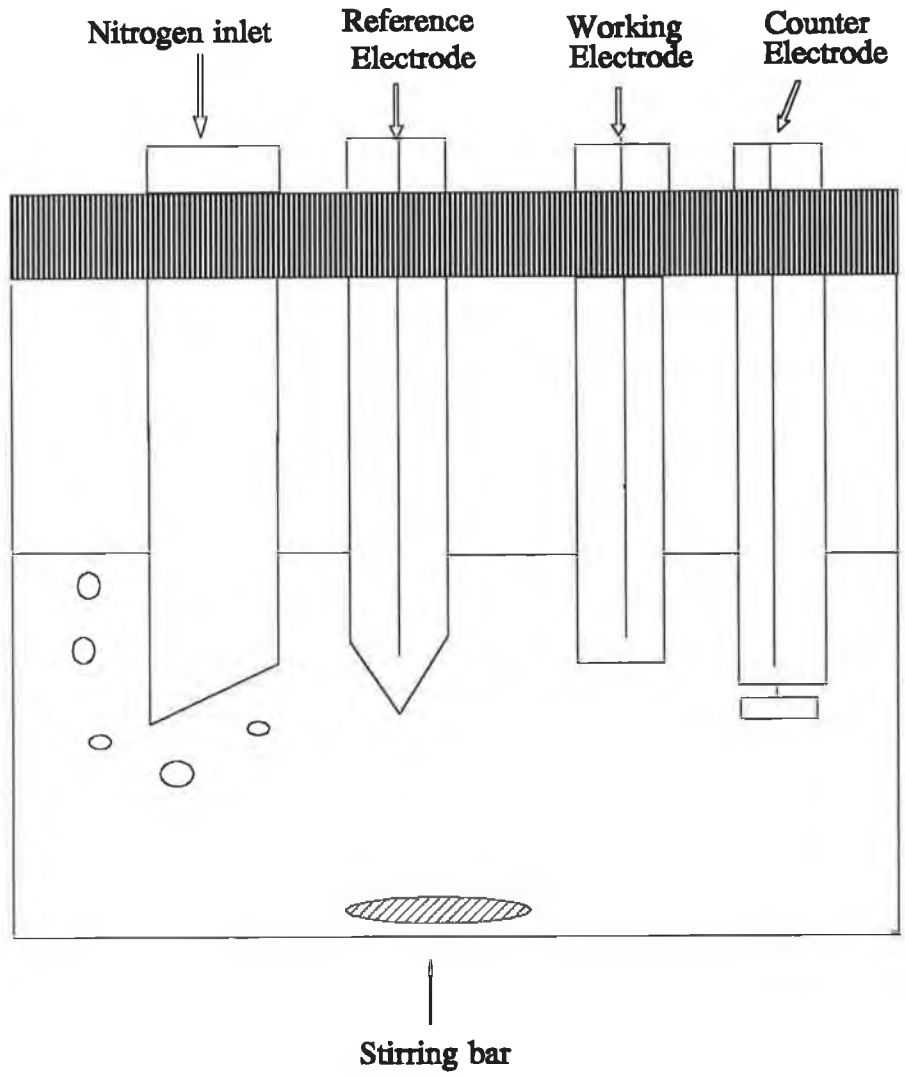


Figure 1.15.4 The electrochemical cell. The reaction takes place at the working electrode as a result of the potential applied. Current flows between the working electrode and the counter electrode and is measured by a potentiostat. A stirring bar provides convective transport if required. Dissolved oxygen is purged from the electrolyte solution using nitrogen gas.

Convective transport is provided (if required) by a magnetic stirring bar. The relative placement of the three electrodes is important. They should be arranged in a way that provides a symmetrical electric field and uniform current distribution across the working electrode surface (Smyth and Vos, 1992).

The redox reaction takes place at the surface of the working electrode. It is vital that the electrode itself does not interfere with either the redox reaction or with the electron transfer process. They are usually made from inert metals (*e.g.*, platinum) or carbon (glassy carbon has excellent electrochemical properties).

The reference electrode monitors the potential of the working electrode. The potential of the reference electrode must be known and remain constant. There are two main reference electrodes: the saturated calomel electrode (SCE) and the silver/silver chloride electrode.

The counter or auxiliary electrode completes the electrical circuit and allows current to flow between it and the working electrode. They are usually made from platinum or carbon and are in the form of a gauze or foil. This gives them a relatively large surface area compared with the working electrodes.

The removal of oxygen from the solution is essential. Oxygen is reduced at a potential of -0.01. Attempts to reduce species at potentials more negative than this will also result in the reduction of oxygen, giving large background currents. A further problem arises from the products of oxygen reduction. These may react with the analyte (*e.g.*, formation of hydroxides in the determination of heavy metals). To alleviate these problems, the solution is purged with an inert gas (such as nitrogen) for 20-30 minutes prior to the electrochemical reaction.

1.15.5 Immobilisation of Biological Components onto the Electrode Surface

Modification of an electrode is achieved by immobilising a biological molecule onto the surface. A number of factors are important in the immobilisation of a biomolecule. A high level of activity must be retained so the immobilisation technique used, the thickness of the immobilised layer and the loading onto the surface are important (Bardeletti *et al.*, 1991).

Immobilisation can be carried out in a number of different ways:

(i) Covalent attachment

(ii) Adsorption

(iii) Polymeric coating

(i) Covalent attachment can be achieved by silanisation or direct binding. Silanisation involves the formation of surface hydroxy or oxide groups which are reacted with trialkoxy or trichloro silanes to form one to three bonds to the underlying electrode material (Srinivasan *et al.*, 1977; Moses *et al.*, 1978; Diaz *et al.*, 1977). These modifications generally give rise to monolayers, although the formation of multilayers is possible (Lenhard and Murray, 1978). Bifunctional reagents (*e.g.*, glutaraldehyde) can also be used to form inter-molecular cross-links, binding a protein to the solid support. The reaction can however be difficult to control and a large amount of the biological component is sometimes required (Bardeletti *et al.*, 1991). Direct binding can be achieved to the platinum or carbon of the electrode by a variety of methods such as mechanical abrasion and vacuum pyrolysis (Smyth and Vos, 1990).

(ii) A variety of substances adsorb enzymes and other proteins onto their surface. This provides an excellent method for immobilisation, as no reagents are required and the process is less disruptive to the protein than chemical methods of attachment. The adsorption involves forces such as hydrogen bonding and Van der Waal's forces. The disadvantage of this method is that these forces are weaker than chemical linkages and are more susceptible to changes in pH temperature and ionic strength (Bardeletti *et al.*, 1991).

(iii) The entrapment of biomolecules behind or within a polymer film has a number of advantages -it provides a stable environment and it is porous, allowing for the movement of both substrate and electrolyte. Physical entrapment in a gel matrix (*e.g.*, gelatin) which is then cast over the electrode surface and held in place by a dialysis membrane, use of membranes (cellulose acetate, polyvinylalcohol and polyurethane) and entrapment within polymer matrices (polyacrylamide and agarose) have all been reported (Gorton *et al.*, 1990).

1.15.6 Applications of Biosensors

Biosensors are set to make a significant impact in the future. They offer the prospect of measuring biomolecules important in medicine, biotechnology and other fields, in a convenient, rapid and relatively inexpensive way. A wide range of substances can currently be measured using biosensors, from enzymes in clinical chemistry and the food and cosmetic industries, to environmental monitoring.

(i) Medical applications

Clinical diagnostics have been the focus of biosensor application over the last number of years, approximately 20 self-contained analysers based on enzyme electrodes for the determination of a range of analytes are currently available (Bardeletti *et al.*,1991). *In vitro* diagnostic biosensors have a worldwide market worth an estimated US$9 billion per annum (Griffiths and Hall, 1993). Biosensors can be used in a number of situations from in-hospital use, to use by the general public. A wide range of analytes can be determined using enzyme-based sensors. The Exac Tech™ glucose monitors manufactured by Medi-Sense (Abington, UK) is a pen-size sensor for the determination of glucose levels in blood, and is the world's smallest blood glucose monitor (Bardeletti *et al.*, 1991). Glucose and lactate levels in blood, as well as uric acid, can be successfully monitored using a number of commercially available biosensor devices (Bardeletti *et al.*, 1991). Biosensors are also available for other clinically important analytes, including cholesterol (Bertrand *et al.*, 1979; Wollenberger *et al.*, 1983), acetylcholine (Mascini *et al.*, 1986) and bilirubin (Renneberg *et al.*, 1982). Blood-glucose monitoring kits and pregnancy tests are the most common consumer biosensors (Griffiths and Hall, 1993).

The development of miniaturised voltammetric probes aimed at *in vivo* monitoring is an area of intense research activity (Vadgama and Desai, 1991). Research into brain neurotransmitters is an area of particular interest, and recent developments, including the use of ion-selective membranes, have lead to great advances in this area (Wightman *et al.*, 1988). The problems associated with *in vivo* monitoring are numerous. Biocompatibility and allergenicity together with technical and ethical problems are major difficulties. However, future work will most likely focus on the development of selective probes for organic drugs and analytes of clinical importance, the coupling of enzymic and immunochemical reactions with *in vivo* electrodes, the design of new non-invasive

and multispecies probes, and the search for means of protecting *in vivo* electrodes against biological matrix effects (Vadgama and Desai, 1991).

(ii) Food Industry

Biosensors are used in the monitoring of food samples during the manufacturing and processing stages. Mannino and Wang (1992) recently reviewed the status of electroanalysis in the food industry. Specific enzyme-based sensors have been developed for sugars (Schubert *et al.*, 1986), as well as other important analytes such as ethanol (Kitagawa *et al.*, 1989) and ascorbate (Matsumoto *et al.*, 1981). The determination of L-glutamate is important in fermentation control as many food stuffs contain L-glutamate as a flavouring agent (Palleschi *et al* 1992).

(iii) Environmental monitoring

Biosensors are widely used in the monitoring of pollution. Kolvoda (1990) recently reviewed current technologies used in environmental monitoring. Monitoring of pesticides and herbicides can be carried out using biosensors. The development of reliable, robust, multifunctional and self-contained biosensors capable of working in the gaseous or organic phases will allow for future developments in the areas of monitoring of ozone, acid rain and industrial atmospheric pollutants, as well as air, soil and water analysis.

(iv) Industrial applications

The pharmaceutical industry provides an ideal environment for the use of biosensors. Use in process control is viable as well as in purity and potency testing of its products. Other areas of interest include product testing and monitoring raw materials or product intermediates. Opportunities in other industrial areas are limited at the present time. Technological limits do not allow for the use of biosensors under the harsh conditions required for many industrial processes. This is one area for possible future development.

In conclusion, the potential for biosensor application is enormous. Improvements in existing transduction techniques, as well as the development of novel methods of measurment, will allow for the wide-spread use of biosensors in the future.

1.15.7 Electrochemical Immunoassays

Immunosensors utilise the characteristic selectivity of antibodies for their antigens and combine them with a suitable transducer, to form highly selective and sensitive biosensor. There has been extensive development in this area over the last decade, mainly because of advances in immunotechnology. With current improvements in biosensor-related technologies such as micro- and opto-electronics, the potential for further development is vast. There are two main types of immunosensors - labelled and non-labelled. Labelled immunosensors are similar to ELISAs in that a signal, produced by a labelled antibody or antigen, is measured. In contrast, the antigen-antibody complex is determined directly in non-labelled sensors (Aizawa, 1991).A variety of substances can be used as labels, including enzymes and redox substances. Enzymes can increase the sensitivity of a sensor by amplification of the signal. Enzyme product can be detected with a variety of electronic or optoelectronic devices. Peroxidase, for example, catalyses the luminescent reaction of luminol, producing photons which can be measured in an optical enzyme immunosensor. In homogeneous immunoassays, where there is no separation of the immune-complex from free antibody and antigen, fluorescent and chemiluminescent labels have been described (Aizawa, 1991). Non-labelled immunosensors measure some physical change brought about by the antibody-antigen complex. Antibody (or antigen) is bound to a surface capable of detecting complex formation (eg, piezoelectric crystals). Antigen (or antibody) in solution binds to the surface causing a change in surface characteristics, and generating a signal.

1.16 Immunoassay

An immunoassay is a technique for measuring the presence of a substance using an immunological reaction, generally an antibody-antigen reaction. There are a variety of different immunoassays from simple precipitation to radioimmunoassay (RIA) and enzyme-immunoassay (EIA) (Kemeny, 1991). The choice of assay is dependent on a number of factors - sample concentration, precision, ease of use and facilities available. EIA are based on two events: the antibody-antigen reaction and the detection of this reaction using enzymes as indicators (Campbell, 1984). There are several formats for EIA. The immunological reaction can be heterogeneous, where there is a separation of the immune complex from the immunoreactants, or homogeneous, where no separation takes place (Tijssen, 1985). In heterogeneous immunoassays, either the antibody or antigen can be immobilised onto the solid phase. The sample is added and test substance bound. This is then reacted with a labelled immunoreactant, substrate added, and enzyme product measured. The concentration of the immunoreactant in the test can then be determined. In homogeneous assays there is no requirement for a separation step because the immune reaction influences the enzyme reaction. Both heterogeneous and homogeneous assays can be further categorised into competitive or non-competitive systems (Tijssen, 1985). Competitive assays are usually used for the quantitation of an immunoreactant. The assay is based on the competition between an enzyme-labelled immunoreactant and free immunoreactant in solution or bound to the solid phase. The signal is inversely proportional to the concentration of free immunoreactant in solution (Tijssen, 1985). In non-competitive systems, there is no competition for binding.

Enzyme-linked immunosorbent assay (ELISA) is perhaps the most widely used EIA. They provide an quick, easy to use, sensitive and reliable method for the detection and determination of antibodies and/or antigens.

1.17 Conclusions

FVIII is notoriously unstable. Stability studies can help to understand how and why it denatures and will help in the design of an altered molecule (by genetic or other means) for use in therapeutic or as an *in vitro* test standard. For this reason, analysis of FVIII's stability was undertaken together with a programme of chemical modification in an attempt to increase FVIIIs *in vitro* thermal stability. Current methods for the analysis of FVIII are based on its procoagulant activity. These techniques rely on the ability of FVIII to correct the prolonged clotting time of FVIII deficient plasma. They are not, therefore, specific for the cofactor itself, as the process of blood coagulation is a complex one involving over 13 different reactions, any of which could be involved. There is much scope for improvements and further developments of the techniques. There are substantial risks from infectious agents, associated with the handling of human blood. Any novel techniques which reduce the possibility of infection from contaminated samples would be a major improvement. Monoclonal antibodies could provide an ideal way of isolating particular constituents of blood because of the high specificity they exhibit for their particular antigen. The possibility of viral or bacterial contamination would subsequently be dramatically reduced. ELISAs have been developed for the detection of FVIII but these generally utilise inhibitor or autoantibodies from haemophiliac patients. The incorporation of monoclonal or polyclonal antibodies into an ELISA system for the detection of FVIII would provide a safer alternative to these human-derived antibodies.

Biosensor technology offers the prospect of easy to use, sensitive and rapid techniques for the detection and quantitation of analytes. The prospect of a system for the detection of FVIII in whole blood could revolutionise the diagnosis and treatment of haemophilia. Home testing kits could be developed which would allow doctors or even the patients themselves, to monitor the disease without the need for time-consuming and expensive in-hospital patient care.

AIMS OF THIS PROJECT

General Aims

The general aims of this project were to investigate the effects of chemical modification on recombinant factor VIII (rFVIII) procoagulant activity, to develop novel antibodies and analytical systems to detect and quantitate recombinant factor VIII and anti-rFVIII antibodies.

Specific Aims

1. To investigate the effects of chemical cross-linkers and protein modifying reagents on rFVIII procoagulant activity, and determine whether any modifications caused by these reagents resulted in an increase in the thermostability of rFVIII.

2. To develop monoclonal and polyclonal antibodies against rFVIII, and to investigate the effects of the binding of these antibodies on rFVIII procoagulant activity.

3. To develop assay systems using the above antibodies, for the detection of rFVIII and anti-rFVIII antibodies.
   (i) An ELISA consisting of a number of different formats
   (ii) A biosensor, incorporating antibody recognition with an electrochemical transduction system.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Recombinant Factor VIII (rFVIII) was a gift from Baxter Biotech., Hyland Division, Glendale, California, U.S.A. The following lot numbers were used:

9A03L007,
9A03L023,
9A03L026,
C010Q1803,
C011Q1803,
C006Q1803,

Factor VIII Chromogenic Assay kit (cat. no. 281068), Coag Cal N calibration plasma (cat. no. 291040) and Factor VIII-deficient plasma (cat. no. 281042) were gifts from Baxter Diagnostics AG, Düdingen, Switzerland.

All tissue culture products including media, foetal calf serum and L-glutamine were supplied by Flow Laboratories Ltd, Richmondsworth, Herts. WD3 1PQ, UK.

Cell culture vessels were obtained from Flow, Costar, Cambridge, MA 02140, U.S.A. and Nunc, Denmark.

T24 cells from human bladder were supplied by Dr. J. Tager, University of Amsterdam, 1105 AZ, Amsterdam, Netherlands.
NSO murine plasmacytoma were supplied by ECACC, PHLS, Porton Down, Salisbury, Wiltshire SP4 0JG, UK. (catalogue number 84112004).
NRK cells were obtained from ATCC, 12301 Park Lawn Drive, Rockville, Maryland 20861, U.S.A.

All chemicals were of analytical grade and were obtained from Sigma Chemical Co., Poole, Dorset, UK, BDH Chemicals Ltd., Poole, Dorset, UK, or Riedel de Häen AG, Seelze, Hannover, Germany. Exceptions were activated Sepharose and Sephadex purchased from Pharmacia, Uppsala, Sweden. Bis-(maleimido-methyl) ether was supplied by Boehringer Mannheim, Hannover, Germany. Sheep anti-Factor VIII polyclonal antibody was from Serotec, 22 Bankside, Kidlington, Oxford OX5 1JE UK.

Hoechst 33258 fluorescent stain was purchased from Calbiochem, Behring Diagnostics, La Jolla, CA 92037, U.S.A. Nafion and Hydroquinone was supplied by Aldrich Chemical Company, Gillingham, Dorset SP8 4JL, UK. Nitrocellulose paper (0.45µm) was from Schleicher and Schuell, Postfach 4, D-3354, Dassel, Germany. Microconcentrators were supplied by Amicon Inc, Beverly, MA 01915, U.S.A. Liquid nitrogen was from Coopers Cryoservice Ltd, Dublin, Ireland. CO_2 and N_2 was supplied by Irish Industrial Gasses, Dublin, Ireland.

2.2 Equipment

A Morgan Grundy (Crowley, Middlesex, UK) waterbath with a Techne Tempette Junior TE-8J heating unit were used in denaturation experiments.

A Titretek Twinreader type 381 (Flow Laboratories Ltd., Scotland) was used to read absorbances on microtitre plates.

Holten LaminAir HB 2448K laminar flow cabinet was used for all tissue culture work. Cells were incubated at 37°C in a 5% CO_2 environment in a Jouan EG 115 1R water-cooled incubator.

Heraeus Christ Labofuge 6000 centrifuge was used to centrifuge universal tubes (1 - 20mls) and a Heraeus Biofuge A was used for smaller volumns (0.5 - 1.5mls).

A Beckmann J2-21 Centrifuge was used in conjunction with the Amicon micro-concentrators to concentrate samples of antibody.

Union Carbide freezing trays and liquid nitrogen containers were used to freeze and store cells in liquid nitrogen.

Atto Model AE-6450 electrophoresis equipment was used for gel electrophoresis.

LKB 2050 Midget Electrophoresis Unit and a Bio-rad semi-dry transfer system were used for Western blotting.

A Beckmann System Gold HPLC system with programmable solvent module 126, detection module 166 at absorbance units full scale (AUFS) 0.05, with detection at 280nm was used.

An EG&G PAR (Princeton Applied Research, Princeton, NJ, U.S.A.) Model 264A polargraphic analyser was used with a WPA (Linton, Cambridge, UK) model CQ95 recorder in voltammetric studies.

Name	Source	Address
Dade Factor VIII Chromogenic Assay Kit	Baxter Diagnostics AG.	Düdingen, Switzerland
Bicinchoninic Acid (BCA Protein Assay Kit)	Pierce Chemical Co.	Rockford, Illinois, 61105, U.S.A.
Murine Monoclonal Antibody Isotyping Kit	Sigma Chemical Co.	Poole, Dorset, UK.
Antibody Isotyping Kit	Serotec	22 Bankside, Oxford OX5 1JE, UK.

Table 2.1.1 Analytical diagnostic kits used

2.3 FVIII Chromogenic Assay

Factor VIII activity was measured using the Factor VIII Chromogenic Assay kit from Baxter Diagnostics AG. Reagents were reconstituted according to the manufacturer's instructions. The assay was miniaturised so that it could be performed on a 96-well microtitre plate. 25µl of sample was pipetted into duplicate wells of a microtitre plate held at 30°C. 25µl of each of factor X and factor IXa reagents (prewarmed to 30°C) were added, mixed and incubated for 90 seconds. 125µl of substrate/stopping buffer (also prewarmed to 30°C) was added, mixed, and absorbance at 405nm read every 15 seconds for 2 minutes on a Titertek Twinreader Plus automatic plate reader.

Coag Cal N calibration plasma and Factor VIII-deficient plasma diluted in 0.9% (w/v) NaCl, served as controls and were included in every assay.

2.4 Buffers

The buffers used for modification experiments were 10mM Tris buffer pH 7.5, containing 0.9% (w/v) NaCl, 5mM $CaCl_2$, and 0.05% (w/v) azide and 10mM barbital buffer, pH 7.5 also containing 0.9% (w/v) NaCl, 5mM $CaCl_2$ and 0.05% (w/v) azide. Samples were diluted in 0.9% (w/v) NaCl before being assayed.

2.5 Denaturation of rFVIII

Denaturation studies were carried out as follows: solutions of native and modified rFVIII were incubated in a 55°C waterbath. Samples were withdrawn at regular intervals (0, 2, 5, 10, 20, 40 and 60 minutes), stored on ice and then assayed in 0.9% (w/v) NaCl.

Coag Cal N (positive) and FVIII-deficient plasma (negative) controls, along with a control containing rFVIII and the diluent in which the cross-linker or the modifying reagent was dissolved, were included in each experiment.

2.6 Transfer of rFVIII into Barbital buffer

For amino-specific reagents, the rFVIII was transferred to barbital buffer. This does not contain free amino groups which could interfere with the cross-linking reaction. Sephadex G-25 was swollen with barbital buffer and packed into a Pasteur pipette, to form a chromatography column. rFVIII was applied in small aliquots (100µls), to the column at 4°C. The protein was subsequently eluted using barbital buffer. The protein concentration of each fraction was estimated using the BCA assay (Section 2.7)

2.7 Determination of Protein Concentration

Protein concentration was determined using the bicinchoninic acid (BCA) method of Smith *et al.* (1985). In this method, protein reacts with Cu^{2+} in an alkaline environment producing Cu^{1+}. BCA is a sensitive, stable and highly specific reagent for Cu^{1+}, forming a water soluble product which exhibits strong absorbance at 562nm. The BCA Assay, supplied in kit form by Pierce Chemical Company, contains two reagents:

Reagent A: an alkaline buffer containing sodium carbonate, sodium bicarbonate, sodium tartrate and BCA reagent.

Reagent B: 4% (w/v) copper sulphate solution

The working solution was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. A range of protein standards was prepared from a 2mg/ml solution of BSA using PBS as diluent. 10µl of each standard or unknown protein sample was pipetted into duplicate wells of a 96-well microtitre plate. Controls consisting of 10µl of diluent were also included. 200µl of working reagent was added and the solutions mixed. The plate was covered and incubated at 37°C for 30 minutes. Absorbance at 560nm was read on a Titertek Twinreader Plus.

2.8 Accelerated Storage Studies

Accelerated storage studies were performed as described by Kirkwood (1984).

Accelerated storage studies were performed on rFVIII diluted in 10mM Tris-HCl buffer with 0.9% (w/v) NaCl, pH 7.5, containing either different concentrations (5mM, 10mM or 20mM) of $CaCl_2$, or different concentrations of BSA (2mg/ml, 5mg/ml or 20mg/ml). Solutions were filter sterilised, aliquotted into sterile microtubes and incubated at particular temperatures.

Temperatures used were 15°C, 27°C, 37°C and 45°C with reference temperatures of 4°C and -20°C.

After certain times, samples were withdrawn and a series of dilutions on 0.9% (w/v) NaCl made. Each of these was assayed in duplicate for FVIII activity.

2.9 CHEMICAL MODIFICATION OF rFVIII

2.9.1 Amino-specific Reagents

2.9.1.1 Modification of rFVIII using Bis-imidates

rFVIII was transferred to 10mM barbital buffer containing 0.9% (w/v) NaCl, pH 7.5, for modification with the bis-imidates. Dilutions of the protein were reacted on ice with final concentrations ranging from 0 to 40mM of the particular bis-imidate in dimethyl formamide (DMF). The reaction was allowed to proceed for thirty minutes before being stopped by the addition of 1/10 volume of ice cold 10mM Tris-HCl buffer, pH 7.5. FVIII activity was then measured in a 1:31 dilution in 0.9% (w/v) NaCl.

2.9.1.2 Modification of rFVIII using Glutaraldehyde

From a stock solution of 100mM glutaraldehyde in water, the following final concentrations were prepared and reacted with rFVIII in barbital buffer: 0, 1mM, 2mM, 3mM. The reaction took place on ice for 1 hour before being terminated on addition of 1/10 volume ice cold 10mM Tris-HCl buffer, pH 7.5, and FVIII activity was measured.

2.9.2 Thiol-specific Reagents

2.9.2.1 Modification of rFVIII using Bis-maleimides

rFVIII, in 10mM Tris-HCl buffer containing 0.9% (w/v) NaCl, pH 7.5, was reacted with a range of concentrations (0 to 100µM) of two members of the bis-maleimide group of cross-linkers. The cross-linkers were dissolved in either acetone (Bis-(maleimido-methyl) ether (BMME), or dimethyl formamide (N-N-bis (3-maleimidopropionyl)-2-hydroxy 1,3-propanediamine (BMPPD)). The reaction took place at room temperature, for ten and sixty minutes. Addition of 5µl of a 100mM solution of 2-mercaptoethanol terminated the reaction and FVIII activity was measured.

2.9.3 Modification of rFVIII using heterobifunctional reagents

A stock solution of the heterobifunctional, N-(γ-maleimidobutyryloxy) succinimide (GMBS), (2.5mM) in DMF was prepared, and from this, final concentrations of 100μM and 250μM were reacted with rFVIII in 10mM barbital buffer containing 0.9% (w/v) NaCl, pH 7.5, at either 4°C for ten minutes, or room temperature for thirty minutes. The reaction was terminated upon addition of 1/10 volume of ice cold 10mM Tris-HCl buffer, pH 7.5. This was then incubated for a further five minutes at 4°C. The solutions were then assayed for FVIII activity.

rFVIII, in 10mM barbital buffer containing 0.9% (w/v) NaCl, pH 7.5, was reacted with a 100-fold molar excess solution of the heterobifunctional succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate, (SMCC) in DMF, for one hour at room temperature (with and without stirring). 1/10 volume of ice-cold 10mM Tris-HCl buffer, pH 7.5, was added and incubated for an additional thirty minutes with stirring, to terminate the amino-specific reaction. FVIII procoagulant activities were then measured.

2.10 Protein Modifying Reagents

2.10.1 Reaction of rFVIII with Polyethylene glycol

rFVIII in 10mM barbital buffer containing 0.9% (w/v) NaCl, pH 7.5, was reacted with cyanuric chloride-activated Polyethylene glycol (PEG) to final molar concentrations of 0.5X and 5X with regard to rFVIII lysines (approx 40). The reaction took place at room temperature for 1 hour, before being terminated by addition of 10% volume of ice-cold Tris-HCl buffer, pH 7.5. This was further incubated for fifteen minutes before being assayed for FVIII activity.

2.10.2 Reaction of rFVIII with Copper/phenanthroline chelate

1mM stock solutions of $CuSO_4$ and o-phenanthroline (o-Ph) in 10mM Tris-HCl containing 0.9% (w/v) NaCl, pH 7.5 were prepared. From these, various combinations of each (10μM, 20μM and 50μM $CuSO_4$ and 25μM, 50μM and 100μM o-Ph) were reacted with rFVIII on ice for twenty minutes. FVIII activity was then assayed and denaturation studies carried out.

2.10.3 Reaction of rFVIII with Hydrogen Peroxide

Final concentrations, ranging from 0 to 1M H_2O_2 were reacted with rFVIII on ice for 15 minutes, 1 hour and 2 hours. The reaction was terminated by addition of 2% (w/v) of a 1 mg/ml solution of catalase enzyme. This was further incubated for 15 minutes on ice and FVIII activity measured.

2.10.4 Reaction of rFVIII with 2-Iminothiolane

rFVIII in 10mM barbital buffer containing 0.9% (w/v) NaCl, pH 7.5, was initially reacted with final concentrations of 0, 0.25mM, 1mM, and 6mM 2-Iminothiolane (2-IT),in water. The reaction took place on ice over a two hour period. 10% volume of ice cold Tris-HCl buffer, pH 7.5, was added to terminate the reaction. FVIII activity was then measured, and denaturation studies carried out. In the second stage of the experiment, the modified rFVIII was reacted with either hydrogen peroxide (40mM solution) or with o-phenanthroline (100µM solution with 50µM $CuSO_4$). These two reactions were carried out in an attempt to promote disulphide bond formation between thiol groups introduced via 2-IT, and free thiol groups on rFVIII. FVIII activity was measured and denaturation studies performed.

2.10.5 Addition of Xylitol

rFVIII in 10mM Tris-HCl buffer containing 0.9% (w/v) NaCl, pH 7.5, was reacted with final concentrations of 10mM, 50mM and 100mM Xylitol. The reaction took place on ice for 30 minutes and FVIII activity was then measured. A control consisting of rFVIII and buffer was also included.

2.11 TISSUE CULTURE TECHNIQUES

2.11.1 Media

The following media were used:

$DMEMS_0$ consisted of Dulbecco's modified Eagles Medium (DMEM) containing 2% (w/v) pyruvate, 1.5% (w/v) $NaHCO_3$ and 10% (w/v) HEPES, pH 7.4.

$DMEMS_{10}$ consisted of $DMEMS_0$ supplemented with 10% (v/v) foetal calf serum (FCS), and 1% (w/v) 200mM L-glutamine.

$DMEMS_{10}$/T24 consisted of $DMEMS_{10}$ supplemented with 5% (v/v) T24 conditioned-medium.

2.11.2 Preparation of T24-Condidioned Medium

T24 cells were grown in 75cm^2 culture flasks at 37°C in 5% CO_2. After passaging twice, the supernatant was removed, centrifuged at 2000 rpm for 10 minutes and filter sterilised. It was then used to supplement $DMEMS_{10}$ at a concentratiom of 5% (v/v) ($DMEMS_{10}$/T24), or stored in small aliquots at -20°C.

2.11.3 Storage and Recovery of Cells

Long term storage of cells was carried out in liquid nitrogen. Cells were centrifuged at 1500 rpm for 10 minutes, the supernatant decanted and the cells resuspended in ice-cold FCS supplemented with 5% (v/v) DMSO to a final concentration of 1 x 10^7 cells/ml. 1ml aliquots were pipetted into cryotubes and placed in a freezing tray which was gradually lowered into the gaseous phase of liquid nitrogen. When frozen, the cells were placed into canes and stored immersed in the liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing rapidly at 37°C. When only a small piece of ice remained, the cells were transferred to a sterile universal containing 10ml of $DMEMS_o$. The cells were washed and centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in fresh $DMEMS_{10}$ medium and transferred to a 24-well plate.

2.11.4 Maintenance of Cell Lines

(i) Non-adherent cell lines

The NS0 non-adherent cell line was maintained in 25cm^2 tissue culture flasks in $DMEMS_{10}$. Cultures were plated out at a density of 1 x 10^5 cells/ml and were maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were harvested by flushing them from the bottom of the flask with media using a sterile pasteur pipette and centrifuging at 1200 rpm for 10 minutes. The supernatant was decanted and the pellet resuspended in fresh $DMEMS_{10}$ and transferred to fresh culture flasks.

(ii) Adherent cell lines

The T24 adherent cell line was maintained in similar conditions to those above. However, because they adhere to the surface of the flask, mild trypsinization was necessary to remove them. This was achieved by decanting the culture medium and adding 1ml trypsin/EDTA solution (0.25% (w/v) trypsin and 0.02% (w/v) EDTA in PBS) to the flask. The flask was rotated gently and the trypsin discarded. A further 2ml of trypsin was added and the flask incubated at 37°C for 10 minutes until the cells became detached. 5ml of $DMEMS_{10}$ was added aseptically and the cells centrifuged at 1200 rpm for 10 minutes in a universal. The supernatant was discarded and the pellet resuspended in $DMEMS_{10}$ and passaged.

2.11.5 Cell Viability Testing

Cell viability was determined using the trypan blue dye exclusion method. A cell suspension was prepared and 50µl removed. An equal volume of trypan blue was added and this was left for 1 minute. A small amount of this suspension was transferred, using a micropipette, to a Neubauer haemocytometer covered with a coverslip. The number of viable cells (not stained) in 5 squares was counted and the total number of cells calculated using the following equation:

Total number of cells =

N (viable cells) x 5 (no. of squares counted) x 2 (dilution factor) x 10^4

2.11.6 Mycoplasma Testing

Cells were routinely tested for the presence of mycoplasma using the fluorescent Hoechst 33258 stain. Sterile coverslips were placed in sterile petri dishes and 1ml of a 5 x 10^3 solution of normal rat kidney (NRK) cells added. These were incubated overnight at 37°C in a humidified incubator containing 5% CO_2. On day 2, 1ml of test sample or control (cell free supernatant) was added to the petri dishes and these were incubated for a further 3 days.

The supernatants were removed and the coverslips washed 3 times in PBS. The cells were fixed for 6 minutes in a 1:1 mixture of methanol:acetone. The coverslips were then transferred to PBS and rinsed twice.

The cells were stained for 10 minutes in Hoechst 33258 stain (0.05 µg/ml in PBS), washed in PBS 3 times, air dried and mounted on slides. Mycoplasma, if present, could then be observed under the 40X magnification of a Leitz Labrolux microscope as defined spots of fluorescence in the cell cytoplasm.

2.12 Production of anti-rFVIII polyclonal antibodies

A New Zealand White male rabbit was immunised intradermally (i.d.) at several sites with 0.3 mg/ml rFVIII emulsified in complete Freund's Adjuvant using a Kikawerk Ultra-Turrax homogeniser. Booster injections of rFVIII in Incomplete Freund's Adjuvant were given at fortnightly intervals until the titre was sufficiently high (1:100 000). Further injections were given two weeks prior to bleeding. 20 ml of blood was taken from the marginal ear vein and anti-rFVIII antibody was purified from this.

2.13 MONOCLONAL ANTIBODY PRODUCTION

2.13.1 Immunisation Protocol

A 0.1mg/ml solution of rFVIII was emulsified in complete Freund's adjuvant as described in Section 2.12. 500µl of this was injected intraperiteally (i.p.) into 8 week old Balb/C mice. Further immunisations were given at fortnightly intervals until a high antibody titre was obtained (*i.e.*, 1:5,000). The final immunisation was given 3-4 days prior to cell fusion.

2.13.2 Testing myeloma cells for HAT-sensitivity

NS0 myeloma cells were cultured in $DMEMS_{10}$ containing 0.1mM 8-azaguanidine for three days. HAT-sensitive cells can grow in this medium whereas HAT-resistant cells die. HAT-sensitive cells are subsequently grown in 8-azaguanidine-free medium for 1 week prior to cell fusion.

2.13.3 Isolation of Immune splenocytes

An immunised mouse was sacrificed by cervical dislocation and placed in 70% alcohol. Under sterile conditions, the spleen was removed and placed in a petri dish with 10mls of $DMEMS_{10}$. Using the plunger of a sterile 20ml syringe, the spleen was pressed through the screen of a sterile dissociation sieve (Sigma, UK) to get a cell suspension. This was transferred to a 50ml centrifugation tube and centrifuged at 2000rpm for 15 minutes. The supernatant was decanted, and the pellet resuspended in 10mls of Gey's haemolysis solution (Mishell and Shiigi, 1981). After 5 minutes the cells were recentrifuged (10 minutes at 1500rpm) and the supernatant discarded.

The cells were washed twice in $DMEMS_0$ and a cell viability count carried out as described in Section 2.11.5.

2.13.4 Preparation of myeloma cells for cell fusion

A $75cm^2$ flask containing HAT-sensitive, mycoplasma-free myeloma cells in the mid-log phase of growth was harvested and centrifuged at 1500rpm for 15 minutes. The cells were washed twice in serum-free medium and a cell viability count carried out as in Section 2.11.5.

2.13.5 Cell Fusion

Immunised splenocytes and myeloma cells were mixed in a ratio of 2:1 in a 50ml centrifuge tube and centrifuged at 1500rpm for 5 minutes. The resulting pellet was washed twice in $DMEMS_0$ to ensure all traces of serum had been removed and was then recentrifuged at 2000rpm for 15 minutes. The supernatant was discarded and the pellet warmed to 37°C for 3 minutes in a waterbath. The cells were transferred back to the laminar flow cabinet and the pellet tapped gently to loosen it from the bottom of the tube. 1ml of a solution of 50% (w/v) PEG_{1500} pre-warmed to 37°C, was added to the cells slowly over 1 minute with continuous gentle stirring. The solution was gently mixed for a further 1 minute. 3mls of $DMEMS_0$ (pre-warmed to 37°C) was added over 3 minutes and the solution mixed. A further 7ml of $DMEMS_0$ was added over the next 5 minutes and the solution left at 37°C for 20 minutes. The pellet was centrifuged (1000rpm, 5 minutes) and the supernatant removed.

The cells were resuspended in $DMEMS_{10}$/T24 conditioned medium to a concentration of 1 x 10^6 cells per well. 100µl of this was dispensed into each well of a 96-well microtitre plate and incubated at 37°C in 5% CO_2.

On Day 2, 100µl of $DMEMS_{10}$ supplemented with 2X HAT selection medium was added to each well and the plates reincubated. Thereafter, the cells were fed at 5 day intervals. 100µl of medium was carefully removed and replaced by 100µl of fresh $DMEMS_{10}$ containing 2X HAT.

After 2 weeks, the unfused myeloma cells should all have died in the presence of aminopterin, and the unfused splenocytes will die off naturally, leaving only the hybridomas. These are weaned off aminopterin by feeding them with medium containing HT for a further two weeks. Thereafter, the cells were fed on $DMEMS_{10}$/T24.

2.13.6 Cloning by Limiting Dilution

To ensure antibodies are monoclonal, the cells must be derived from a homogeneous population. Thus, it is necessary to clone the hybridomas to isolate stable colonies of specific antibody-producing cells.

Cell viability counts (Section 2.11.5) were carried out on each of the wells to be cloned. A solution containing 1 X 10^6 cells/ ml was prepared from each well. From this, a 1:1000 dilution was made by initially making a 1:100 dilution in $DMEMS_0$ and then a 1:10 dilution of this, also in $DMEMS_0$. This yields a solution of 1 cell/1µl. 100µl of this was made up to 20mls of $DMEMS_{10}$/T24 and 200µl aliquoted into each well of a 96-well microtitre plate. These were incubated at 37°C in a 5% CO_2 environment.

2.14 Screening for Antibody Production

2.14.1 Dot Blot

Dot Blotting was carried out according to the method of Hawes (1982). 1µl of a 0.1mg/ml solution of rFVIII was blotted onto nitrocellulose paper (0.45µm pore size, Schleicher and Schuell) which had previously been wetted with PBS (0.15M, pH 7.2). The dots were air dried and free binding sites were blocked for 1 hour at room temperature using a 1% (w/v) solution of bovine serum albumin (BSA) in PBS. The blocking buffer was decanted and the blots were washed 4 times in PBS and 4 times in

PBS containing 0.5% (v/v) Tween 20 (PBS-Tween). Test solution (cell culture supernatants, or purified antibody diluted in blocking buffer) was added and this was incubated at room temperature for 1 hour on a shaker. The blots were washed as before, and anti-mouse (or anti-rabbit) antibody conjugated to alkaline phosphatase, diluted 1:1000 in blocking buffer, was added. These were incubated for 1 hour at room temperature. Unbound secondary antibody was decanted, the blots washed and bound antibody detected using a solution of 0.5M Tris-HCl, pH 8.9 containing 0.46mM 5-Bromo-4-chloro-3-indolyl phosphate, 0.12mM nitroblue tetrazolium, 4mM $MgCl_2$ and 4% (v/v) N,N-dimethyl-formamide. Substrate was incubated for 2-5 minutes, or until the appearance of purple dots. Prolonged incubation periods (*i.e.*, longer than 15 minutes) resulted in false positives. The reaction was terminated by flushing the blots with distilled water.

2.15 Antibody Production

2.15.1 Expansion of Hybridomas *in vitro*

Hybridomas which were actively growing were transferred from 96-well plates to 24-well plates. These were further expanded into $25cm^2$ flasks and $75cm^2$ flasks. Supernatant was harvested, any cells which may have been present were removed by centrifugation and the remaining supernatant stored at -20°C.

2.15.2 Production of Antibody Rich Ascitic Fluid

Balb/c mice were primed by injection of 0.5ml of incomplete Freud's Adjuvant intraperitonially (i.p.). 24 hours later, hybridoma cells from growing homogenous cultures were harvested, centrifuged (2000rpm, 10 minutes) and the supernatant removed. The pellet was resuspended in $DMEMS_0$ to give a final cell concentration of 1×10^6 /ml. 0.5ml of this was injected into each of the primed mice. Ascitic fluid in the form of abdominal swelling became apparent after 10 to 20 days. The mice were sacrificed and fluid drained. Cells were removed by centrifugation and the supernatant was further purified or stored at -20°C.

2.16 Antibody Purification

2.16.1 Saturated Ammonium Sulphate

Antibody was purified according to the method of Hudson and Hay (1980). Ascitic fluid or rabbit serum was purified initially by ammonium sulphate precipitation. The pH of the serum was adjusted to 7.2 and an equal volume of cold 100% saturated ammonium sulphate (SAS) was added dropwise while stirring. The solution was then stirred for 1 hour in an ice bath. The precipitated immunoglobulin was collected by centrifugation (4°C, 5000rpm for 20 minutes). The pellet was resuspended in 50% SAS and recentrifuged. This step was repeated. The pellet was then resuspended in a minimum volume of PBS and exhaustively dialyzed against PBS at 4°C.

2.16.2 Affinity chromatagraphy

0.4g of activated Sepharose (Pharmacia) was suspended in 3ml of 1mM HCl and transferred to a sintered glass funnel. It was washed over 15 minutes, with a further 80ml of 1mM HCl , followed by 2ml of coupling buffer (0.1M $NaHCO_3$ with 0.5M NaCl, pH 8.3). rFVIII (0.8mg/ml), was diluted in coupling buffer to a total volume of 2.8ml and added to the washed gel. This gave a buffer:gel ratio of 2:1. The gel and ligand were then mixed in a shaking waterbath at room temperature for 2 hours. The mixture was returned to the sintered glass funnel and washed with 5ml of coupling buffer. It was then transferred to a conical flask containing 3 ml of blocking buffer (0.2M glycine in coupling buffer, pH 8.3) and left for 1 hour at room temperature. To remove any non-covalently bound protein, the gel was washed with 3 cycles of alternating pH buffers (0.1M sodium acetate with 0.5M NaCl, pH 4.5 and 0.1M Tris-HCl with 0.5M NaCl, pH 8.0). The gel was then suspended in PBS (0.15M, pH 7.2) and used to pour a column in a 1ml syringe barrel with a small piece of cotton wool as a plug. PBS was added to prevent the gel drying out.

The column was washed with PBS to remove any protein that may have leached off. Fractions were monitored for protein content using the BCA protein assay as described in Section 2.7. Antibody sample was slowly added to the column, followed by washing with PBS. 1ml fractions were collected and protein concentration monitored using the BCA protein assay.

Dissociation buffer (0.1M glycine-HCl, pH 2.5) was added and the column clamped and left for 15 minutes. 1ml fractions were collected and monitored for protein as before. The pH of antibody-containing fractions was returned to physiological levels by addition of 100µl of 1M Tris-HCl, pH 10.5.

The column was regenerated by washing with 5ml of each of 0.1M Tris-HCl with 0.5M NaCl, pH 8.5, 0.1M sodium acetate, pH 4.5 and 0.15M PBS, pH 7.2. The column was stored at 4°C in PBS containing 0.02% (w/v) sodium azide.

2.16.3 Protein A Affinity Chromatography

Antibodies were also purified by affinity chromatography on immobilized Protein A. The pre-poured column was equilibrated with 10mM Tris, pH 7.5 (binding buffer). The serum was diluted with binding buffer to within the binding range of the gel and 2ml of this was applied to the column. The column was washed with binding buffer to remove unbound protein. 1ml fractions were collected and the protein concentration monitored using the BCA protein assay (Section 2.7). Bound IgG was eluted with 0.1M glycine buffer, pH 2.5. The pH of the IgG fractions was returned to physiological levels by addition of 100µl of 1M Tris, pH 7.5. Pooled fractions containing eluted immunoglobulins were desalted on a 10ml Sephadex G-25 column.

2.16.4 Gel Filtration

Monoclonal antibodies were also purified by gel filtration through Sepharose 6B (Pharmacia). A 0.9cm x 24cm column with a bed height of 18cm was used, with a flow rate of approximately 0.4ml/minute. 1ml of antibody solution was applied to the column which had been equilibrated with 0.15M PBS, pH 7.2. 1ml fractions were collected and protein content monitored using the BCA protein assay (Section 2.3.5). The immunoreactivity of each fraction was assessed by Dot Blot (Section 2.9.1).

2.17 Isotyping Analysis

(i) Enzyme-linked Immunosorbent Assay (ELISA)

An isotyping kit (Sigma, UK) was used. 2 formats were used: indirect and capture ELISAs. In the indirect system, test antibody was diluted in PBS (0.15M, pH 7.2) to a concentration of 1µg/ml, 100µl applied to wells of a microtitre plate and the plate incubated at 37°C for 1 hour. The wells were washed in PBS containing 0.05% (v/v) Tween 20 and the goat anti-mouse isotype-specific antibodies (diluted in PBS) were added (100µl). Plates were further incubated at room temperature for 30 minutes before being washed as before, and 100µl of horseradish peroxidase-labelled rabbit anti-goat IgG applied for 30 minutes at room temperature. Following washing, 100µl of substrate [10mg o-phenylenediamine and 5µl H_2O_2 in 25ml citrate buffer (0.5M Na_2HPO_4 with 0.1M citric acid, pH 4.5) was added and incubated for 30 minutes at room temperature. Colour was allowed to develop at 37°C for 20 minutes.

In the capture ELISA, the isotype-specific antibodies were coated onto the plate (10µg/ml, 100µl). This was then incubated at 37°C for 1 hour, washed as above, and test antibody added (100µl). The plates were further incubated, (1 hour at room temperature) washed and 100µl of alkaline-phosphatase-labelled goat anti-mouse IgG added.

This was then incubated at room temperature for 30 minutes, washed and substrate added (1mg/ml BCIP in AMP buffer). Colour developed over 20 minutes at 37°C.

(ii) Agglutination

A Serotec antibody isotyping kit was used. The kit is based on the principle of cell agglutination. A highly specific antibody is coupled to sheep red blood cells. This recognises and binds to the particular isotype to which it is directed. This "binding" forms a lattice on the bottom of the microtitre plate well and is termed agglutination. 30µl of diluted antibody (in 0.15M PBS, pH 7.2) was pipetted into each of 8 wells of a 96-well "U" bottomed microtitre plate. 30µl of each specific isotyping reagent was added and the solutions mixed. The plate was covered and incubated on a flat surface for 1 hour at room temperature. A part or full carpet of agglutination indicated a positive reaction. A small red circle or "button" was indicative of a negative reaction. Here, no agglutination occured, so the reagents fall to the bottom of the well forming the red circle.

2.18 Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970) using a Tris-Glycine buffer system in the presence of sodium dodecyl sulphate (SDS). Stock solutions of 1.5M Tris-HCl, pH 8.8, 0.5M Tris-HCl, pH 6.8, 30% (w/v) acrylamide containing 0.8% bisacrylamide, 10% (w/v) SDS, 1% (w/v) ammonium persulphate (APS) freshly prepared, were used to prepare both resolving (10% (w/v) and 7.5% (w/v) acrylamide) and stacking gels (5% (w/v) acrylamide) according to Table 2.18.1.

Samples for electrophoresis were dissolved in sample buffer consisting of 0.08M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 0.2% (w/v) bromophenol blue (2-mercaptoethanol (0.5% (v/v)) was included when reducing conditions were required). and boiled at 100°C for 3 minutes. 20µl was applied to each well on the gel and electrophoresis carried out in electrode buffer (0.192M glycine, 25mM Tris-HCl, 0.1% (w/v) SDS, pH 8.3) at a constant current of 20mA/gel until the dye front approached the bottom of the gel.

Gels were stained for 45 minutes in 0.5% (w/v) Coomassie Brilliant Blue in a 5:1:4 solution of water:acetic acid:methanol. Destaining was carried out overnight in a 5:1:4 solution of water:acetic acid:methanol.

Stock Solution	Resolving gels		Stacking gels
	7.5%	10%	5%
30% (w/v) Acrylamide	3.8ml	5.0ml	0.8ml
Distilled Water	8.0ml	6.8ml	3.6ml
1.5M Tris-HCl, pH 8.8	3.0ml	3.0ml	—
0.5M Tris-HCL, pH 6.8	—	—	0.5ml
SDS	150µl	150µl	50µl
TEMED	9µl	7.5µl	5µl
APS	60µl	50µl	17µl

Table 2.18.1 Resolving and stacking gels used in Electrophoresis and Western blotting.

2.19 Western Blotting

The method was essentially that of Towbin *et al.* (1979). rFVIII (120µg/ml) was incubated with human thrombin (1U/ml) at 37°C for 5 minutes. It was then dissolved in sample buffer and SDS-PAGE, as described in Section 2.18, carried out on a 0.75mm thick, 7.5% resolving gel (Table 2.18.1) using a LKB Mini-gel system. The gel was washed in transfer buffer (25mM Tris-HCl, 192mM glycine, pH 8.3) and placed on a sheet of nitrocellulose (NC) paper (0.45µm pore size; Schleicher and Schuell) cut to the appropriate size and presoaked in transfer buffer. The NC-gel sandwich was then placed between sheets of filter paper (Whatman 3M) soaked in transfer buffer and placed into a blotting tank (Biorad) with the NC facing the anode. Electrophoretic transfer was carried out at 15V (with an upper limit set at 340mA) for 30 minutes.

Blots were removed, washed in TBS (10mM Tris-HCl, 150mM NaCl, pH 7.5) and free binding sites on the NC blocked using 1% (w/v) BSA in TBS. After 2 hours incubation at room temperature with agitation, the blots were washed 3 times as before and monoclonal antibody, diluted in blocking buffer, added. The blots were incubated on a shaking waterbath for 2 hours at room temperature. The NC was washed 5 times in TBS to remove unbound antibody, and alkaline phosphatase-labelled anti-mouse IgG, (Sigma, UK), diluted in blocking buffer, was added. This was incubated, with shaking, for 2 hours at room temperature. The blots were washed 5 times as before, with the final wash in alkaline phosphatase buffer (100mM Tris-HCl, with 100mM NaCl and 5mM $MgCl_2$, pH 9.5). Substrate solution, consisting of 66µl nitroblue tetrazolium (0.5g in 10ml 70% (v/v) dimethyl formamide (DMF)), and 33µl 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (BCIP) (0.5g in 10ml 100% (v/v) DMF) in 10 ml alkaline phosphatase buffer. Colour development was allowed to proceed at room temperature for no more than 30 minutes. The reaction was stopped by addition of 0.15M PBS, pH 7.2 containing 20mM EDTA.

2.20 HPLC Analysis of antibodies

10µl of purified antibody diluted in PBS (0.15M, pH 7.2) was applied to a Waters Protein Pak 300SW 10µm column pre-equilibrated with mobile phase buffer (0.1M phosphate buffer pH 7.2) (filtered and degassed). A flow rate of 0.5ml/minute was used and the presence of protein was monitored by reading absorbance at 280nm.

2.21 Conjugation of Horseradish peroxidase to anti-rFVIII IgG

The method of Tijssen and Kurstak (1984) was followed. There are three steps involved in the procedure (i) activation of horseradish peroxidase (HRP) (ii) conjugation of activated HRP to the immunoglobulin (iii) purification of the conjugate.

(i) Activation of HRP: 2mg of HRP was dissolved in 5mls of freshly prepared 100mM $NaHCO_3$. 5mls of 8mM $NaIO_3$ was added to this, the tube sealed and stored in the dark at room temperature for 2 hours.

(ii) Conjugation: 1.5mls of a 5mg/ml solution of anti-rFVIII antibody was dialysed against 20mM Na_2CO_3 buffer, pH 9.5 overnight at 4°C. The solution was then added to 1ml of activated HRP. The mixture was transferred to a glass wool-plugged 1ml pasteur pipette with the tip sealed by a flame and dry Sephadex G-25 (5mg) added. The pipette was covered with foil and incubated at room temperature for 3 hours. The reaction mixture was eluted from the column and 1/20 volume (100µl) of freshly prepared $NaBH_4$ (5mg/ml in 0.1mM NaOH) added. This was incubated for 30 minutes at room temperature before a further 1/10 volume (200µl) of $NaBH_4$ (freshly prepared) was added. The mixture was incubated for 1 hour at 4°C.

(iii) Purification of the conjugate: free HRP is soluble in ammonium sulphate up to 70-80% (w/v) saturation. Therefore, an equal volume of SAS was added gradually with stirring and the mixture incubated for 1 hour. The precipitate was collected by centrifugation (5000rpm, 20 minutes), washed with 50% SAS and recentrifuged. The precipitate was suspended in a minimum volume of PBS and dialysed against PBS overnight at 4°C.

2.21.1 Measurement of the enzymic activity of the antibody/enzme conjugate

The conjugate was diluted in PBS (1:1000), and 20µl of this was added to 3mls of freshly prepared substrate solution (10mg of o-phenylenediamine in 25mls of citrate/phosphate buffer (0.1M sodium citrate, 0.1M disodium hydrogen phosphate, pH 4.5) with 5µl of H_2O_2). The mixture was covered in foil and incubated at room temperature. 200µl samples were withdrawn after intervals of 0, 2, 5, 10, 15, 20, 30 minutes and transferred to duplicate wells of a microtitre plate. The reaction was stopped by addition of 25µl of 20% (v/v) H_2SO_4. The plate was stored in the dark between sample additions. Absorbance at 492nm was read on a Titertek Twinreader Plus.

The conjugate was stored at -20°C in the presence of 50% (v/v) glycerol.

2.22 Binding of antibodies to rFVIII

The effect of antibody binding on the procoagulant activity of rFVIII was examined. rFVIII (0.66mg/ml) was incubated with equal volumes of a series of antibody concentrations at room temperature and 37°C for 2 hours. The activity of each sample was then assayed.

Samples were then subjected to denaturation studies as described in Section 2.5.

2.23.1 Enzyme-linked Immunosorbent Assay

5µg/ml of rFVIII in carbonate/bicarbonate buffer (0.03M $NaHCO_3$, 0.01M Na_2CO_3, pH 9.6) was coated onto the surface of duplicate wells of a microtitre plate (Nunc, Denmark) (1 hour incubation at 37°C). The plates were washed 3 times in PBS (0.15M, pH 7.2) and 3 times in PBS containing 0.05% (v/v) Tween-20 (PBS-Tween) with the final wash in PBS. Free binding sites were blocked with 200µl of a solutiom of 1% (w/v) BSA in PBS. After an hour at 37°C, the plate was washed as above, and 100µl of primary antibody diluted in blocking buffer added. The plate was incubated at 37°C for 1 hour. After washing, alkaline phosphatase-labelled anti-rabbit antibody, diluted 1:4000 in blocking buffer, was added (100µl) and incubated for 1 hour at 37°C. The plate was washed, and substrate added (1mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer, pH 9.5). After 30 minutes at 37°C, absorbance at 620nm was read on a Titertek Twinreader Plus.

2.23.2 IgG ELISA

10µg/ml of anti-rFVIII antibody in carbonate/bicarbonate buffer (0.03M $NaHCO_3$, 0.01M Na_2CO_3, pH 9.6) was coated onto the surface of duplicate wells of a microtitre plate (Nunc, Denmark) (1 hour incubation at 37°C). The plates were washed 3 times in PBS (0.15M, pH 7.2) and 3 times in PBS containing 0.05% (v/v) Tween-20 (PBS-Tween) with the final wash in PBS. Free binding sites were blocked with 200µl of a solution of 1% (w/v) BSA in PBS. After an hour at 37°C, the plate was washed as above, and 100µl of alkaline phosphatase-labelled anti-rabbit antibody, diluted 1:4000 in blocking buffer, was added and incubated for 1 hour at 37°C. The plate was washed, and substrate added

(1mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer, pH 9.5).

After 30 minutes at 37°C, absorbance at 620nm was read on a Titertek Twinreader Plus.

2.24 Concentration of samples

Samples of rFVIII and anti-rFVIII antibodies were concentrated using microconcentrators (Amicon). 425µl of a 0.05mg/ml solution of rFVIII or 300µl of a 0.1mg/ml solution of anti-rFVIII monoclonal antibody, was applied to a microconcentrator.

These were centrifuged in a fixed-angle centrifuge (Beckman J2-21) at 5000rpm for 30 minutes. 60µl of rFVIII and 40µl of antibody were recovered. The protein concentration of the samples before and after concentration were assay using the BCA protein assay (Section 2.7).

2.25 Thrombin Activation of FVIII

100µl of Coag Cal N calibration plasma or normal human plasma in plastic test tubes was reacted with 10U/ml of thrombin (Sigma, UK) and incubated at 37°C for 90 seconds. 100µl of heparin was added to a final concentration of 100U/ml to prevent the formation of a clot. This was then added to wells of a microtitre plate which had been coated with anti-rFVIII antibodies, and an ELISA carried out as described in Section 2.23.1.

2.26 ELECTROCHEMICAL METHODS

2.26.1 Electrode Preparation

Before use, the bare glassy carbon electrode was polished with alumina slurry, after which it was sonicated in distilled water and allowed to dry in air. Modification of the electrode was achieved via drop coating of a 10µl aliquot of the appropriate concentration of rFVIII in Nafion onto the electrode surface. This was allowed to dry in air for 15 minutes and then further dried by gentle heating under an air gun.

2.26.2 Amperometric Voltammetry

10mls of 0.1M phosphate buffer (0.02M KH_2PO_4, 0.08M K_2HPO_4, pH 7.3) was placed in the reaction cell and purged with oxygen-free nitrogen for 20 minutes. The modified electrode was introduced into the cell and connected to the potentiostat. DC constant voltammetry was carried out at a potential of -0.03 vs saturated calomel electrode (SCE). The hydroquinone mediator (0.1M in phosphate buffer) was injected into the cell to a final concentration of 2mM immediatly prior to use. Hydrogen peroxide substrate was then added (to a final concentration of 1mM) and the reduction current of the mediator measured.

2.27 Partial purification of FVIII samples and separation of the FVIII/vWF complex

This method was based on that described by Rick and Hoyer (1973). The FVIII/vWF complex was separated in the presence of high ionic strength buffers by filtration through a Sepharose 6B gel filtration column. A 0.9cm x 24cm column with a bed height of 17cm was used, with a flow rate of approximately 0.4ml/minute. 0.5ml of Coag Cal N calibration plasma or normal human plasma was applied to the column which had been equilibrated with 0.1M imidazole buffer, pH 7.2 containing 0.24M $CaCl_2$ and 1ml fractions were collected. The presence of FVIII in each fraction was measured using the FVIII chromogenic assay (Section 2.3), along with the protein content (measured by the BCA protein assay (Section 2.7)).

CHAPTER 3

CHEMICAL MODIFICATION OF rFVIII

3.1 Introduction

The use of chemical cross-linkers to modify protein structure is a widespread and powerful technique. The chemistry of such reactions is well understood allowing for specific side chains to be targeted. Such modifications can be used to enhance the stability of a protein by introducing inter- and intra-molecular cross-links. These "bridges" act by reinforcing the active conformation of the protein, decreasing the entropy and reducing the rate of denaturation (Wong and Wong, 1992).

Bifunctional cross-linking reagents are the most successfully used cross-linkers. They can be divided into three main subgroups (Ji, 1983):

(i) zero-length

(ii) homobifunctional

(iii) heterobifunctional

Zero-length cross-linkers induce direct conjugation between two chemical groups, without introducing extrinsic material. Examples include carbodiimides and carbonyldiimidazole, which induce condensation of a carboxyl and amine group forming an amide bond (Ji, 1983).

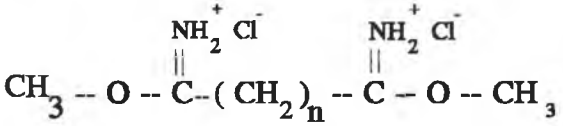
Homobifunctional cross-linkers contain two identical functional groups, separated by a spacer of varying length. The two functional groups react with similar amino acid side-chains, forming a link of defined length.

Heterobifunctional cross-linkers contain two different functional groups at either end of the molecule.

Acylating and alkylating agents are the most commonly used functional groups (Wong and Wong, 1992). The acylating agents are generally considered to be amino-specific while the alkylating agents are thiol-specific. Thus, homobifunctional reagents contain either acylating or alkylating agents, whereas heterobifunctionals contain a combination of both of these types. Examples of homobifunctional agents are the amino-specific bis-imidate series (Ji, 1983) and the thiol-specific bis-maleimides (Weston *et al*, 1980) while succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate (SMCC) (Yoshitaki *et al*, 1982) is an example of a heterobifunctional cross-linker (Figure 3.1).

FVIII is stabilised *in vivo* by the large multimeric protein von Willebrand factor (vWF). In its absence FVIII becomes highly unstable. The effects of cross-linkers and other protein modifying reagents on rFVIII were investigated.

Bis-imidates

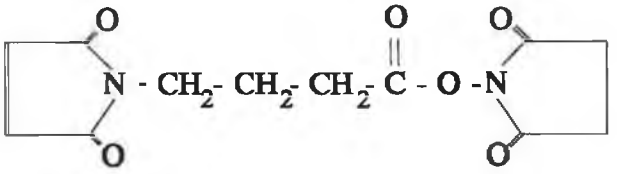


n = 4 Dimethyl adipimidate

n = 5 Dimethyl pimelimidate

n = 6 Dimethyl suberimidate

Heterobifunctionals

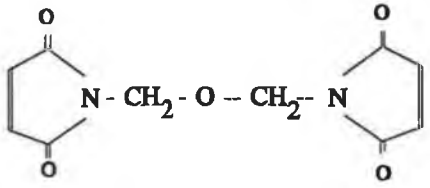


N- (γ-maleimidobutyryloxy) succinimide

(GMBS)

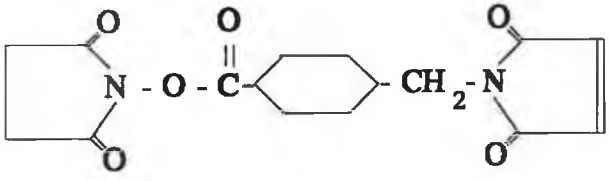
Spacer arm = 10.2A

Bis-maleimides



Bis (maleimido-methyl) ether

(BMME)



Succinimidyl 4-(N-maleimidomethyl)

cyclohexane carboxylate

(SMCC)

Spacer arm = 11.6A

Figure 3.1 Bifunctional cross-linking reagents

Bis-imidates and Bis-maleimides are examples of homobifunctional reagents

3.2 Miniaturisation of FVIII chromogenic assay

The Baxter Factor VIII Chromogenic assay was used for the photometric determination of FVIII activity. The assay is based on the detection of Factor X (FX). Thrombin activates FVIII in the sample and this in turn acts as a cofactor, along with Ca^{2+} and phospholipids, in the activation of FX by activated Factor IX (FIXa). Activated FX (FXa) then hydrolyses a p-nitroanilide substrate releasing a coloured product, p-nitroaniline, which can be detected photometrically at 405nm. The colour is directly proportional to FXa activity and, thus, to FVIII activity in the sample (Figure 3.2.1).

The kit was capable of performing 40 macroassays and in order to maximise efficiency and save reagents, it was miniaturised. The volumes of each component were reduced by a factor of 4, to 25µl of sample, 25µl of FX and FIXa reagents and 125µl of substrate, and the assay carried out in a 96-well microtitre plate held at 30°C. The absorbances were read on a Titretek Twinreader Plus. Using this method, each kit was capable of performing approximately 160 assays.

Reagents were added to the plate by means of a multichannel pipette. An automatic dispenser was incorporated into the machine, but the time taken to dispense automatically into each well was longer than the time period of the assay.

The advantages of such a microassay include the capacity for a large number of samples, ease of performance of replicate assays, economy in its use of reagents, small sample volumes, a controlled temperature environment and automatic mixing.

Using the kinetic assay procedure with Coag Cal N calibration plasma and FVIII-deficient plasma as positive and negative controls respectively, FVIII activity was calculated as follows:

% FVIII activity of calibration plasma = A (= 107%)

ΔA/min calibration plasma = C

ΔA/min zero standard = B

ΔA/min sample = S

Calibration factor = F

$$\frac{A}{C - B} = F$$

% FVIII activity in sample:

$$= (S - B) \times F$$

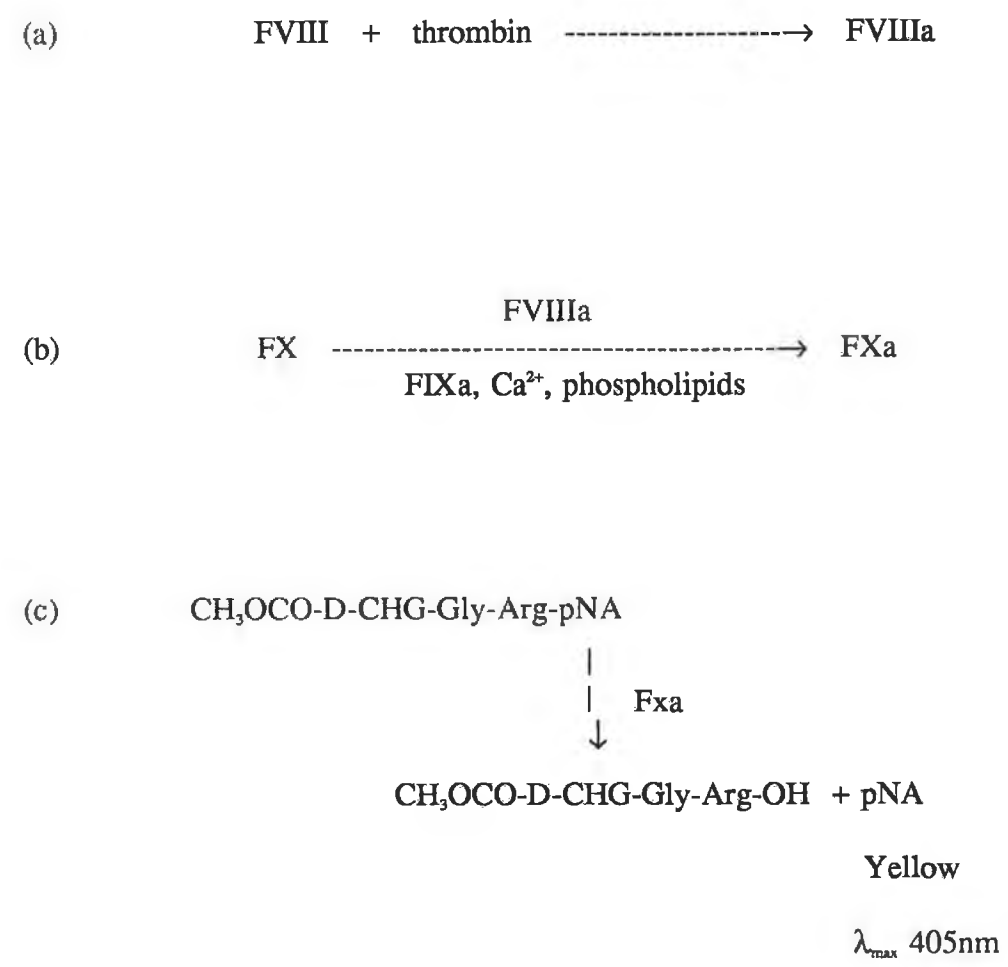


Figure 3.2.1 The chromogenic assay for the detection of FVIII activity.

FVIII was initially activated by proteolytic cleavage carried out by thrombin (a). Activated FVIII (FVIIIa) then acted as a cofactor in the enzymic activation of Factor X (FX) by activated Factor IX (FIXa) in the presence of Ca^{2+} and phospholipids (Pl) (b).

Substrate was added and hydrolysed by FXa (c), forming a coloured product which can be detected at 405nm.

The rate of increase in absorbance for each dilution of Coag Cal was linear over 2 minutes up to a dilution of 1:124 (equivalent to less than 1% FVIII activity) (Figure 3.2.2). The change in absorbance for each dilution is shown in Figure 3.2.2.

3.3 Accelerated Storage studies

(i) The effects of Calcium on rFVIII activity.

The concentration of Ca^{2+} has an enormous effect of FVIII activity as shown in Figures 3.3.1 and 3.3.2. Any concentration greater than 5mM causes a marked decrease in FVIII activity.

An immediate decrease in FVIII activity was noticable in the presence of 10mM Ca^{2+}. At temperatures of 27°C and above, there was no activity recorded after Day 1, whereas at these temperatures the control exhibited activity up to Day 3. At 4°C no activity was recorded after Day 3, compared with the control (5mM Ca^{2+}) which was active until Day 7.

At -20°C and-70°C, activity was again decreased as compared with the control.

No activity was recorded in any of the samples incubated in 20mM Ca^{2+}.

These results show that the concentration of Ca^{2+} in the buffer is critical for the expression of FVIII activity. Any concentration above 5mM caused a pronounced decrease and eventual loss of FVIII procoagulant activity.

(ii) The effects of albumin on rFVIII activity.

Results from this study indicate that in the presence of additional protein, FVIII activity is maintained over a number of temperatures for a longer period of time. At the higher temperatures (37°C and 45°C), activity persisted longer in the test samples as compared with the control (no excess protein).

The period over which activity remained in the test, but not in the control, depended on the amount of added protein *i.e.* the more protein present, the longer the activity persisted. (Figure 3.3.3)

The presence of protein in the buffer, therefore, appears to stabilise rFVIII.

This is the normal method of storage of FVIII for clinical applications in the treatment of haemophilia A.

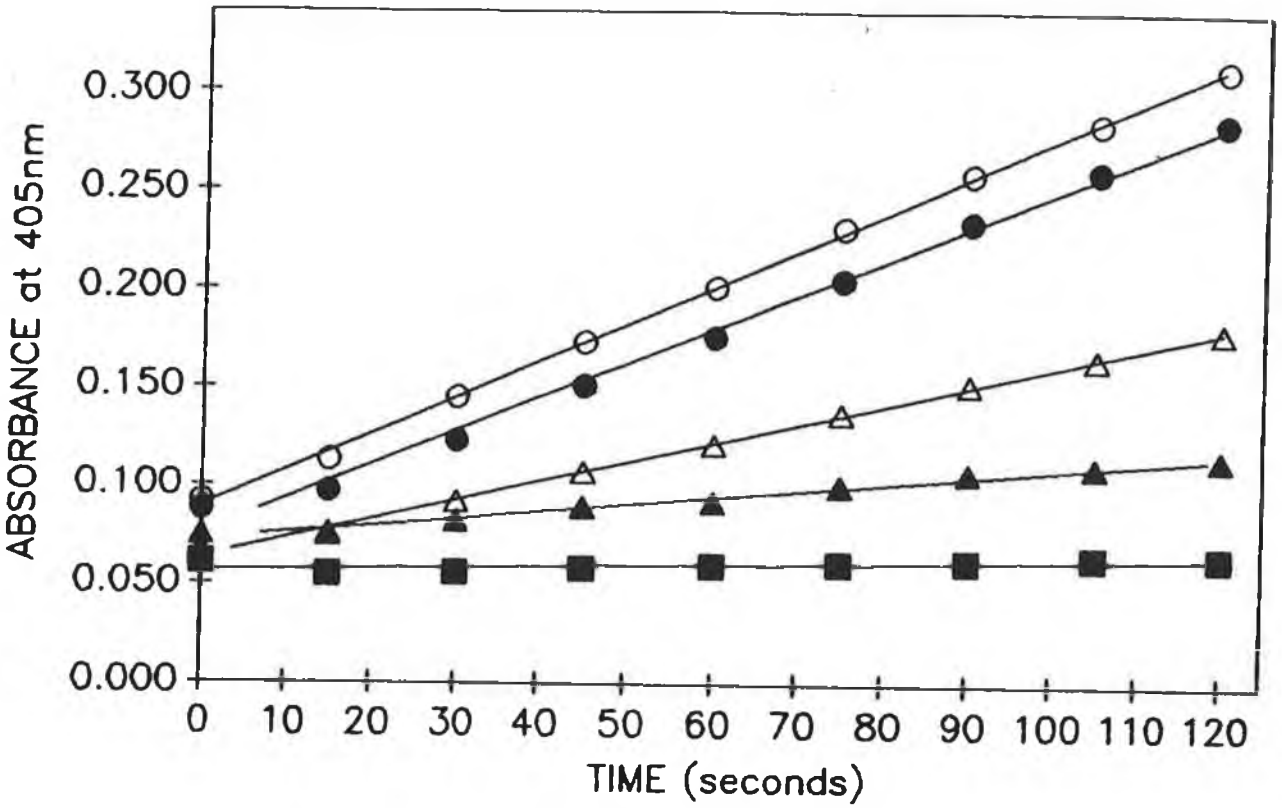


Figure 3.2.2 Time course of the chromogenic assay for the detection of FVIII in a microtitre plate. A series of dilutions of Coag Cal N calibration plasma (*i.e.*, normal human plasma) were prepared in 0.9% (w/v) NaCl (O, 1:21 dilution; ●, 1:31 dilution; Δ, 1:62 dilution; ▲, 1:124 dilution; ■, FVIII-deficient plasma) and assayed. Absorbance was measured at 405nm at 15 second intervals over 2 minutes. Each point represents the mean of quadruple duplicates.

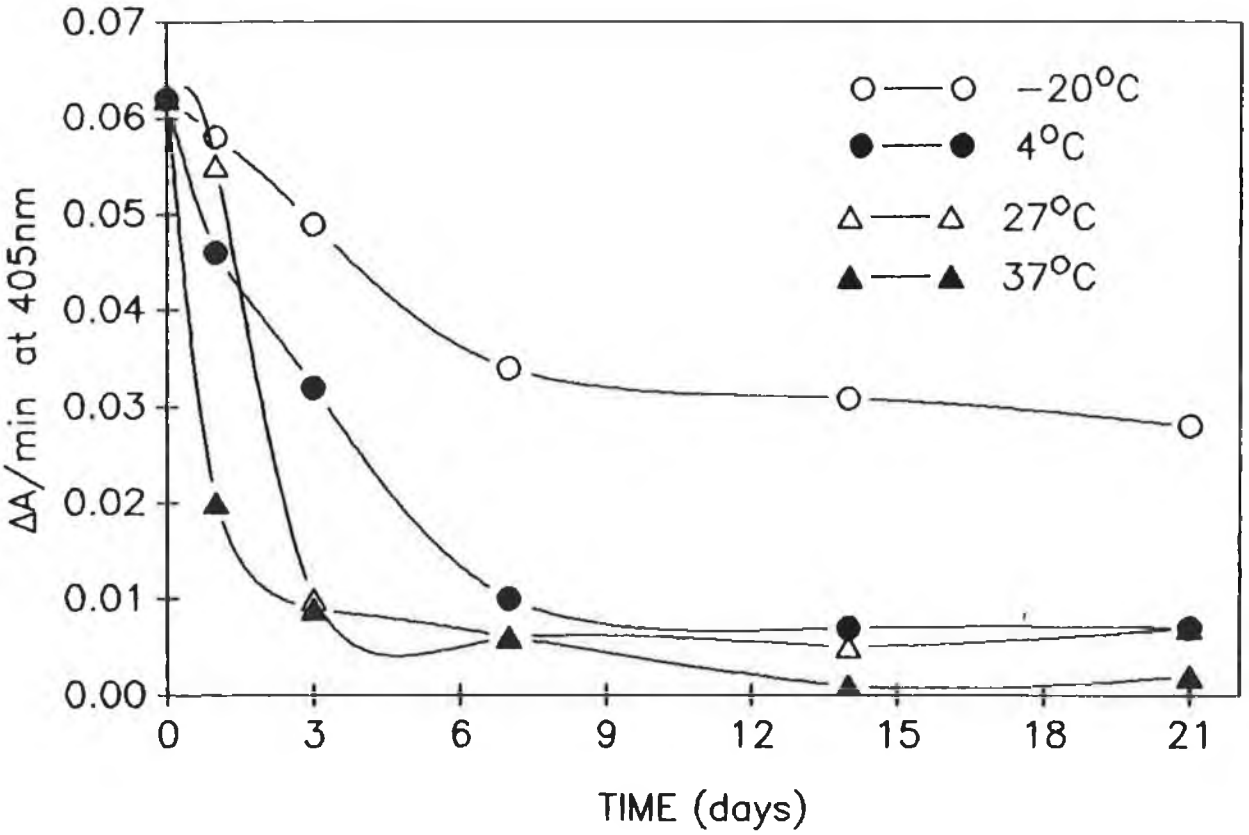


Figure 3.3.1 Accelerated storage studies on rFVIII in the presence of 5mM Ca^{2+}.

rFVIII was incubated in Tris-HCl buffer containing 5mM Ca^{2+} at the indicated temperatures. Samples were withdrawn at regular intervals and assayed for FVIII activity. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

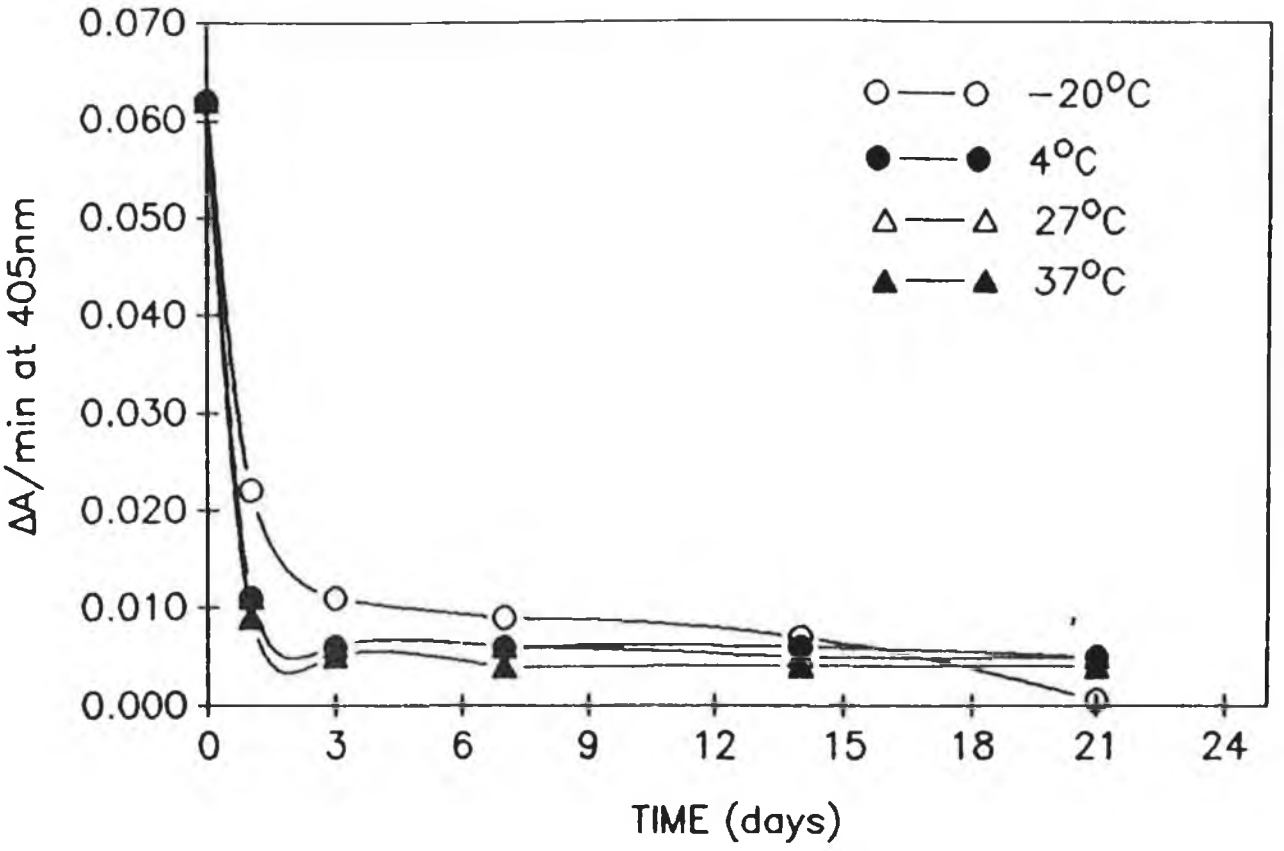


Figure 3.3.2 Accelerated storage studies on rFVIII in the presence of 10mM Ca^{2+}.

rFVIII was incubated in Tris-HCl buffer containing 10mM Ca^{2+} at the indicated temperatures. Samples were withdrawn at regular intervals and assayed for FVIII activity. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

3.4 Diluent for FVIII assay

0.9% (w/v) NaCl and FVIII-deficient plasma were compared as diluents for the FVIII assay. The results are shown in Table 3.4. When assayed in 0.9% (w/v) NaCl, the rate of increase in absorbance at 405nm for each dilution was higher than the corresponding dilution in FVIII-deficient plasma. For this reason and for economy and ease of handling, 0.9% (w/v) NaCl was chosen as diluent for all FVIII activity assays, with FVIII-deficient plasma being used as a negative control.

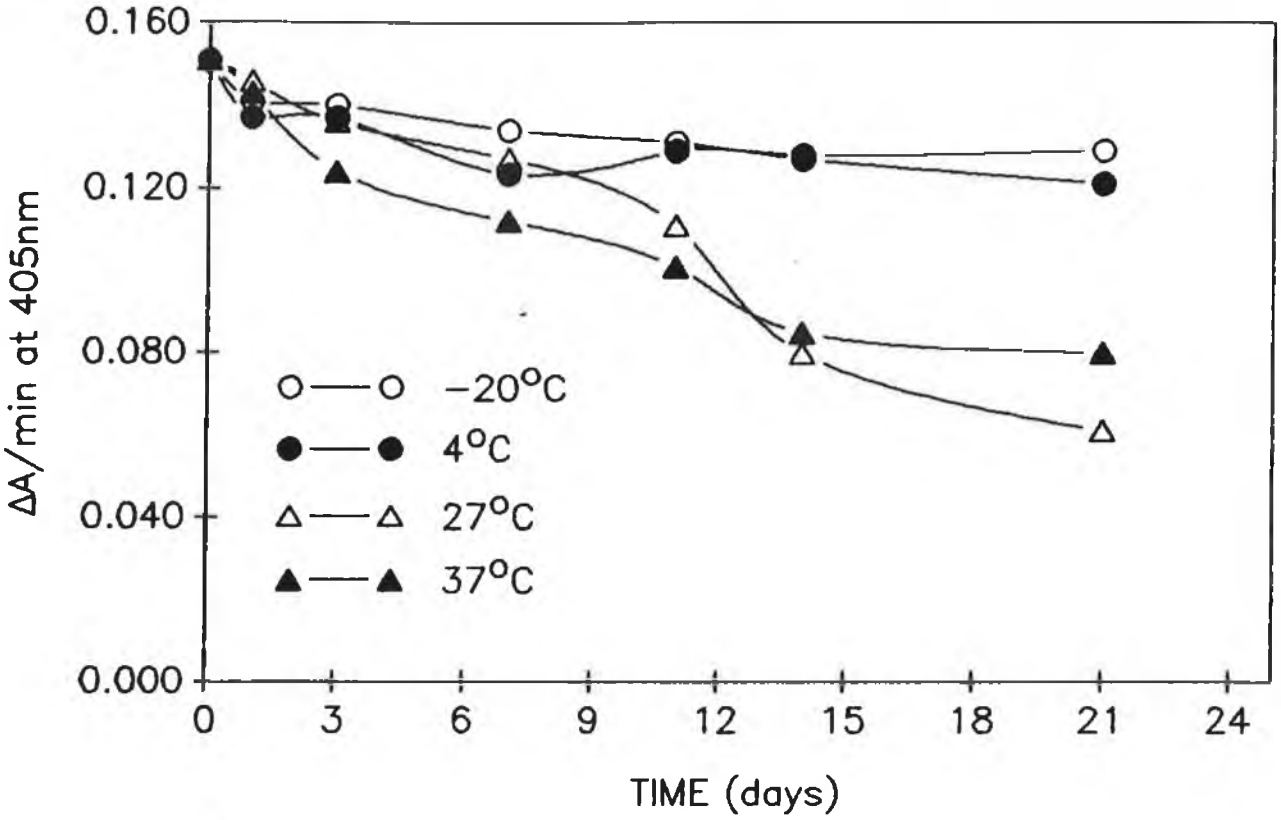


Figure 3.3.3 Accelerated storage studies on rFVIII in the presence of additional protein.

rFVIII was incubated in Tris-HCl buffer containing 20mg/ml bovine serum albumin (BSA), at the indicated temperatures. Samples were withdrawn at regular intervals and assayed for FVIII activity. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

Dilution of Coag Cal N	ΔA/min ± S.D. at 405nm in FVIII-deficient plasma	ΔA/min ± S.D. at 405nm in 0.9% (w/v) NaCl
1:21	.024 ± .002	.081 ± .006
1:31	.022 ± .003	.073 ± .005
1:62	.018 ± .001	.041 ± .002
1:124	.013 ± .001	.020 ± .001
Positive control	.105 ± .011	.125 ± .020
Negative control	.002 ± .000	.005 ± .001

TABLE 3.4 Comparison between FVIII-deficient plasma and 0.9% (w/v) NaCl as diluent for FVIII chromogenic assay.

Dilutions of Coag Cal N were assayed in each diluent and the absorbance at 405nm was measured at 15 second intervals over 2 minutes. The change in absorbance per minute (ΔA/min) was then calculated.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

3.5 Stability of rFVIII in Tris-HCl and Barbital buffers

rFVIII, in both Tris-HCl and Barbital buffers, was assayed to ensure that neither buffer interfered with the assay in any way. Denaturation studies, as described in Section 2.5, were then carried out on rFVIII in each buffer. Chemical modification experiments (except those involving amino-specific reagents) were carried out with rFVIII in Tris-HCl buffer. An alternative buffer was used for amino-specific reagents, as the quarternary amino groups in Tris could interfere with the modification process. Barbital buffer was chosen as the alternative.

Figure 3.5 shows that while rFVIII was denatured at similar rates in both buffers, the residual FVIII activity appeared to be higher in Trsi-HCl buffer than in barbital buffer.

Therefore, it was concluded that these buffers were suitable for use in the modification experiments.

3.6 Chemical Cross-linking of rFVIII

A wide range of cross-linkers were used, including both homobifunctional and heterobifunctional reagents. The concentrations of the cross-linkers used were in relation to the moles of lysine (approximately 40) or cysteine (23) residues (Vehar *et al.*, 1984) present in the recombinant FVIII molecule. Thus, the concentrations were in the mM to μM range.

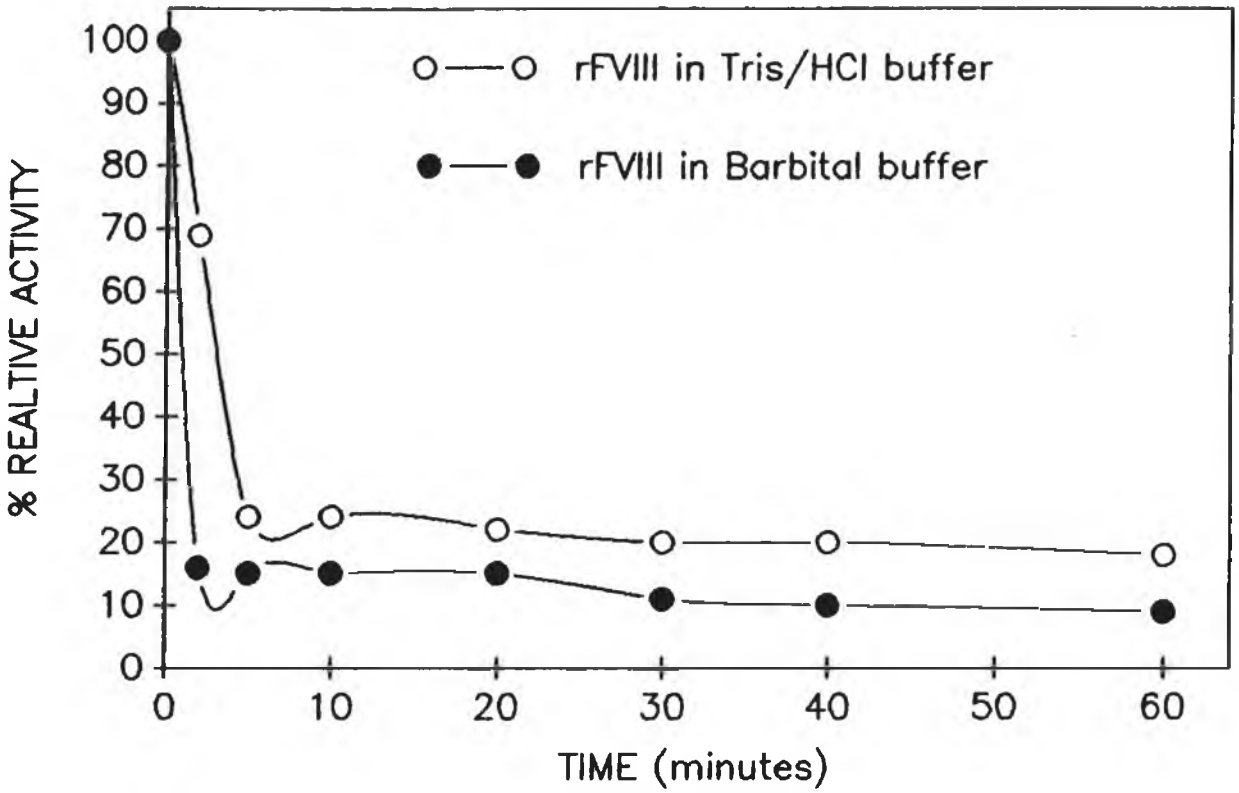


Figure 3.5 Comparison between the rate of denaturation of rFVIII in Tris-HCl and barbital buffers.

rFVIII was diluted in each buffer and subjected to and incubated at 55°C for 1 hour. Samples were withdrawn at regular intervals and assayed for FVIII activity. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

3.6.1 Amino-specific reagents

3.6.1.1 Bis-imidates

Three of the bis-imidate series of cross-linkers, dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP) and dimethyl suberimidate (DMS) (Ji, 1983), were reacted with rFVIII in barbital buffer. They contained 4, 5 and 6 methyl groups, respectively between the reactive ends of the molecule (Figure 3.1). The reaction resulted in an almost complete loss of FVIII activity in the test samples, as compared with the control rFVIII (Table 3.6.1.1).

Denaturation studies were carried out on the control and test samples containing rFVIII and 40μM DMS and rFVIII with 40μM DMA, to determine whether there was any increase in thermostability (Figure 3.6.1.1). No increase was detected.

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII control	0.172 ± .010	100
rFVIII + 40μm DMA	0.011 ± .001	6
rFVIII + 40μM DMP	0.010 ± .001	6
rFVIII + 40μM DMS	0.010 ± .001	6
Positive control	0.132 ± .007	
Negative control	0.004 ± .001	

TABLE 3.6.1.1 Reaction of rFVIII with three of the amino-specific bis-imidate series of cross-linkers with rFVIII. rFVIII was reacted with the bis-imidates on ice for 30 minutes. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

The addition of any of the cross-linkers caused almost complete loss of FVIII activity.

DMA: Dimethyl adipimidate

DMP: Dimethyl pimelimidate

DMS: Dimethyl suberimidate

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

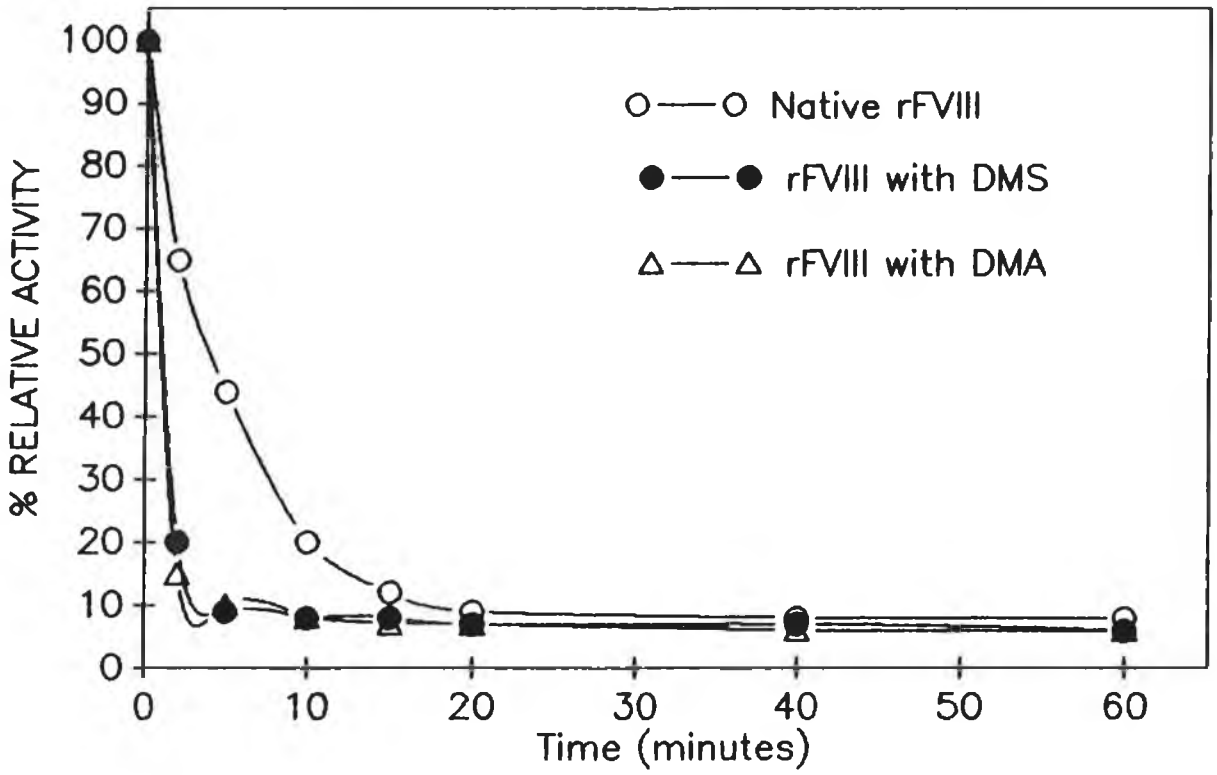


Figure 3.6.1.1 Denaturation of native rFVIII, dimethyl suberimidate-(DMS) and dimethyl adipimidate-(DMA) modified rFVIII.
Samples of rFVIII and rFVIII modified with 40μM DMA and 40μM DMS were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity. Relative percentage activity was calculated as the percentage of activity at T0, remaining at the particular time.

3.6.1.2 Glutaraldehyde

The addition of glutaraldehyde (GA), a non-specific cross-linker (Steck, 1972), in concentrations greater than 1mM resulted in loss of rFVIII activity (Table 3.6.1.2). Denaturation studies were carried out on the control containing rFVIII only and rFVIII with 1mM GA. The modified sample did not prove to be any more thermostable than the native protein (Figure 3.6.1.2). GA reacts with a number of different amino acid side chains, including those of lysine, cysteine, histidine and tyrosine. In dilute solution, GA exists in a number of different polymeric forms, and, therefore, the length of any cross-link formed cannot be estimated (Ji, 1983).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII Control	.308 ± .002	100
rFVIII + 1mM GA	.205 ± .002	66
rFVIII + 2mM GA	.025 ± .001	8
rFVIII + 3mM GA	.041 ± .002	13
Positive control	.137 ± .010	
Negative control	.005 ± .000	

TABLE 3.6.1.2. Reaction of rFVIII with glutaraldehyde (GA). rFVIII was reacted with increasing concentrations of GA on ice for 1 hour. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

At concentrations greater than 1mM FVIII activity is greatly reduced.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma, were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

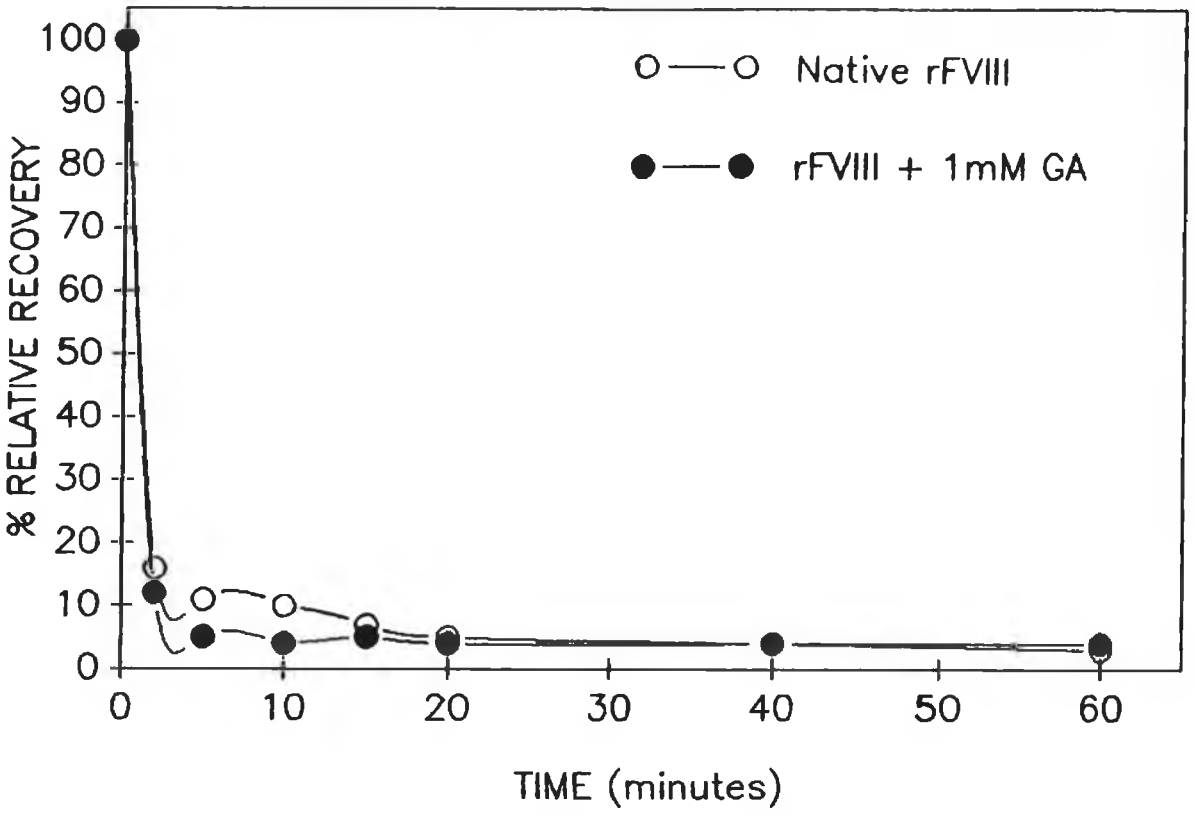


Figure 3.6.1.2 Denaturation of native rFVIII and Glutaraldehyde (GA).
Samples of rFVIII and rFVIII with 1mM GA were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.
Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.2 Thiol-specific reagents

3.6.2.1 Bis-maleimides

N-substituted bis-maleimide derivatives provide highly specific and mild bifunctional cross-linking reagents for sulphydryl groups (Ji, 1983). Two of this series of compounds were used in an attempt to form cross-links by an alkylation reaction. The first of these was bis-(maleimido)-methyl-ether (BMME) (Weston *et al.*, 1980).
Results showed that, unlike the amino-specific reagents, FVIII activity was not lost after addition of the cross-linker. For a range of BMME concentrations, 0-100μM, the FVIII activity was comparable with the control (Table 3.6.2.1).
rFVIII and rFVIII which had been reacted with 10μM and 40μM BMME, respectively, was subjected to denaturation (Figure 3.6.2.1.1).
No increase in thermostability was recorded.

A second bis-maleimide, N-N-bis (3-maleimidopropionyl)-2-hydroxy-1,3-propane-diamine (BMPPD), was used. Addition of the cross-linker proved to have no adverse effect on the activity of rFVIII (Table 3.6.2.1).
Denaturation studies, however, showed that bis-maleimide treated rFVIII was no more thermostable than the native rFVIII (Figure 3.6.2.2).

Concentration of Bis-maleimide	ΔA/min ± S.D. at 405nm	% Recovery
(i) *BMME*		
0 (FVIII control)	.315 ± .009	100
10μM	.271 ± .013	86
20μM	.221 ± .015	63
40μM	.295 ± .006	94
80μM	.288 ± .012	91
100μM	.234 ± .018	74
(ii) *BMPPD*		
0 (FVIII control)	.315 ± .009	100
10μM	.310 ± .008	98
20μM	.312 ± .004	99
40μM	.325 ± .010	103
80μM	.244 ± .005	77
100μM	.305 ± .004	97
Coag Cal N (positive FVIII control)	.134 ± .008	
FVIII-deficient plasma (negative control)	.007 ± .001	

TABLE 3.6.2.1 Reaction of rFVIII with two of the thiol-specific bis-maleimide series of cross-linkers. The reaction took place at room temperature for 10 minutes. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.
The presence of the cross-linker does not cause any significant loss of FVIII procoagulant activity, even at the higher concentrations. Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were included as positive and negative FVIII controls, respectively.
S.D. is the standard deviation where n = 3.

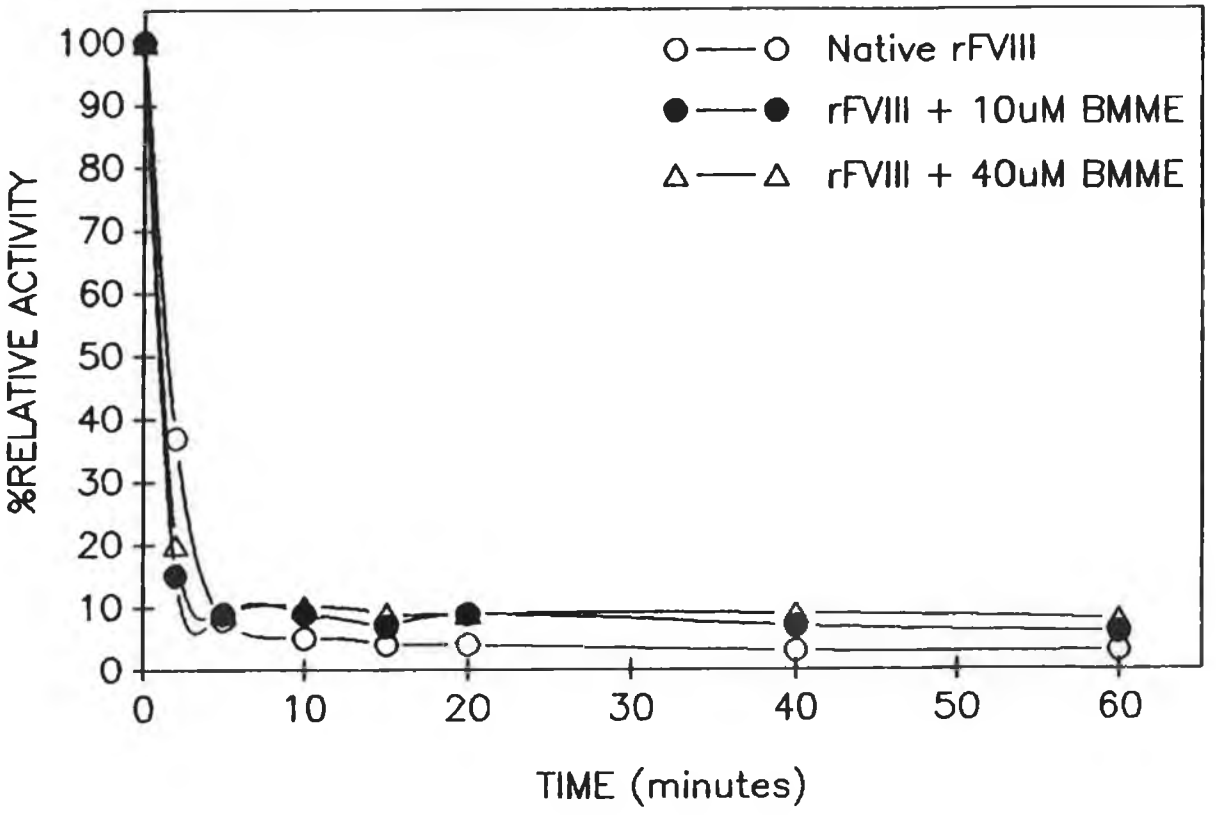


Figure 3.6.2.1.1 Denaturation of native rFVIII and bis-(maleimido-methyl) ether (BMME)-modified rFVIII.

Samples of rFVIII and rFVIII modified with 10μM and 40μM BMME were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

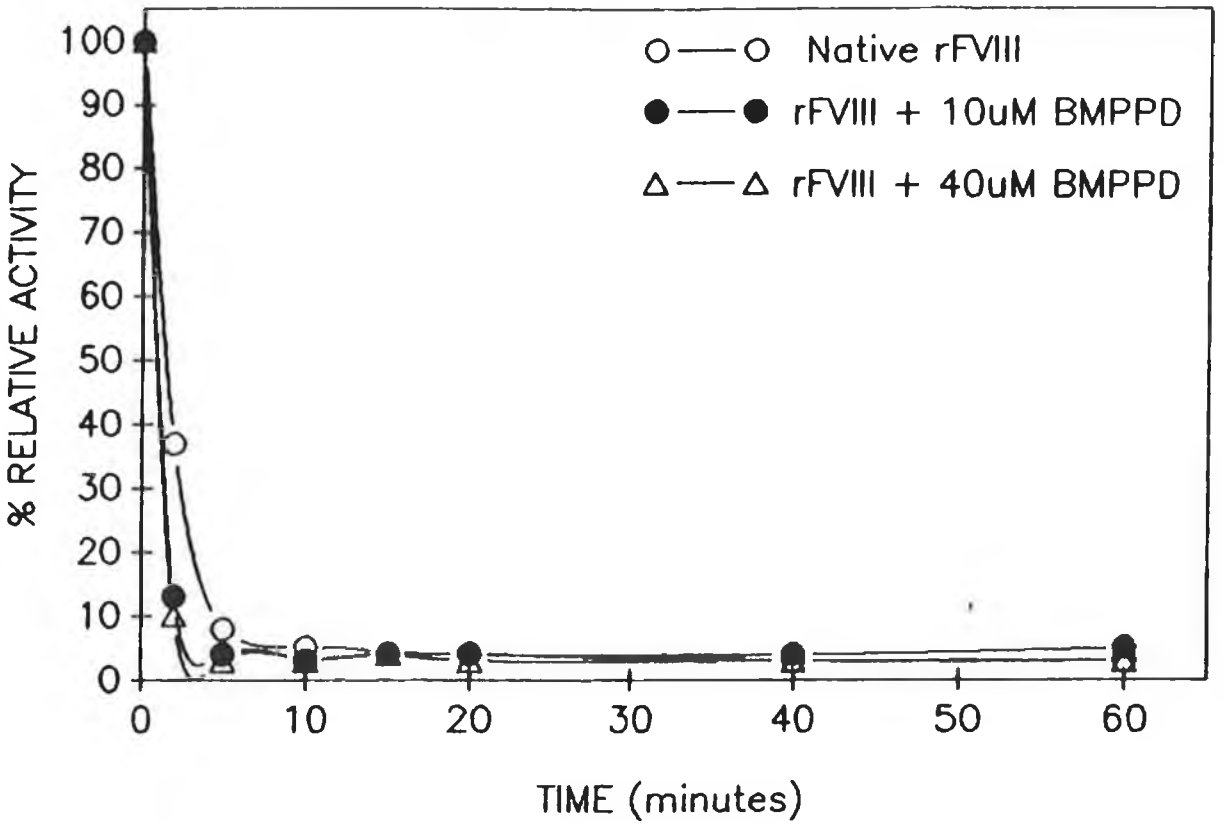


Figure 3.6.2.1.2 Denaturation of native rFVIII and N-N-bis (3-maleimidopropionyl)-2-hydroxy 1,3-propanediamine (BMPPD)-modified rFVIII.

Samples of rFVIII and rFVIII modified with 10μM and 40μM BMPPD were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.3 Heterobifunctional cross-linkers

Two heterobifunctional cross-linkers, N-(γ-maleimidobutyryloxy) succinimide (GMBS) (Fujiwara *et al.*, 1981), and succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate (SMCC) (Yoshitaki *et al.*, 1982), were used. Each contained an amino-specific succinimide moiety at one end of the molecule and a thiol-specific maleimide group at the other (Figure 3.1). Addition of GMBS caused a marked loss of FVIII activity when compared with the controls (Table 3.6.3). A control containing rFVIII and the diluent (dimethyl formamide, DMF) was included to ensure that any loss of activity was caused solely by the cross-linker. Addition of the other heterobifunctional reagent, SMCC, caused complete loss of activity (Table 3.6.3) similar to that observed with other amino-specific reagents.

Denaturation studies were carried out on rFVIII, rFVIII plus DMF and rFVIII with GMBS (Figure 3.6.3). No increase in thermostability was observed.

Concentration of Heterobifunctional	ΔA/min ± S.D. at 405nm	% Recovery
(i) *GMBS*		
0 (FVIII control)	.284 ± .011	100
FVIII + DMF (diluent)	.225 ± .012	80
0.05mM	.091 ± .007	32
0.1mM	.051 ± .007	18
0.5mM	.031 ± .002	5
(ii) *SMCC*		
0 (FVIII control)	.212 ± .005	100
FVIII + DMF (diluent)	.123 ± .006	58
0.25mM	.015 ± .002	7
0.5mM	.008 ± .001	4
2.5mM	.006 ± .001	3
Positive control	.125 ± .010	
Negative control	.005 ± .001	

TABLE 3.6.3 Reaction of rFVIII with two heterobifunctionals, N-(γ-maleimidobutyryloxy) succinimide (GMBS) and succinimidyl 4-(N-maleimidomethyl) cyclohexane carboxylate (SMCC). The reactions took place at room temperature for 30 minutes. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity. Both cross-linkers were dissolved in dimethyl formamide (DMF) so a control containing rFVIII in DMF was included in each experiment.
Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively. S.D. is the standard deviation where n = 3.

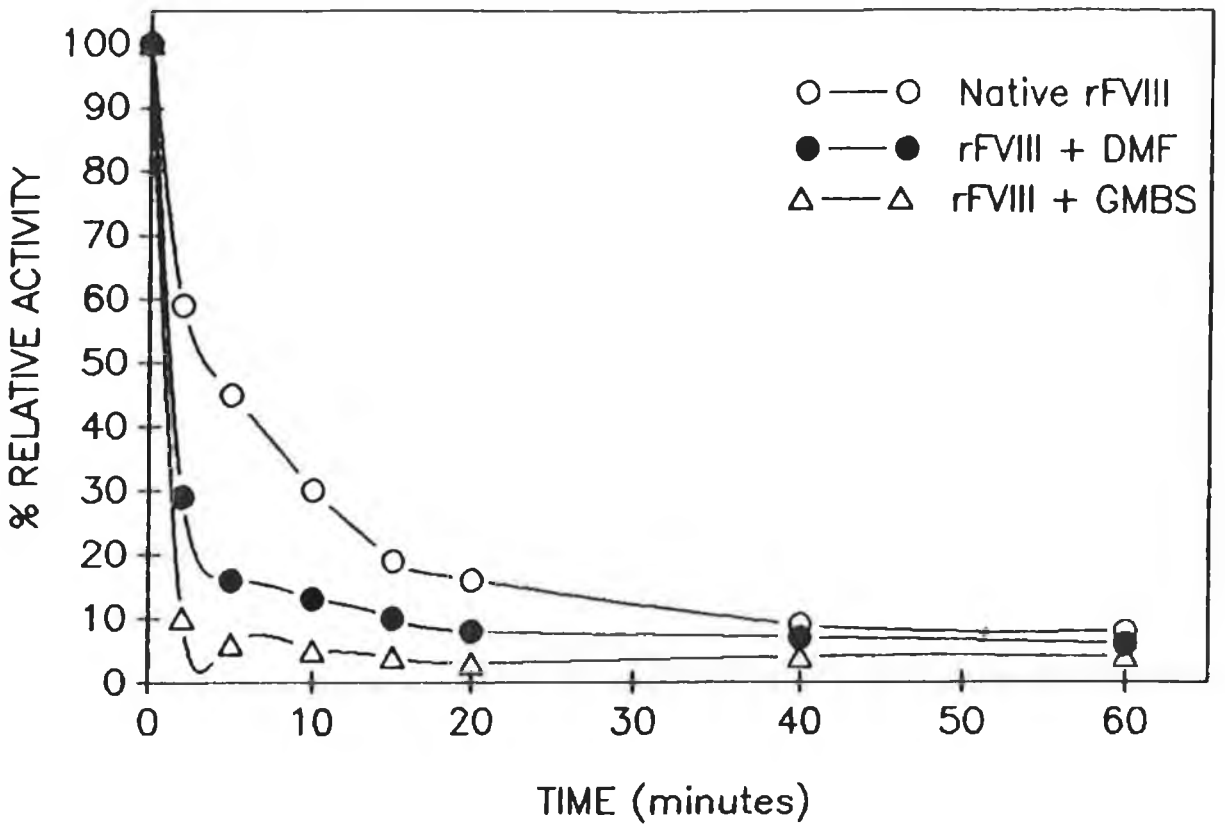


Figure 3.6.3 Denaturation of native rFVIII and N-(γ-maleimidobutyryloxy) succinimide (GMBS)-modified rFVIII.

Samples of rFVIII, rFVIII in dimethyl formamide (DMF) and rFVIII modified with 0.05mM GMBS were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.4 Protein Modifying Reagents

3.6.4.1 Oxidation using Hydrogen peroxide

The rFVIII molecule contains a number of inactivation sites, at least one of which is known to contain a methionine residue (Vehar *et al.*, 1984; Toole *et al.*, 1984). Oxidation of this residue to form the sulphoxide, with a view to altering the inactivation site, was attempted using hydrogen peroxide (H_2O_2) (Neuman, 1972). Concentrations of H_2O_2 above 100mM caused loss of procoagulant activity, whereas at 50mM and 100mM concentrations, around 55% of the control activity remained (Table 3.6.4.1).

Denaturation studies were carried out using these two concentrations, along with a control (rFVIII alone). The modified samples showed no increase in thermostability (Figure 3.6.4.1).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII Control	.266 ± .003	100
rFVIII + 10mM H_2O_2	.116 ± .004	44
rFVIII + 50mM H_2O_2	.145 ± .006	55
rFVIII + 100mM H_2O_2	.143 ± .001	54
rFVIII + 200mM H_2O_2	.012 ± .003	4
rFVIII + 500mM H_2O_2	.013 ± .002	5
rFVIII + 1000mM H_2O_2	.015 ± .001	6
Positive control	.145 ± .020	
Negative control	.004 ± .001	

TABLE 3.6.4.1 Reaction of rFVIII with hydrogen peroxide. (H_2O_2). rFVIII was reacted with final concentrations of (H_2O_2) ranging from 0 to 1M, on ice for either 1 or 2 hours. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity. Concentrations above 100mM caused significant loss of procoagulant activity.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

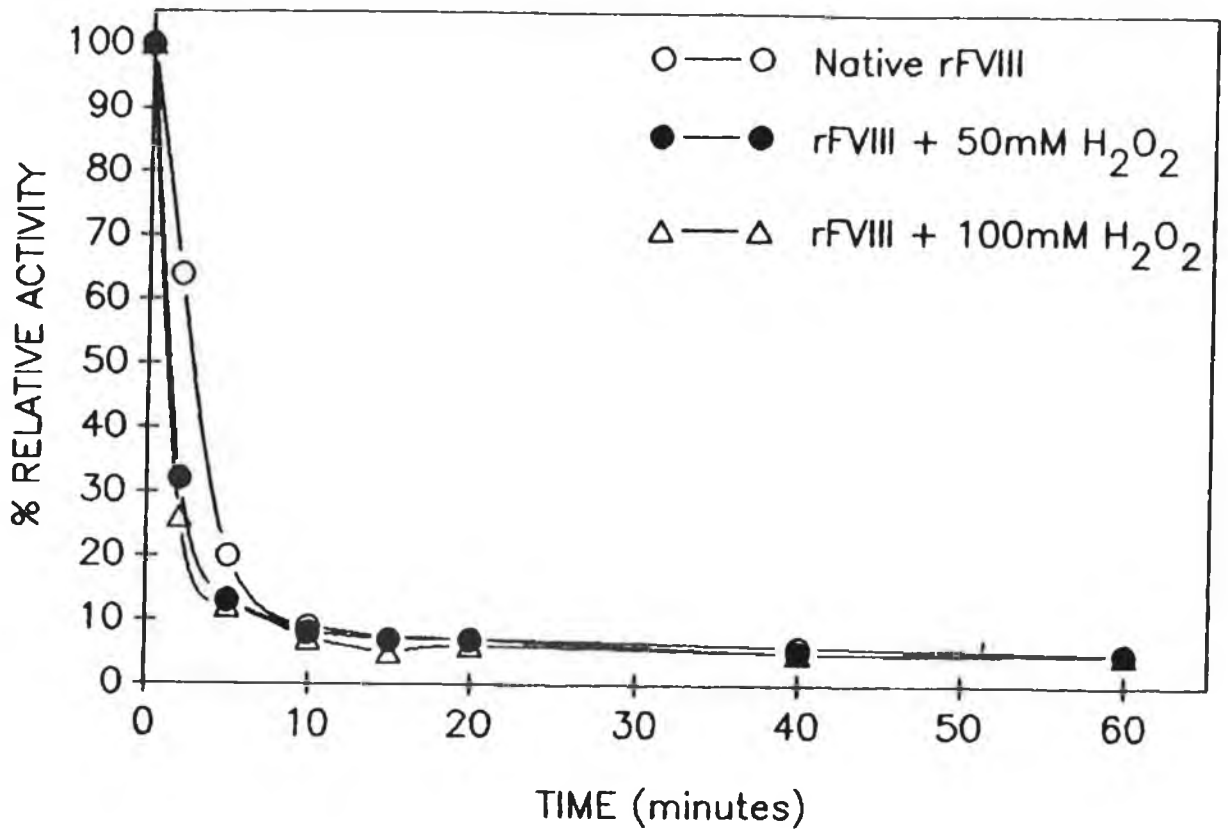


Figure 3.6.4.1 Denaturation of native rFVIII and hydrogen peroxide (H_2O_2)-modified rFVIII.

Samples of rFVIII and rFVIII modified with 50mM and 100mM H_2O_2 were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity. Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.4.2 Copper/o-Phenanthroline

Copper/o-Phenanthroline chelate (Cu/o-ph) can be used to enhance the oxidation of sulphydryl groups to the disulphide form (Steck, 1972). The addition of each component of the mixture individually, and in the form of a chelate, did not cause any significant loss of FVIII procoagulant activity, even at higher concentrations (Table 3.6.4.2).

Denaturation studies were carried out on each of the concentrations, along with controls. None of the modified samples exhibited any increase in thermostability as compared with the control (Figures 3.6.4.2.1 and 3.6.4.2.2).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII	.339 ± .016	100
rFVIII + 50μM o-ph	.267 ± .021	78
rFVIII + 50μM Cu^{2+}	.315 ± .021	93
rFVIII + X	.330 ± .030	97
rFVIII + Y	.327 ± .014	96
rFVIII + Z	.331 ± .022	97
Postive control	.136 ± .013	
Negative control	.005 ± .001	

TABLE 3.6.4.2 Reaction of rFVIII with varying concentrations of Cu/o-Ph. rFVIII was reacted with each combination below (A, B and C), on ice for 20 minutes.

X: 10μM $CuSO_4$ and 50μM o-Ph

Y: 50μM $CuSO_4$ and 25μM o-Ph

Z: 20μM $CuSO_4$ and 100μM o-Ph

The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively. S.D. is the standard deviation where n = 3.

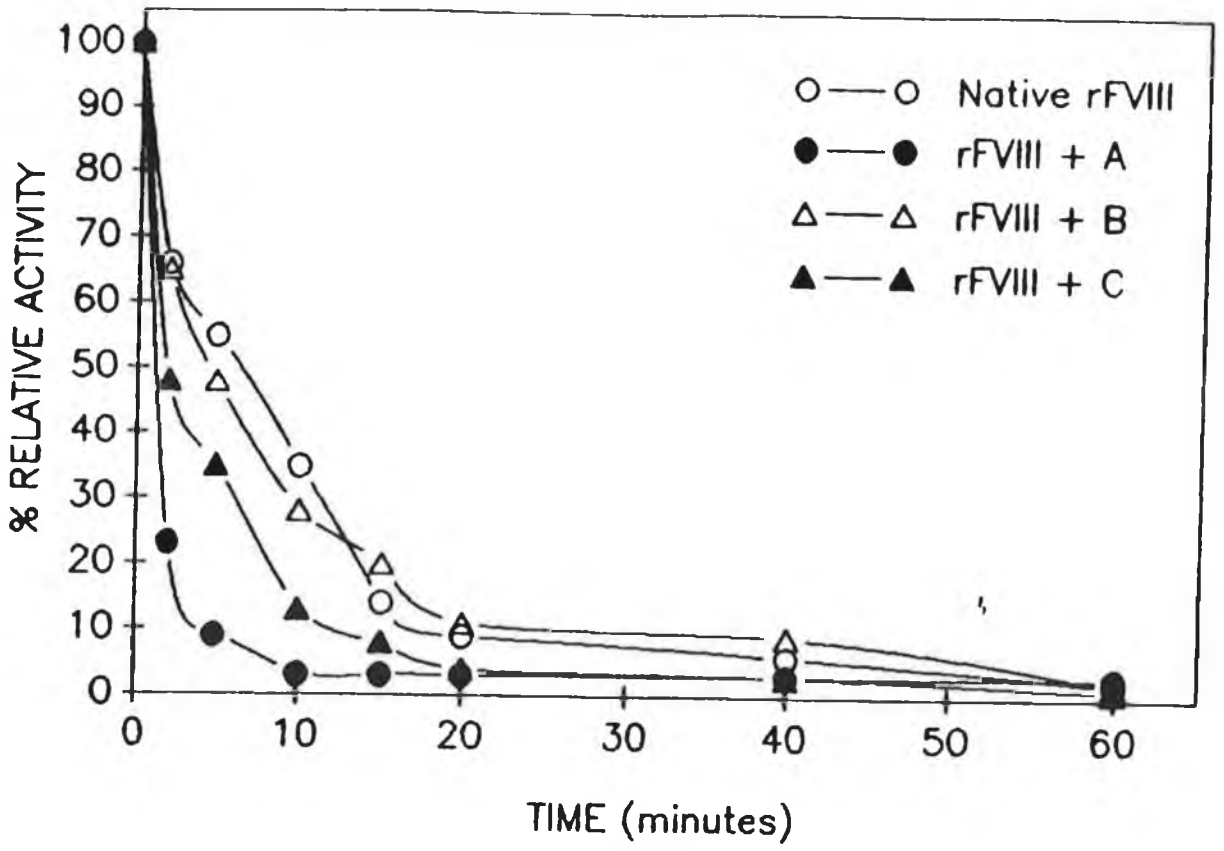


Figure 3.6.4.2.1 Denaturation of native rFVIII and rFVIII modified with the Copper/o-Phenanthroline chelate (Cu/o-ph).

Samples of rFVIII and rFVIII modified with 10μM Cu^{2+} + 50μM o-ph (C), along with controls, were incubated at 55°C for 1 hour. The controls consisted of rFVIII in the presence of 10μM Cu^{2+} (A) and rFVIII with 50μM o-ph (B). Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

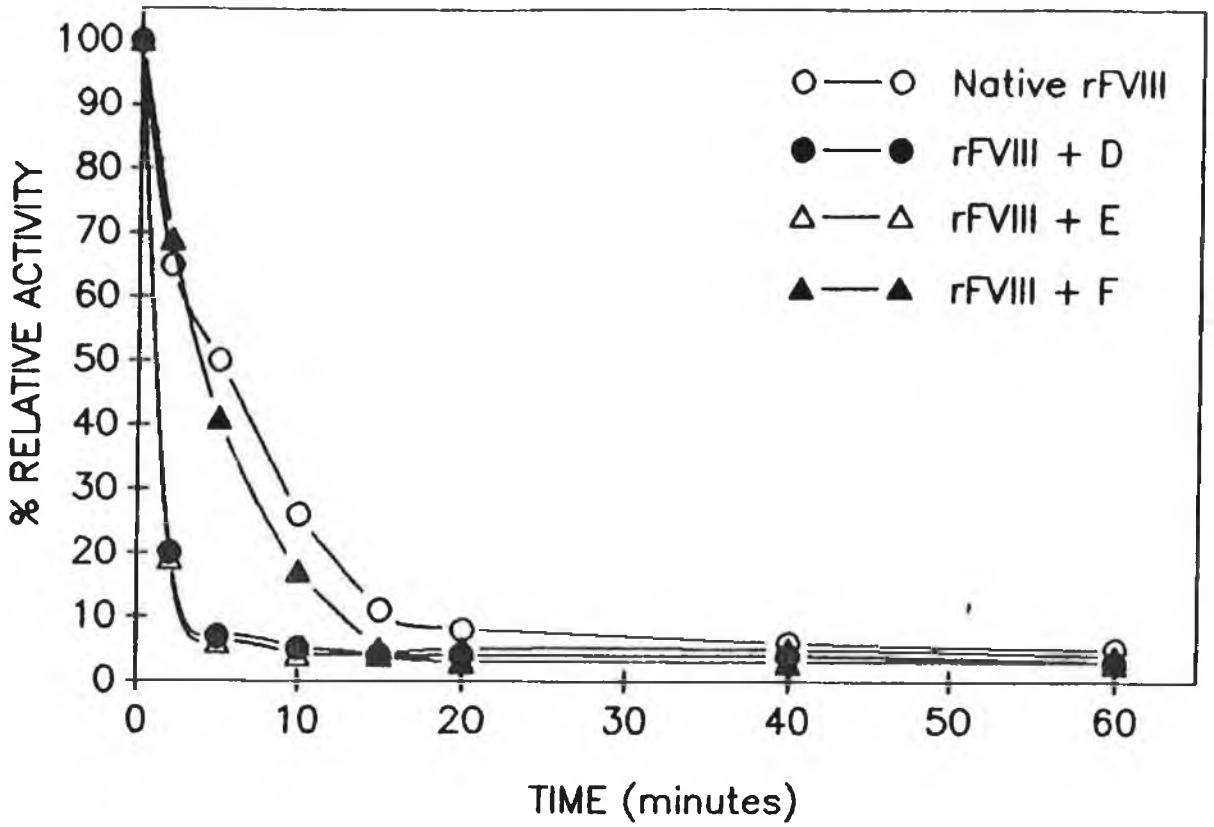


Figure 3.6.4.2.2 Denaturation of native rFVIII and rFVIII modified with the Copper/o-Phenanthroline chelate (Cu/o-ph).
Samples of rFVIII and rFVIII modified with 20µM Cu^{2+} + 100µM o-ph (F), along with controls, were incubated at 55°C for 1 hour. The controls consisted of rFVIII in the presence of 20µM Cu^{2+} (D) and rFVIII with 100µM o-ph (E). Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

3.6.4.3 2-Iminothiolane

2-Iminothiolane (2-IT) reacts with the amino group of lysine residues to form amidine derivatives, introducing an additional thiol group (Kenny *et al.*, 1979). Disulphide bonds may be formed when the derivative is subjected to oxidation if a second free thiol lies in close proximity. The addition of 2-IT to rFVIII does not cause any loss of activity over the given range of concentrations (0-6mM) (Table 3.6.4.3.1).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII (Control)	.268 ± .009	100
rFVIII + 0.25mM 2-IT	.264 ± .012	98
rFVIII + 1.0mM 2-IT	.240 ± .020	90
rFVIII + 6.0mM 2-IT	.123 ± .003	46
Positive control	.128 ± .010	
Negative control	.004 ± .000	

Table 3.6.4.3.1 Reaction of rFVIII with 2-Iminothiolane (2-IT). rFVIII was reacted with the above final concentrations of 2-IT on ice for 2 hours. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.
Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

Denaturation studies showed that the 2-IT modified rFVIII was no more stable than was the untreated rFVIII (Figure 3.6.4.3.1).

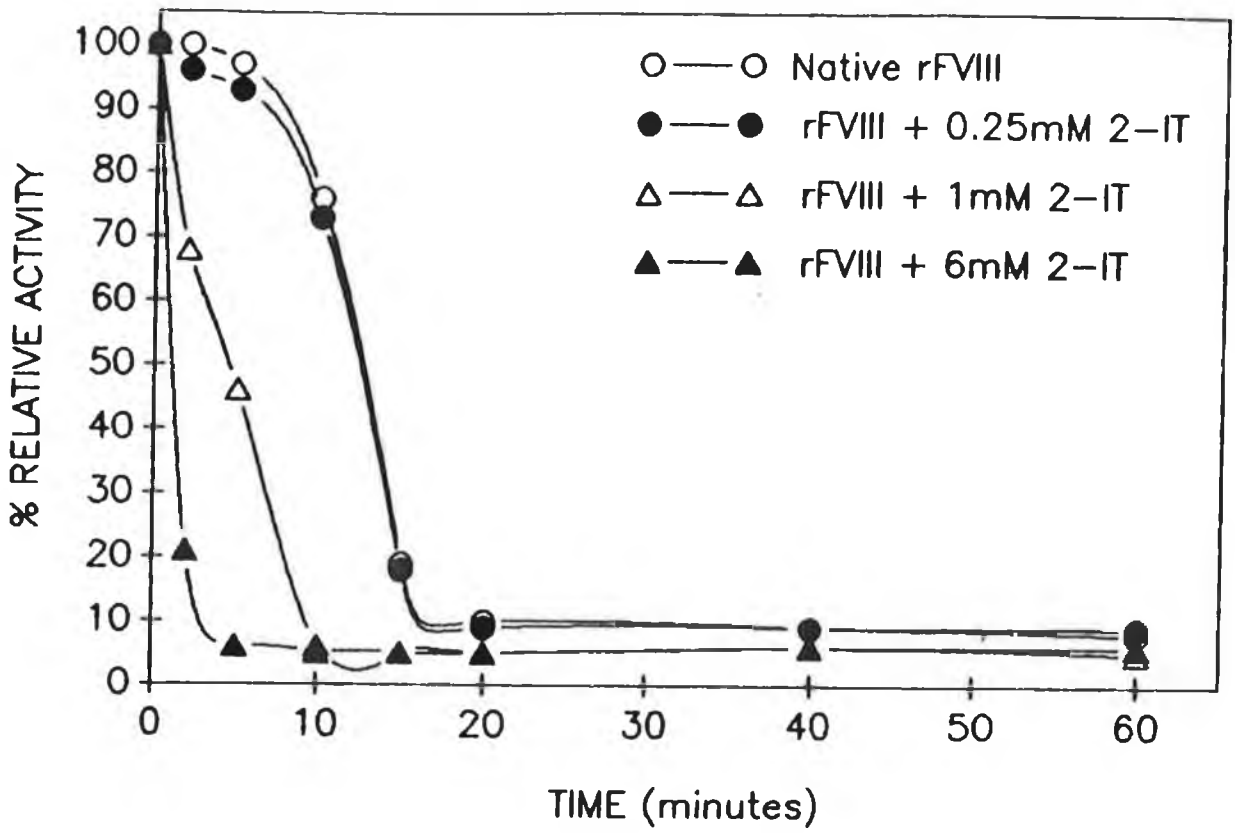


Figure 3.6.4.3.1 Denaturation of native rFVIII and rFVIII modified with 2-Iminothiolane (2-IT).

Samples of rFVIII and rFVIII modified with 1mM 2-IT were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

The second stage of the experiment involved the oxidation of the thiolated amidine derivative in an attempt to form additional disulphide bridges. Two reagents were employed, hydrogen peroxide (H_2O_2) and the copper/o-phenanthroline chelate (Cu/o-ph). The addition of H_2O_2 destroyed rFVIII activity (Table 3.6.4.3.2). Approximately 55% FVIII activity was retained, however, after the addition of Cu/o-ph. (Table 3.6.4.3.3). Figure 3.6.4.3.2 shows the results of the denaturation studies on the native and modified rFVIII (rFVIII + 2-IT + Cu/o-ph). While neither of the modification steps caused any major loss of activity, neither did they alter the stability of rFVIII.

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
0 (control)	.247 ± .015	100
FVIII + 40mM H_2O_2	.014 ± .001	6
FVIII + 0.25mM 2-IT + 40mM H_2O_2	.012 ± .002	5
FVIII + 1mM 2-IT + 40mM H_2O_2	.012 ± .001	5
FVIII + 6mM 2-IT + 40mM H_2O_2	.011 ± .001	5
Positive control	.133 ± .012	
Negative control	.006 ± .000	

TABLE 3.6.4.3.2 Reaction of rFVIII with 2-Iminothiolane (2-IT), followed by oxidation using 40mM H_2O_2.

rFVIII was initially reacted with varying concentrations of 2-IT on ice for 2 hours. These were then reacted with 40mM H_2O_2 on ice for 15 minutes. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity. Addition of H_2O_2 causes substantial loss of FVIII procoagulant activity.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
0 (rFVIII control)	.334 ± .026	100
rFVIII + 1mM 2-It	.233 ± .020	70
rFVIII + 1mM 2-It + 10μM Cu^{2+}	.166 ± .010	50
rFVIII + 1mM 2-It + 50μM o-ph	.172 ± .015	52
rFVIII + 1mM 2-It + Cu/o-ph	.192 ± .020	58
Positive control	.125 ± .007	
Negative control	.005 ± .000	

TABLE 3.6.4.3.3 Reaction of rFVIII with 2-Iminothiolane (2-It) followed by oxidation with copper/o-phenanthroline chelate (Cu/o-ph) [10μM $CuSo_4$ and 50μM o-Ph].

rFVIII was initially reacted with 1mM 2-IT on ice for 2 hours. It was then reacted with Cu/o-ph on ice for 20 minutes. The change in absorbance per minute was measured and used as an indicator of FVIII activity.

Controls consisting of rFVIII with each component of the chelate, as well as Coag Cal calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma (positive and negative FVIII controls, respectively) were included.

S.D. is the standard deviation where n = 3.

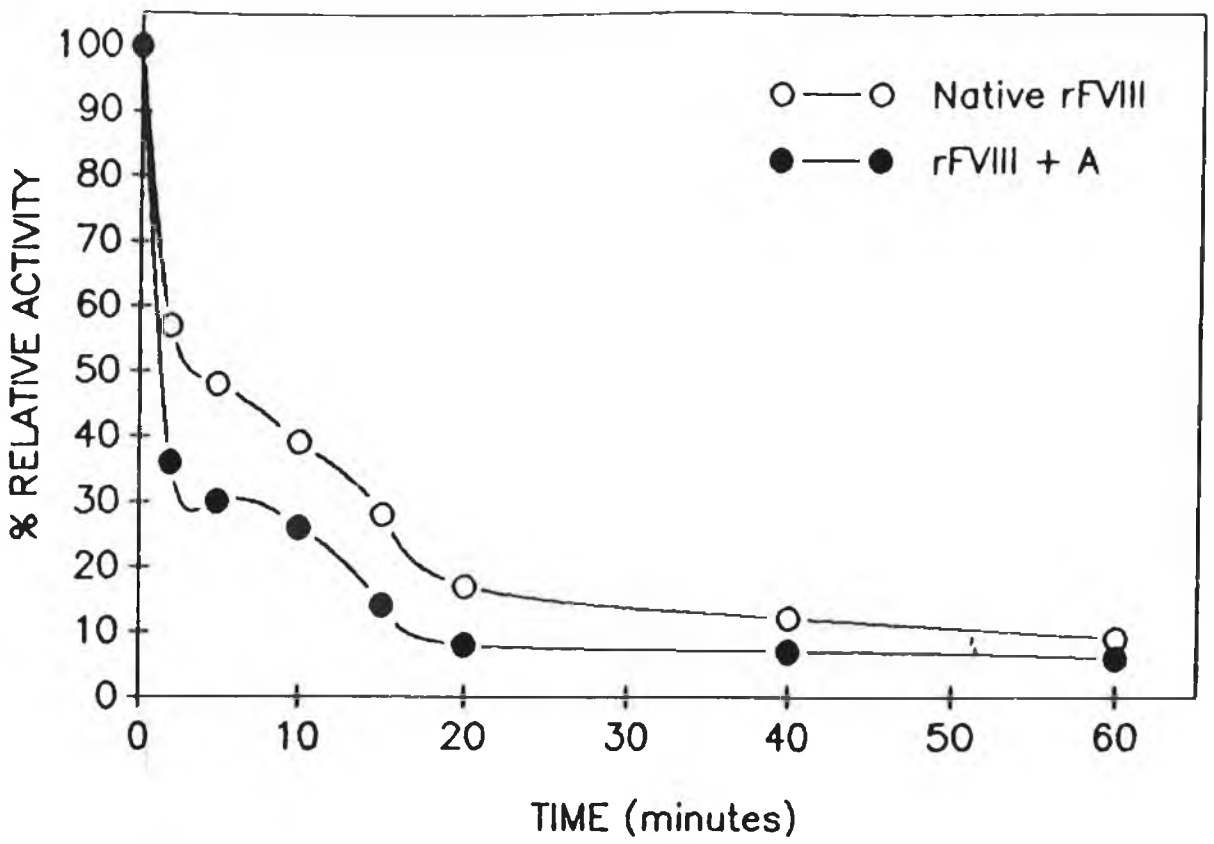


Figure 3.6.4.3.2 Denaturation of native rFVIII and rFVIII modified initially with 2-Iminothiolane (2-IT), and then with Copper/o-phenanthroline (Cu/o-ph).

Samples of rFVIII and rFVIII modified with 1mM 2-IT and followed by 10µM Cu^{2+} + 50µM o-ph (A), were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.4.4 Polyethelene Glycol

Proteins to which polyethelene glycol (PEG) has been attached have been shown to be stable and active in organic solvents, and to be less antigenic and allergenic (Imoto and Yamada, 189). PEG can be attached to a protein amino group (usually lysine) using cyanuric chloride-activated PEG (a conjugate of cyanuric chloride and PEG) (Jackson *et al.*, 1987).

Approximately 80% of the control value remained after the addition of PEG to rFVIII (Table 3.6.4.4). Denaturation studies again showed that no increase in thermostability was obtained (Figure 3.6.4.4).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII (Control)	.109 ± .001	100
rFVIII + 0.5X PEG	.089 ± .001	81
rFVIII + 5X PEG	.088 ± .001	80
Positive control	.132 ± .010	
Negative control	.005 ± .001	

Table 3.6.4.4 Reaction of rFVIII with polyethylene glycol (PEG).

rFVIII was reacted with final molar concentrations of 0.5X and 5X PEG with regard to lysines (approx. 40), at room temperature for 1 hour. The change in absorbance per minute (ΔA/min) was measured, and used as an indicator of activity.

The modification with PEG did not cause substantial loss of FVIII activity when compared with the control.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

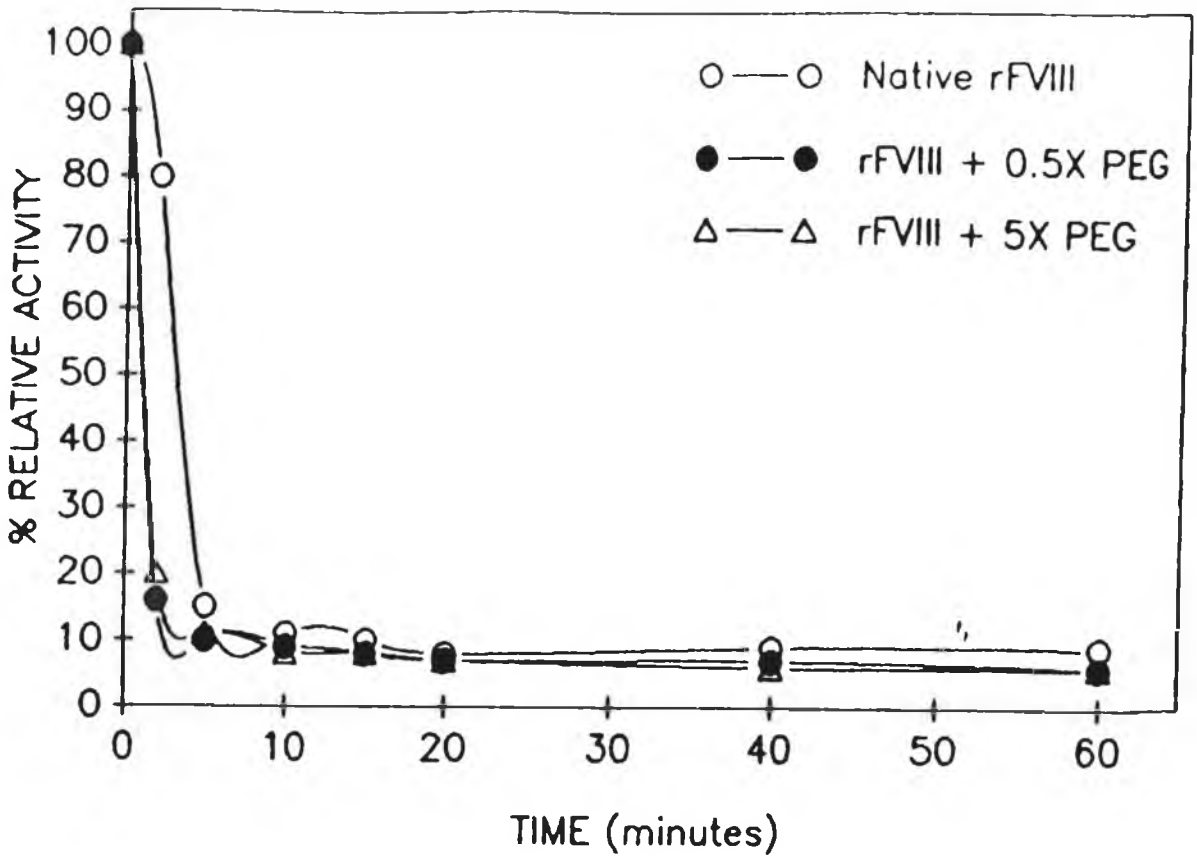


Figure 3.4.4.4 Denaturation of native rFVIII and Polyethylene glycol (PEG)-modified rFVIII.

Samples of rFVIII and rFVIII modified with final concentrations of 0.5X and 5X (with regard to lysine residues) PEG were incubated at 55°C for 1 hour. Aliquots were withdrawn at regular intervals and assayed for FVIII activity.

Relative percentage activity was calculated as the percentage of activity at T0, remaining at a particular time.

3.6.4.5 Xylitol

Xylitol is a five carbon sugar alcohol which is used as a stabilising additive. It has little direct interaction with the protein but affects the bulk solution properties of water. It acts by stabilising the molecular lattice structure of water. This in turn stabilises the hydration shell around the protein and protects against aggregation (Schein, 1990).

The addition of xylitol to rFVIII causes substantial loss of activity, even at the lowest concentrations (Table 3.6.4.5).

Reactants	ΔA/min ± S.D. at 405nm	% Recovery
rFVIII (Control)	.252 ± .024	100
rFVIII + 10mM Xylitol	.032 ± .003	28
rFVIII + 50mM Xylitol	.031 ± .004	32
rFVIII + 100mM Xylitol	.021 ± .002	
Positive control	.125 ± .011	
Negative control	.005 ± .001	

TABLE 3.6.4.5 Reaction of rFVIII with Xylitol. rFVIII was reacted with 10mM, 50mM and 100mM Xylitol on ice for 30 minutes. The change in absorbance per minute (ΔA/min) was measured and used as an indicator of FVIII activity.

The presence of xylitol causes substantial loss of FVIII procoagulant activity.

Coag Cal N calibration plasma (*i.e.* normal human plasma) and FVIII-deficient plasma were used as positive and negative FVIII controls, respectively.

S.D. is the standard deviation where n = 3.

3.7 Discussion

The ε-amino group of lysine residues is usually located on the surface of a protein due to the positive charge it exhibits under physiological conditions. It's strongly nucleophilic side chain is, thus, an ideal candidate for selective modification using bifunctional and other modifying reagents. The thiol group of cysteine residues is another major target for cross-linking experiments. This group is the strongest nucleophile among the functional groups of amino acids, reacting with most thiol-specific cross-linkers at an alkaline pH (Wong and Wong, 1992).

FVIII contains a number of activation sites, where arginine residues are predominant (Vehar *et al.*, 1984) and so reagents which would alter these sites in any way were avoided. Carboxyl-specific reagents were not employed at any time since clusters of negatively charged carboxyl groups may bind the calcium ions essential for FVIII activity (Mikaelsson, 1983).

The buffer systems had to be compatible with the reagent being used. Thus, for most of the reactions, rFVIII was dissolved in Tris buffer. However, for the amino-specific reagents the protein was transferred to barbital buffer. Tris buffer contains quaternary amino groups which could interfere with the FVIII-modifier reaction.

The pH tolerance of FVIII is narrow. Inactivation occurs at pH values less than about 6 and greater than 8. Thus, the pH of the reactions was controlled within the range pH 6-8. This reduced the number of cross-linkers which could be used, as many reagents act optimally outside this narrow pH range. This was particularly important for the heterobifunctional reagents, where each end of the molecule is selectively activated at different pH values. The range of such compounds which could be useful was, therefore, severely restricted.

Most chemical modifications of proteins are performed at protein concentrations about ten-fold higher than carried out here. While the clotting activity of rFVIII used was high, its protein concentration was about 0.15mg/ml (approximately 2μM). The amount of rFVIII available was greatly restricted because of the cost involved as well as the demand for the protein for use in replacement therapy in the treatment of haemophilia A. Therefore, concentration of the protein could not be carried out. This, together with activated FVIII's extremely narrow pH tolerance, forced us to operate under very limited conditions, some of which were not ideal for the reagents in

question. Imidoesters, for example, react with unprotonated amino groups and so reaction is favoured at mildly alkaline pH values which FVIII will not tolerate. The low protein concentration also prevented the characterisation of the chemical derivatives produced.

The only experimental parameter of activity or functional stability, therefore, was the FVIII procoagulant activity as determined by chromogenic assay. However, it is this activity of FVIII that is primarily important in any diagnostic or theraputic application of rFVIII or any derivative.

There is a noteworthy difference between the effects of the amino and the thiol-specific reagents on rFVIII. With the exception of 2-IT, all of the amino-group modifers, whether cross-linkers or not, inactivate most of the procoagulant activity. It is not clear why 2-IT alone did not inactivate rFVIII. In contrast, the thiol-specific reagents did not lead to rFVIII inactivation. This suggests that some free amino groups are essential for rFVIII integrity and activity. At least some of the numerous free thiol groups can be modified, however, without adverse effects.

Oxidation-type treatments were either neutral (attempts to create new disulphide links with Cu/o-ph) or deleterious (attempts to oxidise methionine residues with H_2O_2). A methionine residue is located at Arg 336 - Met 337 of native FVIII, one of a number of inactivation sites present in the FVIII molecule (Vehar *et al.*, 1984). This is the site where activated Protein C, thrombin and activated Factor X can cleave the FVIII heavy chain, leading to inactivation (Manning *et al.*, 1993). Attempts were made to oxidise this to the bulky sulphoxide form and thereby prevent inactivation cleavage. H_2O_2 is quite a strong oxidant, however, and may have reacted with residues other than methionine.

Attempts to alter the properties of the protein in solution caused total loss of FVIII procoagulant activity. The protein is inherently unstable in the absence of vWF and, thus, any changes in the hydration shell around the protein caused destabilisation leading to loss of activity.

Factor VIII undergoes many cleavages, some of which are necessary for molecular activation while others lead to inactivation. The activation cleavage sites at least must be preserved in any functional derivative which is to be prepared. Since it is also a

large and complex molecule, any successful cross-linking or other modification are likyely to be extremely subtle.

These experiments have, nevertheless, demonstrated a qualitative difference between different amino acid side chains within FVIII in terms of their importance to it's function. Amino groups seem to be essential for FVIII activity while thiol groups may be modified without causing adverse effects on FVIII activity. It appears therefore, that amino-groups play an important role in FVIII integrity and activity.

CHAPTER 4

PRODUCTION OF MONOCLONAL AND POLYCLONAL ANTI-rFVIII ANTIBODIES

4.1 Introduction

The development of techniques which immortalise antibody-producing cells (Köhler and Milstein, 1975), allowing them both to survive and produce antibody in culture, has proved to be one of the most important developments of recent times. Today, monoclonal antibodies have a wide range of applications, from clinical use in the treatment and diagnosis of diseases, to preparative uses and use in basic research.

Monoclonal antibodies have been used in the purification of a number of blood clotting proteins, including factors XII (Pixley *et al.*, 1993), IX (Bajaj and Birktoft, 1993) and V (Kalafatis *et al.*, 1993) as well as factor VIII (Lollar *et al.*, 1993). They provide an ideal means of obtaining high purity blood products for use in the treatment of blood clotting disorders because of their specificity. The risks associated with the use of human-derived products are huge. Blood-borne viruses including hepatitis and human immunodeficiency virus (HIV) are well documented, but other potential infectious agents (perhaps as potent as these) may also be present. It is vital, therefore, that products for replacement therapy be contaminant free. Recombinant proteins are being increasingly used in replacement therapy. They provide a safe alternative to plasma-derived products, being largely contaminant free. However, some patients are prone to developing inhibitor antibodies against the recombinant protein as new antigenic determinants are exposed. In this chapter, the development of novel monoclonal and polyclonal antibodies against a recombinant FVIII is described.

4.2 Polyclonal Antibody Production

Polyclonal antibodies were produced as described in Section 2.12 (Figure 4.2.1). The rabbit was bled two weeks after booster injections and the titre measured by ELISA (Section 2.23.1). A titre of 1:100,000 was obtained (Table 4.2).

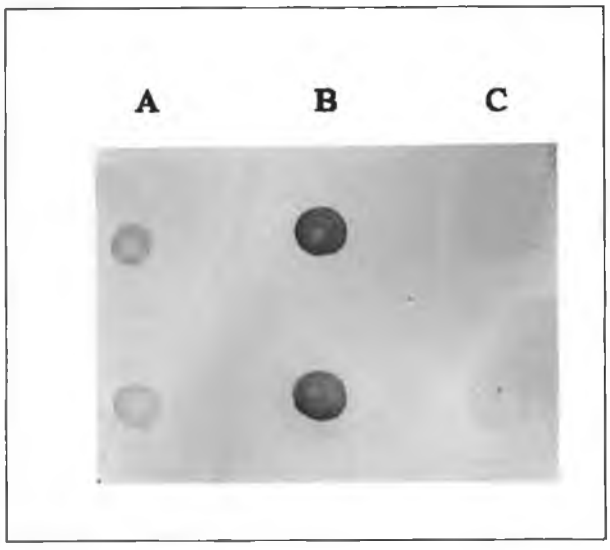


Figure 4.2.1 Dot blot on immunised rabbit serum.

rFVIII was dotted onto nitrocellulose paper and free binding sites blocked with a 1% (w/v) solution of bovine serum albumin. Duplicate samples of immunised rabbit serum (B) and controls consisting of normal rabbit serum (A), and PBS (0.15M, pH 7.2) (C) were added. A substrate which produced an insoluble end-product (5-bromo-4-chloro-3 indolyl phosphate) was used, and the appearance of intense dark dots was indicative of the presence of anti-rFVIII antibodies.

Dilution	Immunized Rabbit Serum Absorbance ± SD at 620nm	Normal Rabbit Serum Absorbance ± SD at 620nm
1:1000	.451 ± .033	.055 ± .001
1:5000	.411 ± .017	.050 ± .001
1:10,000	.355 ± .031	.046 ± .001
1:50,000	.237 ± .021	.044 ± .001
1:100,000	.175 ± .006	.045 ± .001
PBS	.000 ± .000	.000 ± .000

Table 4.2 The titre of serum from the immunised rabbit was measured by ELISA. 5µg/ml rFVIII was bound to the plate and free binding sites on the plate blocked. Dilutions of immunised and normal rabbit serum were added. Bound antibody was probed with alkaline phosphatase-labelled antibody. Substrate was added and absorbance measured at 620nm.
SD: Standard deviation where n=3.

4.2.1 Purification of Polyclonal Antiserum

The rabbit serum was purified initially by ammonium sulphate precipitation (Section 2.16.1). The partially purified antibody was dialysed against 0.15M PBS, pH 7.2 at 4°C overnight, and the protein concentration measured.

The second stage of purification involved Protein A affinity chromatography (Section 2.16.3). The antibody was diluted in binding buffer, to within the binding range of the column (1:4). 2ml of this was then applied to the column and a total of 13 x 1ml fractions collected. Figure 4.2.1.1 shows the elution profile of the column. Bound antibody eluted in two fractions (fractions 9 and 10) and these were desalted on a Sephadex G-25 column (Figure 4.2.1.2). The protein concentration of each fractions was monitored and two were found to contain protein (anti-rFVIII antibody). The protein concentration was estimated to be 5mg/ml.

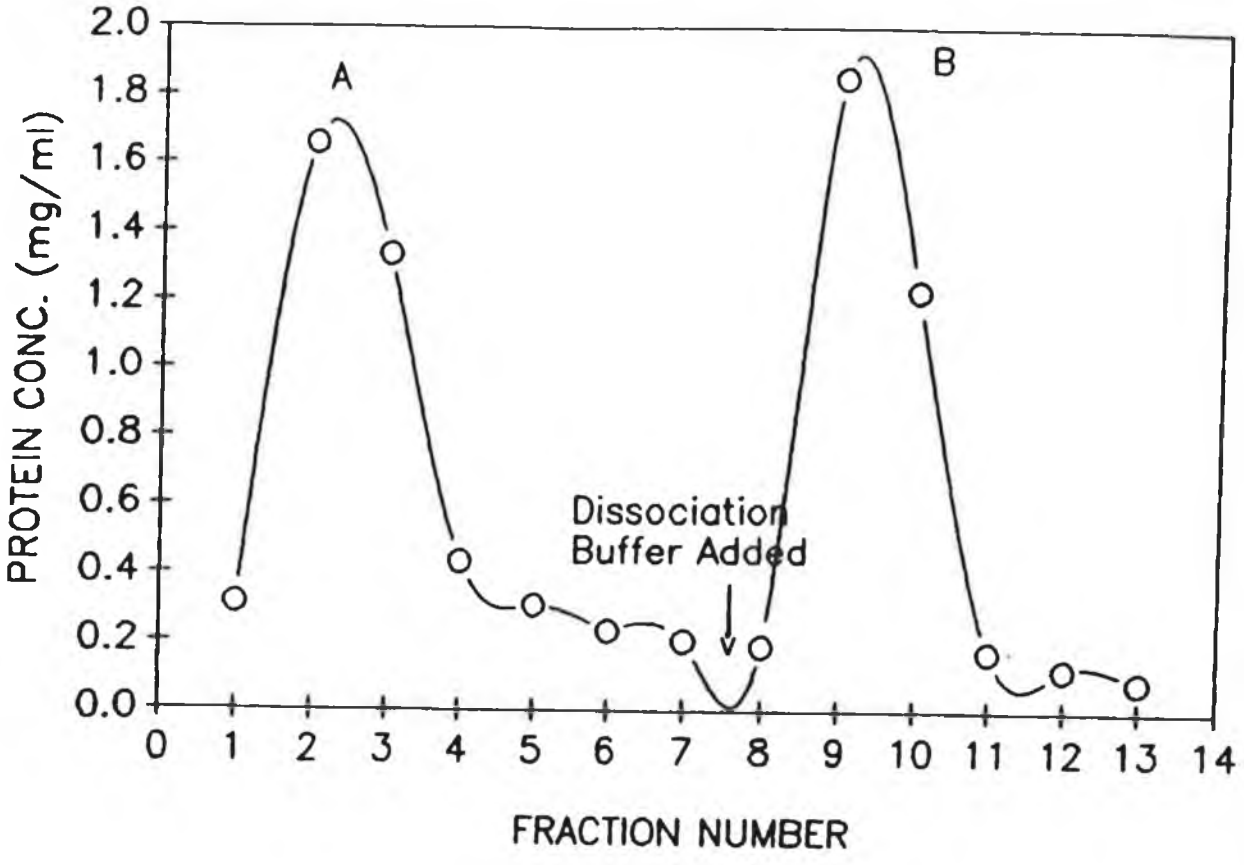


Figure 4.2.1.1 Elution of polyclonal anti-rFVIII antibody from a Protein A affinity column.

The antibody was eluted with a 0.1M glycine buffer, pH 2.5 and 13 fractions were collected. The concentration of protein in each fraction was determined using the BCA assay (Section 2.7). Peak B represents the eluted antibody fractions.

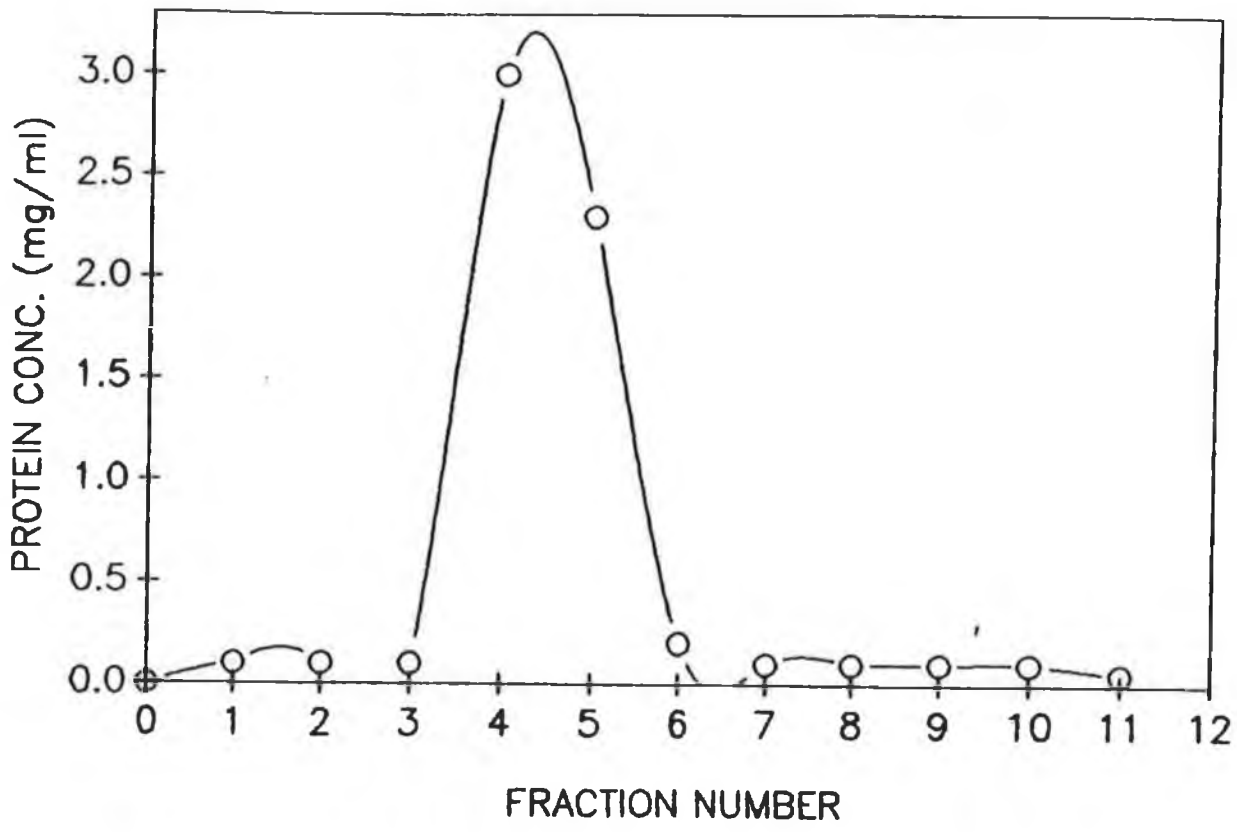


Figure 4.2.1.2 Desalting of antibody-containing fractions on a 10ml Sephadex G-25 column. The antibodies were eluted with 0.1M PBS, pH 7.2, and 11 fractions were collected. The concentration of protein in each fraction was determined using the BCA protein assay (Section 2.7).

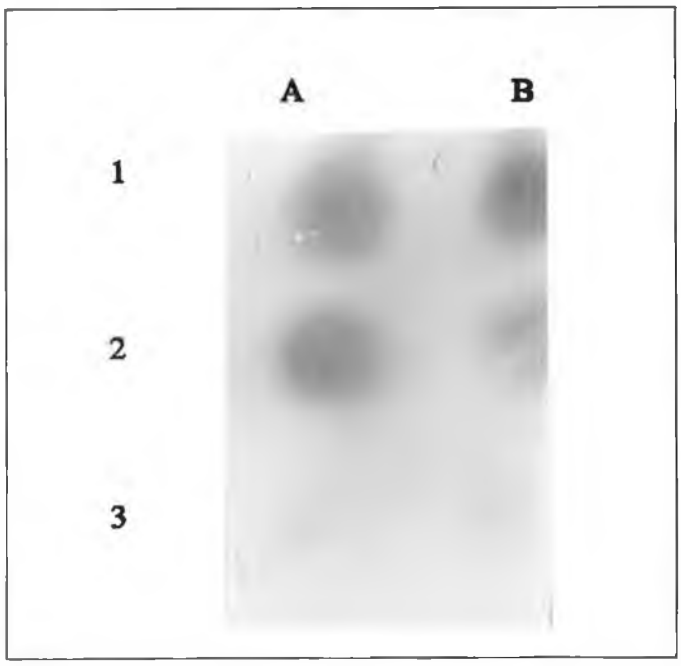
4.2.2 Conjugation of horseradish peroxidase to anti-rFVIII polyclonal antibody

Horseradish peroxidase (HRP) was conjugated to rabbit anti-rFVIII antibody as described in Section 2.21. A dot blot was carried out based on that described in Section 2.14.1 to ensure that conjugation had taken place. rFVIII was dotted onto nitrocellulose paper and the free binding sites blocked. The conjugate was added and, after incubation, unbound conjugate removed by washing and substrate (3,3'- diaminobenzidine tetrahydrochloride, DAB) added. This produced an insoluble end-product which could be observed visually (Figure 4.2.2.1).

The enzymic activity of the conjugate was also measured as described in Section 2.21.1. An ELISA (Section 2.23.1) was carried out to estimate the working dilution of the conjugate (Table 4.2.2).

The protein concentration of the conjugate was estimated to be 1mg/ml.

The purity of the conjugate was assessed using SDS-PAGE (Section 2.18) and is shown in Figure 4.2.2.2.



	A rFVIII	B NO rFVIII
1	DUPLICATE CONJUGATE SAMPLES	
2	DUPLICATE CONJUGATE SAMPLES	
3	PBS	PBS

Figure 4.2.2.1 Immunological activity of horseradish peroxide-labelled anti-rFVIII antibodies. rFVIII was dotted onto nitrocellulose paper (NC) and the free binding sites blocked. Duplicate samples of the antibody conjugate were added to the rFVIII (lane A), and to control blots (lane B) where no rFVIII had been blotted. A control consisting of PBS (0.15M, pH 7.2) was also included (A3 and B3). Bound antibody detected using 3,3′-diaminobenzidine tetrachloride, (DAB) which produced an insoluble end-product. Some degree of non-specific binding was obtained because of the high binding capacity of NC.

Dilution	Absorbance ± SD at 492nm
1:100	1.828 ± .027
1:500	1.112 ± .044
1:800	1.090 ± .042
1:1000	.799 ± .056
1:1100	.494 ± .043
1:1300	.252 ± .024
PBS	.000 ± .000

Table 4.2.2 Results of an ELISA to determine the working dilution of the polyclonal anti-rFVIII IgG/HRP conjugate.

5µg/ml of rFVIII was immobilised onto the surface of wells of a microtitre plate and the remaining free binding sites were blocked using 1% (w/v) BSA. Dilutions of the conjugate were then added. Substrate (o-phenylenediamine) was added and the reaction terminated after 30 minutes by addition of 20% (v/v) H_2SO_4.

SD: Standard deviation where n=3.

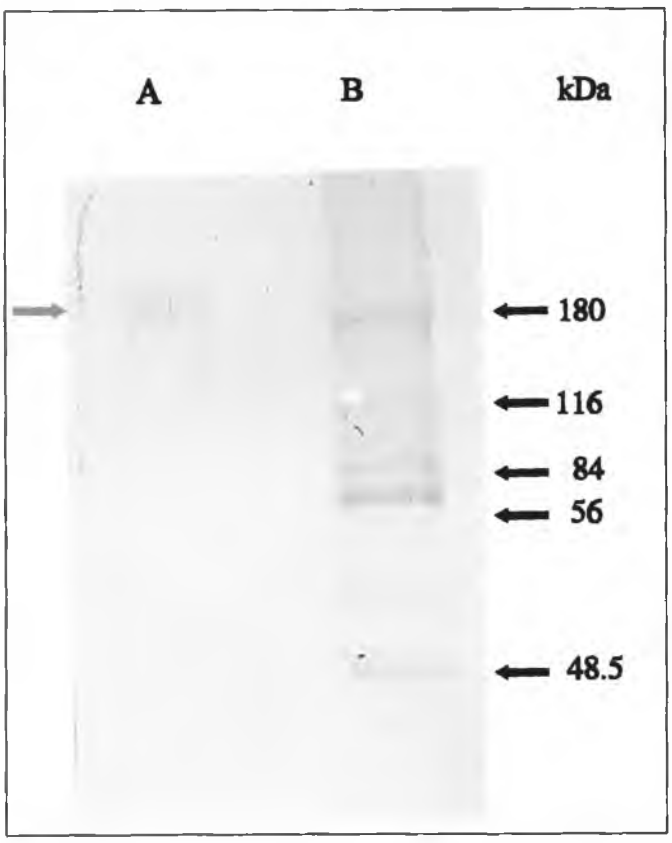


Figure 4.2.2.2 The purity of the anti-rFVIII antibody-horseradish peroxide conjugate assessed using SDS-PAGE.

The conjugate (lane A) produced a single band with a molecular weight of around 190 kDA. This corresponds to a conjugate consisting of IgG (Mr, 150 kDa) and horseradish peroxidase (Mr, 44 kDa).

Molecular weight markers are shown in lane B.

4.3 Monoclonal Antibody Production

4.3.1 Immunisation

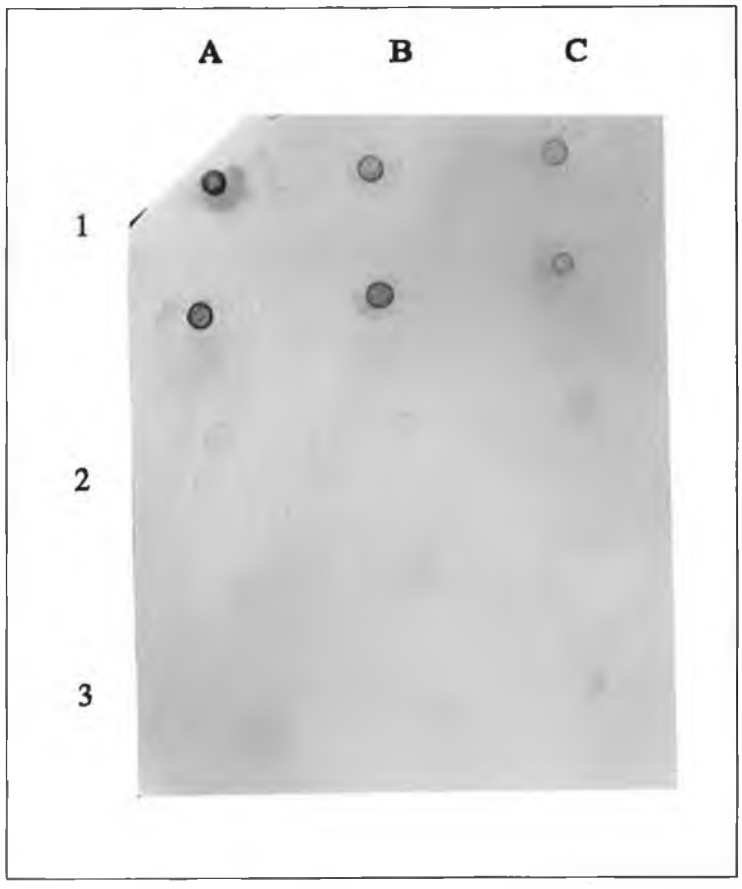
Male Balb/c mice were immunised with rFVIII as described in Section 2.13.1. The animal was bled at fortnightly intervals from the tail vein and the titre estimated by dot blotting (Section 2.14.1). When a sufficiently high titre was obtained (*i.e*, 1:5,000, Figure 4.3.1) the mouse was sacrificed, and a splenectomy performed. The splenocytes were subsequently used for fusing to generate monoclonal antibodies.

4.3.2 Fusion Results

Two fusions were carried out between immunized mouse splenocytes and NSO murine myeloma cells. In the first fusion an average of 14 wells per plate contained hybridomas from 10 fusion plates. 148 hybridomas were screened for anti-rFVIII antibody production by dot blot, and eight strongly positive clones were identified. These were cloned out by limiting dilution (Section 2.13.7) on two separate occasions to ensure monoclonality. Two monoclonal antibodies were isolated from these, M1 and M2.

Some cells were lost during expansion *in vitro* due to bacterial contamination. Infected wells could be identified by the acidic, cloudy medium. Samples were withdrawn from the suspected wells and a Gram stain carried out. This indicated the presence of both Gram positive and Gram negative bacteria. Thus a broad spectrum antibiotic (gentamycin) was used. Efforts to remove the infection by culturing in the presence of gentamycin (200µg/ml) proved unsuccessful. Because of the importance of the cells, if only one or two individual wells within a plate were found to be infected, the contents of these wells were aspirated and the well washed several times with 5M NaOH. The remaining wells were carefully monitored for signs of contamination and if any appeared, the plates were autoclaved and discarded.

The second fusion produced approximately 11 clones per plate from a total of 10 fusion plates. 115 hybridomas were screened for anti-rFVIII antibody production by dot blotting. 13 strongly positive hybridomas were identified and after cloning out by limiting dilution (twice) 1 monoclonal antibody was obtained (M10).



	A	B	C
1	NEAT MOUSE SERUM	1:100 DILUTION	1:500 DILUTION
2	1:1000 DILUTION	1:2000 DILUTION	1:5000 DILUTION
3	PBS	PBS	

Figure 4.3.1 The titre of mouse serum prior to fusion.

rFVIII was dotted onto nitrocellulose paper and free binding sites blocked with a 1% (w/v) solution of bovine serum albumin. Duplicate samples of a series of dilutions of immunised rabbit serum were added as shown. A substrate which produced an insoluble end-product (5-bromo-4-chloro-3 indolyl phosphate) was used, and the appearance of dark dots was indicative of the presence of anti-rFVIII antibodies.

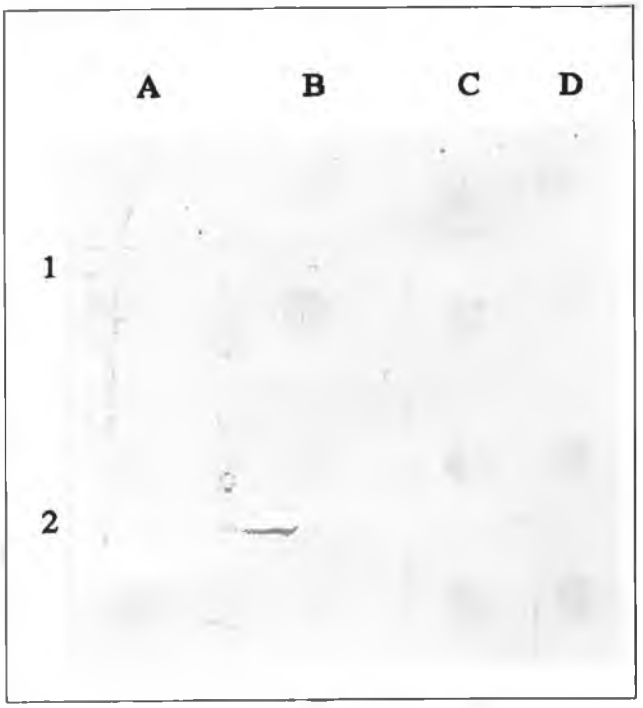
Thus, from two fusions, 263 hybridomas were obtained, yielding three monoclonal anti-rFVIII antibodies.

The monoclonal antibodies were expanded *in vitro* as described in Section 2.15.1. Some of these were used to produce ascitic fluid (Section 2.15.2), while remaining stocks were frozen in liquid nitrogen (Section 2.11.3) for long-term storage.

Figure 4.3.2.1 shows the results of dot blots identifying hybridomas which are secreting anti-rFVIII antibodies.

4.4 Purification of Antibody from Ascitic Fluid by Ammonium Sulphate Precipitation

The monoclonal antibodies were partially purified from ascitic fluid by precipitation with ammonium sulphate as described in Section 2.16.1. A dot blot was performed on the supernatants after each centrifugation step, as well as the final precipitate. After dialysis against 0.15M PBS, pH 7.2, the protein concentration was measured using the BCA protein assay (Section 2.7). Samples were stored at -20°C in small aliquots to prevent repeated freezing and thawing.



	A	B	C	D
1	X	✓✓	✓✓	✓
2	✓	X	✓✓	✓✓

Figure 4.3.2.1 Identification of clones secreting anti-rFVIII antibody after a fusion. Supernatants were withdrawn from 8 wells of a microtitre plate post-fusion and duplicate samples were dotted onto nitrocellulose paper (A1, A2, B1, B2, C1, C2) and tested for the presence of antibody by dot blotting. A substrate which produced an insoluble end-product (5-bromo-4-chloro-3 indoyl phosphate) was used, and the appearance of dark dots was indicative of the presence of anti-rFVIII antibodies. Strongly positive (✓✓), weak (✓), and negative (X) clones could be identified.

4.5 Determination of Antibody Class

The isotype of the three monoclonal antibodies was determined using two isotyping kits (Section 2.17). The first, an ELISA based kit, indicated that the antibodies were IgM, with IgG_1 also present. The second kit, based on the principle of agglutination, also indicated that the antibodies were IgM. IgM is a pentameric molecule consisting of five IgG-like molecules held together by disulphide bonds between the $C\mu_3$ domains. These disulphide bonds are easily reduced, causing destruction of the pentameric structure and forming IgG-like molecules. This accounts for the identification of IgG_1 along with IgM in the samples. The results are summarized in Table 4.5.

Antibody	Isotype according to Kit 1	Isotype according to Kit 2
M1	IgM	IgM
M2	IgM	IgM
M10	IgM + IgG_1	IgM

Table 4.5 Isotypes of the three monoclonal anti-rFVIII antibodies.
All three were identified as IgMs with M10 having IgG_1 also present. IgM consists of 5 IgG_1-like antibodies linked together. Thus the presence of IgG_1 suggests that the IgM has been broken down in some way.

Kit 1: Sigma Mouse Isotyping kit, based on ELISA.
Kit 2: Serotec Isotyping kit, based on agglutination

4.6 Affinity Purification of M2 Monoclonal Antibody

The M2 monoclonal antibody was further purified by affinity chromatography. An affinity column consisting of rFVIII immobilised onto Sephadex G-25 was prepared as described in Section 2.16.2. Specific anti-rFVIII antibodies bind to the immobilised rFVIII, while non-specific contaminants were removed by washing. The antibodies were then eluted from the column. After checking for protein leaching off the column, partially-purified antibody (after $(NH_4)_2SO_4$ precipitation) was added (300µl of a 1mg/ml solution). Seven 1 ml fractions were collected, and the protein concentration of each monitored using the BCA protein assay. Specific antibody was then eluted (4 x 1ml fractions). The elution profile of the column is shown in Figure 4.6.1.

Fractions were dialysed overnight at 4°C against 0.15M PBS, pH 7.2.

A dot blot was performed on all protein-containing fractions, and anti-rFVIII antibodies were identified in fraction 4, (Figure 4.6.2).

The protein concentration of the antibody-containing fraction was estimated (using the BCA protein assay) to be 0.2mg/ml.

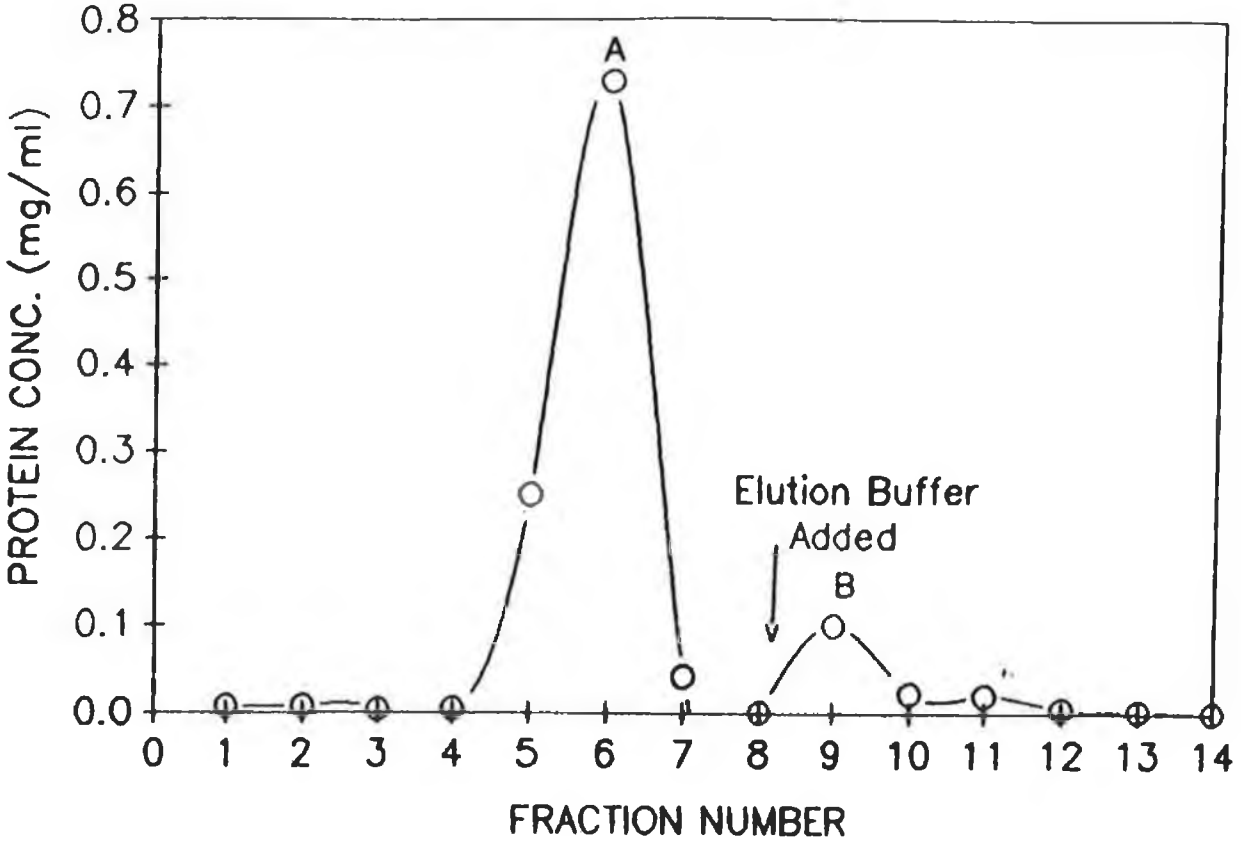
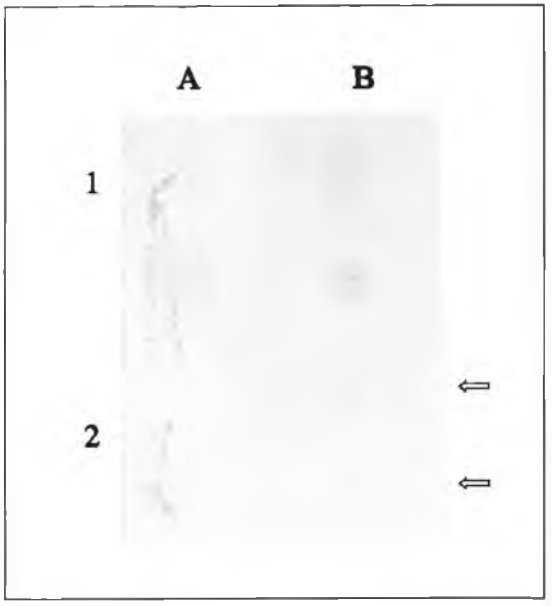


Figure 4.6.1 Elution of M2 monoclonal anti-rFVIII antibody from a FVIII affinity column.

The antibody was eluted with a 0.1M glycine buffer, pH, 2.5, and 7 fractions were collected. The concentration of protein in each fraction was determined using the BCA protein assay (Section 2.7). Peak A represents the unbound protein, and Peak B represents the eluted antibody fraction.



	A	B
1	ASCITIC FLUID	ANTI-rFVIII CONTROL
2	FRACTION 3	FRACTION 4

Figure 4.6.2 Dot blot identifying the fractions containing anti-rFVIII antibody after affinity purification of M2 antibody.

rFVIII was dotted onto nitrocellulose paper and free binding sites blocked with a 1% (w/v) solution of bovine serum albumin. Duplicate samples of $(NH_4)_2SO_4$-purified ascitic fluid (A1), a control consisting of anti-rFVIII polyclonal antibodies (B1), fraction 3 (A2) and fraction 4 (B2) after affinity purification were added. A substrate which produced an insoluble end-product (5-bromo-4-chloro-3 indolyl phosphate) was used, and the appearance of dark dots was indicative of the presence of anti-rFVIII antibodies. Antibodies were identified in fraction 4 (indicated by the arrows) from the affinity cloumn.

4.7 Protein A Purification of M10 Monoclonal Antibody

The M10 monoclonal antibody was further purified using a Protein A affinity column as described in Section 2.16.3.

The protein concentration of the ascitic fluid after ammonium sulphate precipitation was calculated to be 5mg/ml. Since the maximum binding capacity of the column was 6-8mg/ml, the antibody was diluted 1:1 with binding buffer, and 1.4ml of this was applied to the column.

1ml fractions were collected and protein concentration monitored.

Figure 4.7.1 shows the elution profile of the column.

Fractions containing protein after elution were desalted on a Sephadex G-25 column (Figure 4.7.2).

The protein concentration of the unbound fractions was calculated to be 4mg/ml and that of the eluted fractions (fractions 3 and 4) was 0.4mg/ml each. A dot blot was performed on all fractions and anti-rFVIII antibodies identified in fraction 3 (Figure 4.7.3).

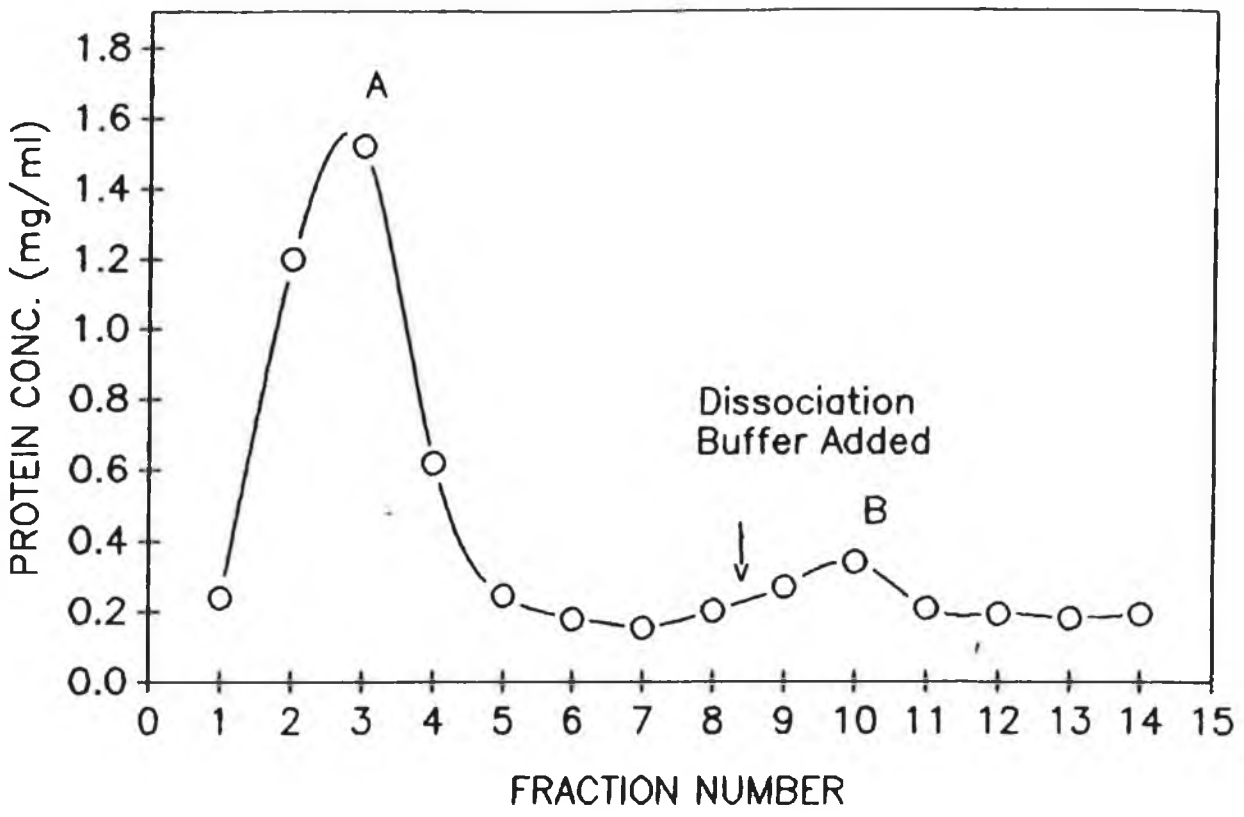


Figure 4.7.1 Elution of M10 monoclonal anti-rFVIII antibody from a Protein A affinity column.

The antibody was eluted with a 0.1M glycine buffer, pH 2.5 and 13 fractions were collected. The concentration of protein in each fraction was determined using the BCA assay (Section 2.7). Peak A is the unbound proteins, while peak B represents the eluted antibody fractions.

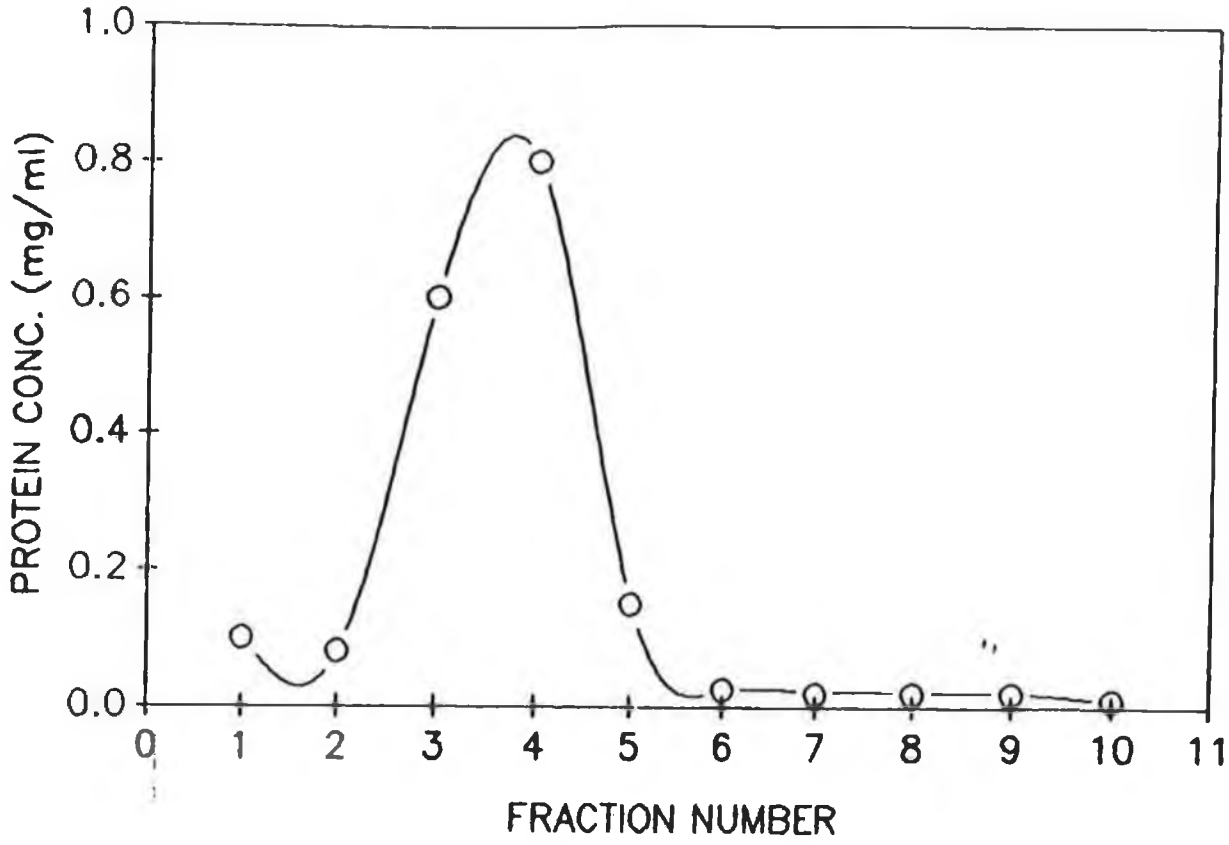
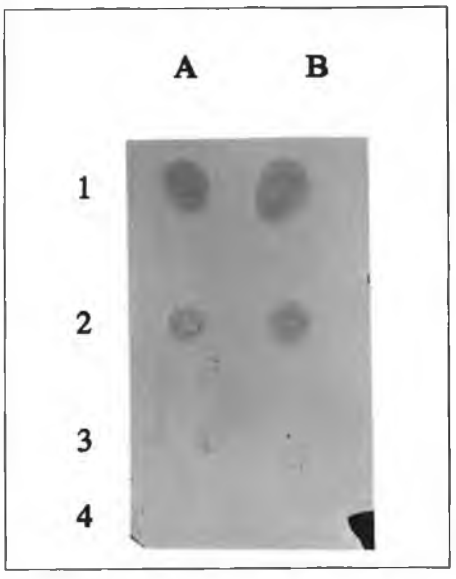


Figure 4.7.2 Desalting of antibody-containing fractions on a Sephadex G-25 column. The M10 monoclonal antibodies were eluted with 0.1M PBS, pH 7.2, and 10 fractions were collected. The concentration of protein in each fraction was determined using the BCA protein assay (Section 2.7).



	A	B
1	ASCITIC FLUID	
2	ANTI-rFVIII CONTROL	
3	FRACTION 3	
4	FRACTION 4	

Figure 4.7.3 Dot blot identifying the fractions containing anti-rFVIII antibody after Protein A affinity purification of M1 antibody.

rFVIII was dotted onto nitrocellulose paper and free binding sites blocked with a 1% (w/v) solution of bovine serum albumin. Duplicate samples of $(NH_4)_2SO_4$-purified ascitic fluid (A1, B1), a control consisting of anti-rFVIII polyclonal antibodies (A2, B2), fraction 3 (A3, B3) and fraction 4 (A4, B4) after Protein A purification were added. A substrate which produced an insoluble end-product (5-bromo-4-chloro-3 indolyl phosphate) was used, and the appearance of dark dots was indicative of the presence of anti-rFVIII antibodies. Antibodies were identified in fraction 3 from the Protein A column.

4.8 Purification of M1 Monoclonal Antibody by Gel Filtration

The M1 monoclonal was purified by passage through a size-exclusion column (Sepharose-6B) as described in Section 2.16.4. The IgM antibody thus elutes in the void volume.

1ml of antibody was applied and a total of 9 x 1ml fractions were collected (Figure 4.8.1). The protein concentration of each fraction was measured. A dot blot was carried out on all fractions, and anti-rFVIII activity was identified in fraction 3 (Figure 4.8.2).

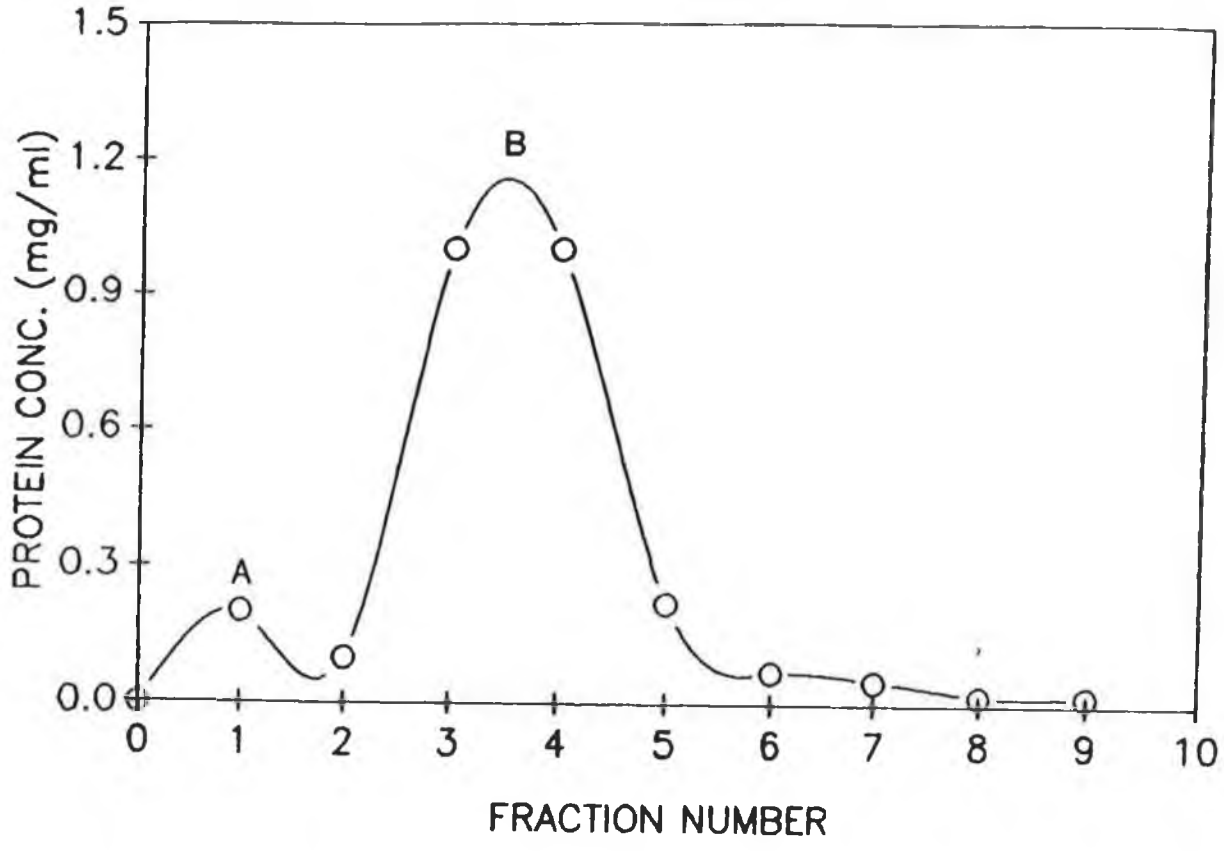
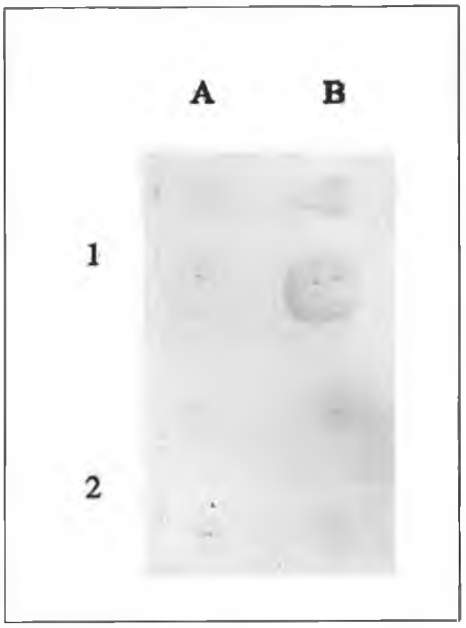


Figure 4.8.1 Elution of M1 monoclonal anti-rFVIII from a gel filtration column. The antibody was eluted with a 0.1M imidazole buffer, pH, 7.2 and 9 fractions were collected. The protein concentration of each fraction was determined using the BCA protein assay. The IgM antibody eluted in the void volumn (Peak A) beacuse of its size (900 kDa), while the remaining proteins eluted in later fractions (Peak B).



	A	B
1	ANTI-rFVIII CONTROL	ASCITIC FLUID
2	FRACTION 3	FRACTION 4

Figure 4.8.2 Dot blot to identify the fractions containing anti-rFVIII antibody after gel filtration purification of M10 antibody.

rFVIII was dotted onto nitrocellulose paper and free binding sites blocked with a 1% (w/v) solution of bovine serum albumin. Duplicate samples of controls consisting of polyclonal anti-rFVIII antibodies (A1), and $(NH_4)_2SO_4$-purified ascitic fluid (B1) as well as fractions 3 (A2) and fraction 4 (B2) from the desalting column were added.

Anti-rFVIII antibody was identified in fraction 4.

4.9 HPLC Analysis of Antibodies

Both monoclonal and polyclonal antibodies were analysed by HPLC as described in Section 2.20. Standards consisting of commercial rabbit IgG (Figure 4.9.1) and commercial mouse IgM (Figure 4.9.3) were also run. Table 4.9 summarises the retention times of the standards, the polyclonal and the monoclonal antibodies.

A sample of the polyclonal anti-rFVIII antibody was analysed after Protein A purification (Figure 4.9.2).

Commercial IgM and samples of M2 and M10 monoclonal antibodies after purification were also examined by HPLC (Figures 4.9.4 and 4.9.5).

Antibody	Retention Time (minutes)
Commercial IgM (Standard)	9.8
M2 Monoclonal	9.4
M10 Monoclonal	9.2
Commercial IgG (Standard)	14.5
Polyclonal after $(NH_4)_2SO_4$ precipitation	14.0
Purified Polyclonal after Protein A	14.3

Table 4.9 Retention times of standards (commercial antibodies), purified monoclonal antibodies and polyclonal antibody samples after each stage of purification, on HPLC. 10µl of each sample was run on a Protein Pak SW 300, 10µm column. The mobile phase was 0.1M phosphate buffer, with a flow rate of 0.5ml/min. Protein was detected by monitoring absorbance at 280nm. The IgM antibodies elute in the void volume because of their size.

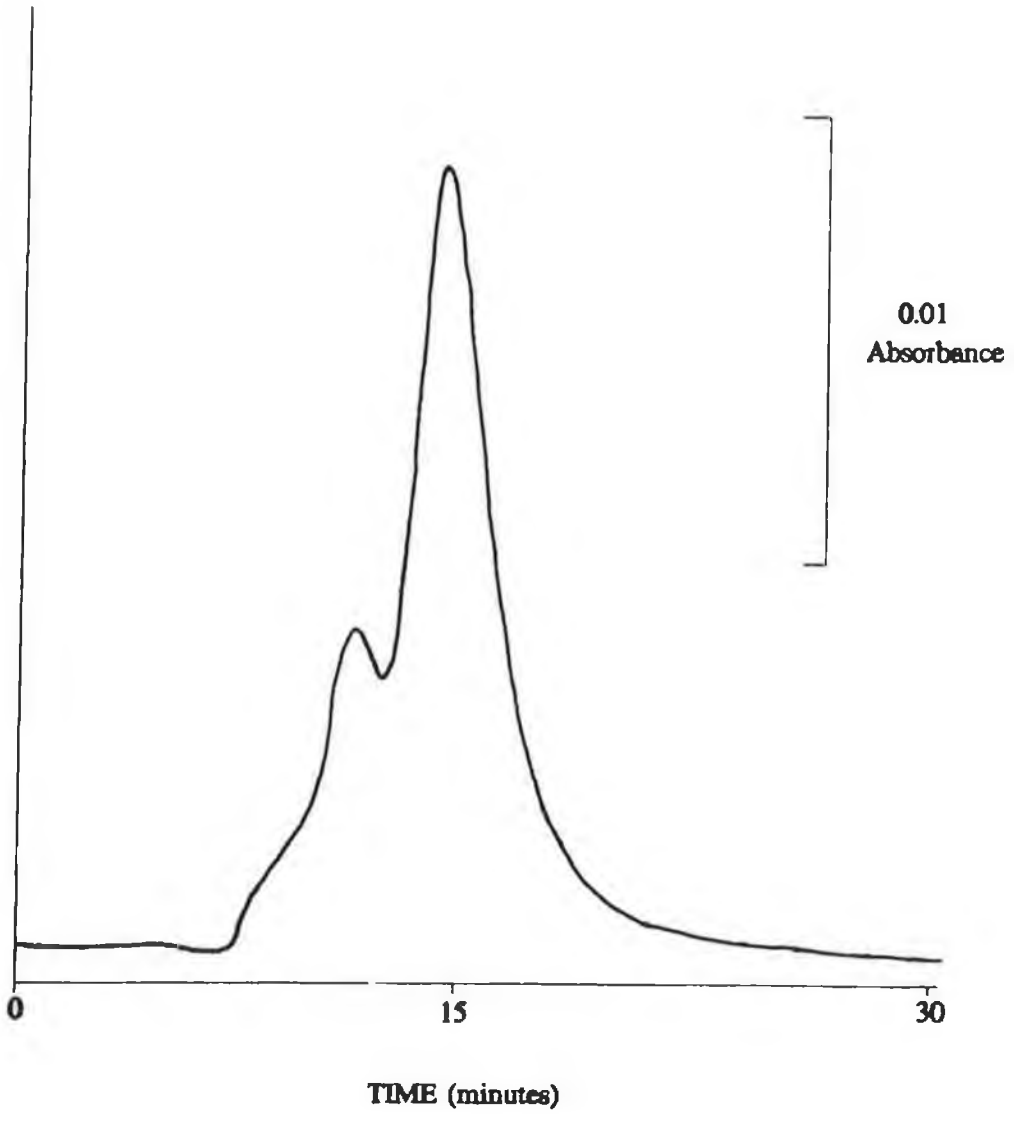


Figure 4.9.1 HPLC chromatogram of commercial IgG antibodies. 10µl of sample was applied to a Waters Protein Pak SW 300. The mobile phase was 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. The presence of protein was detected by monitoring absorbance at 280nm. A retention time of 14.5 minutes was obtained for IgG. A smaller peak was also obtained which could have been caused by the protein added by the manufacturer as a stabilising additive.

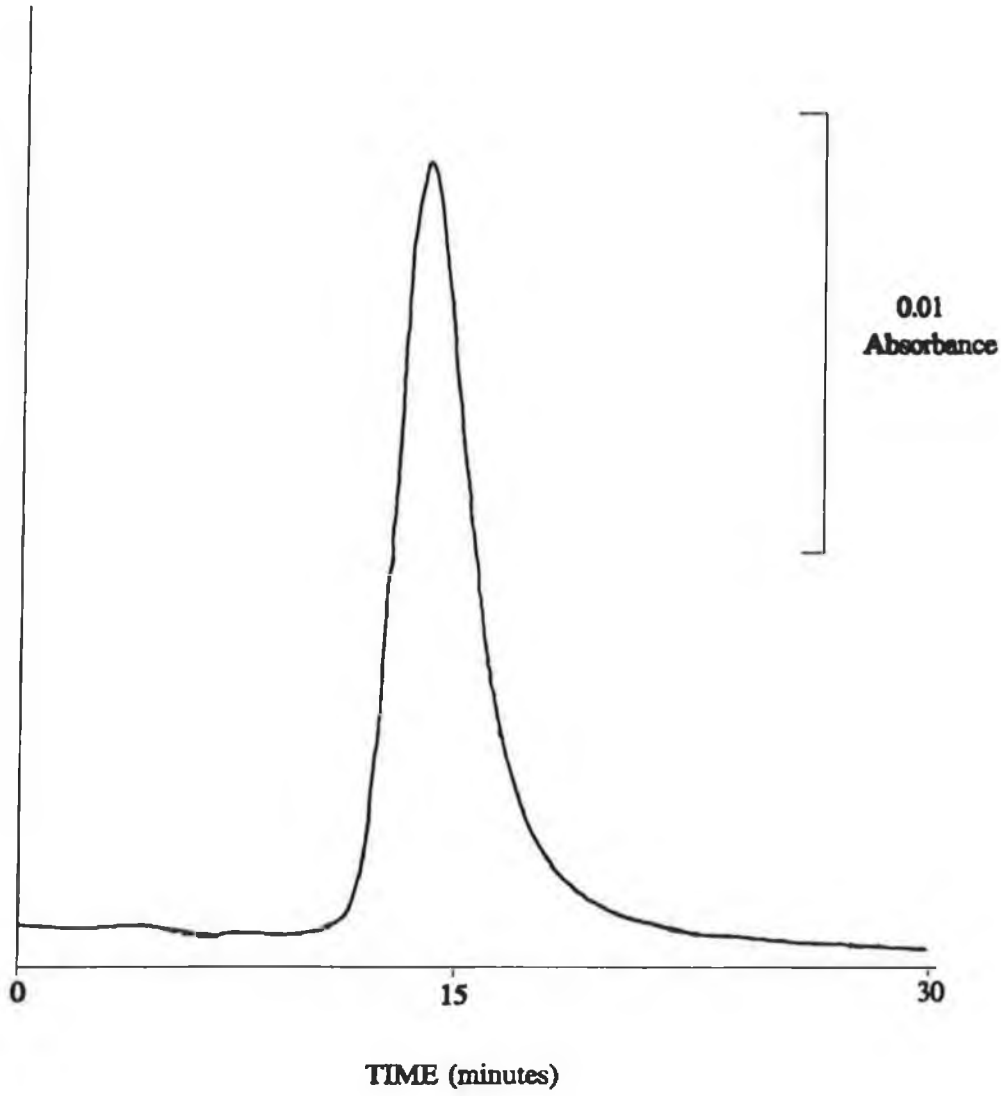


Figure 4.9.2 HPLC chromatogram of polyclonal anti-rFVIII antibodies after Protein A affinity purification.

10µl of sample was applied to a Waters Protein Pak SW 300. The mobile phase was 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. The presence of protein was detected by monitoring absorbance at 280nm. A peak with a retention time of 14.3 minutes was obtained corresponding to pure IgG.

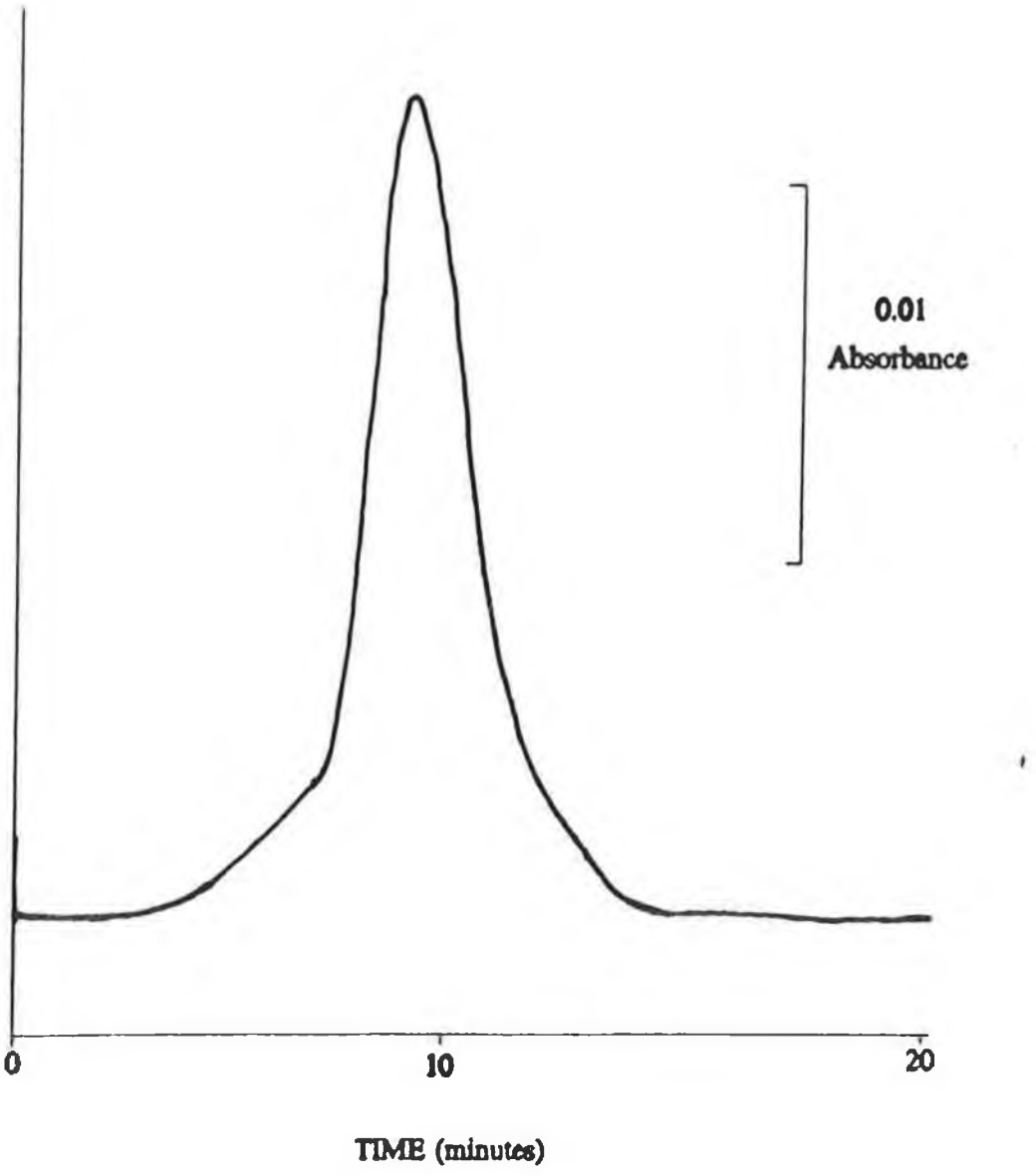


Figure 4.9.3 HPLC chromatogram of commercial IgM antibodies.
10µl of sample was applied to a Waters Protein Pak SW 300. The mobile phase was 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. The presence of protein was detected by monitoring absorbance at 280nm. A peak with a retention time of 9.8 minutes was obtained for IgM.

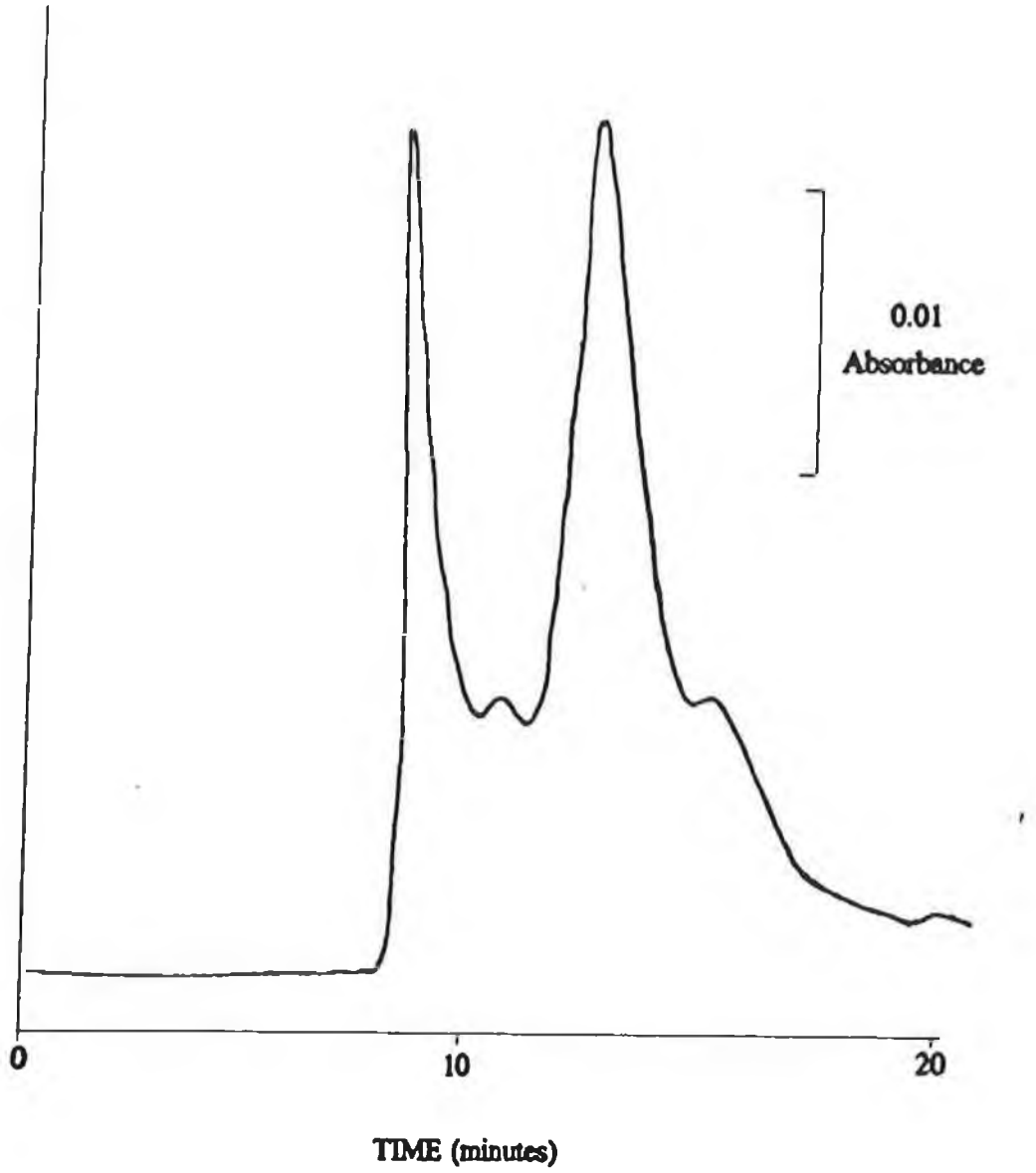


Figure 4.9.4 HPLC chromatogram of M10 monoclonal anti-rFVIII antibody after Protein A affinity purification.

10µl of sample was applied to a Waters Protein Pak SW 300. The mobile phase was 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. The presence of protein was detected by monitoring absorbance at 280nm. Two peaks were present with retention times of 9.2 and 14.4 minutes respectively. The first peak corresponds to IgM. The second peak, with a retention time of 14.4 minutes, was most likely fragments of the IgM molecule. These are IgG-like molecules with a molecular weight of around 200 kDa formed when the IgM molecule was reduced.

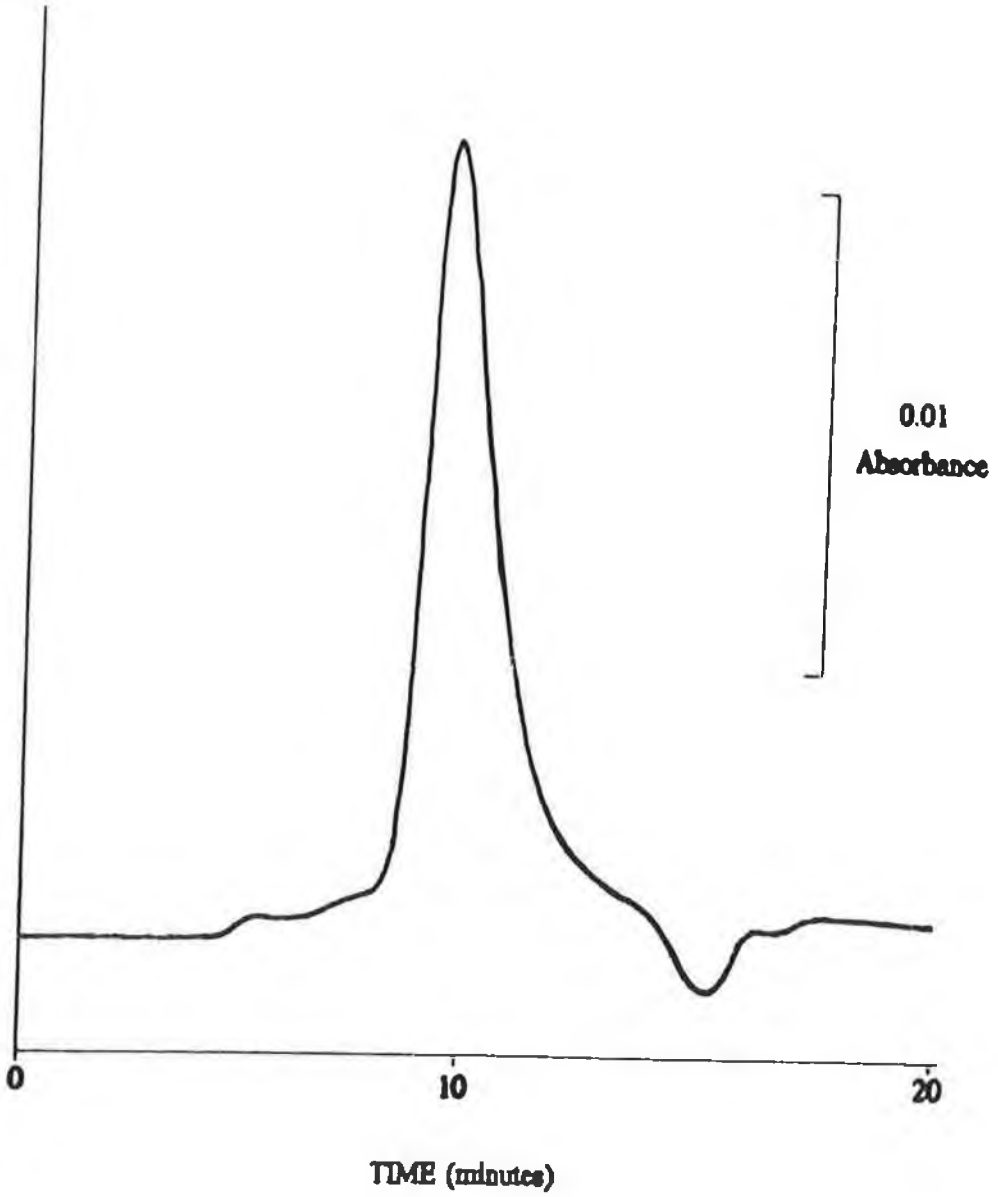


Figure 4.9.5 HPLC chromatogram of M2 monoclonal anti-rFVIII antibody after affinity purification.

10µl of sample was applied to a Waters Protein Pak SW 300. The mobile phase was 0.1M phosphate buffer, pH 7.0, with a flow rate of 0.5ml/min. The presence of protein was detected by monitoring absorbance at 280nm. A peak with a retention time of 9.4 minutes was obtained indicating IgM.

4.10 Electrophoresis

The purity of M10 monoclonal antibody was assessed using SDS-PAGE (Section 2.18). The unreduced form of the antibody was too large to enter the gel, but when run under reducing conditions in the presence of 2-mercaptoethanol, bands corresponding to molecular weights of approximately 80 and 30 kDaltons (kDa) were observed (Figure 4.10). These are characteristic of reduced fragments of IgM. Another band was visible, corresponding to a molecular weight of around 180 kDa. This could be an IgG-like fragment resulting from incomplete reduction of the IgM molecule.

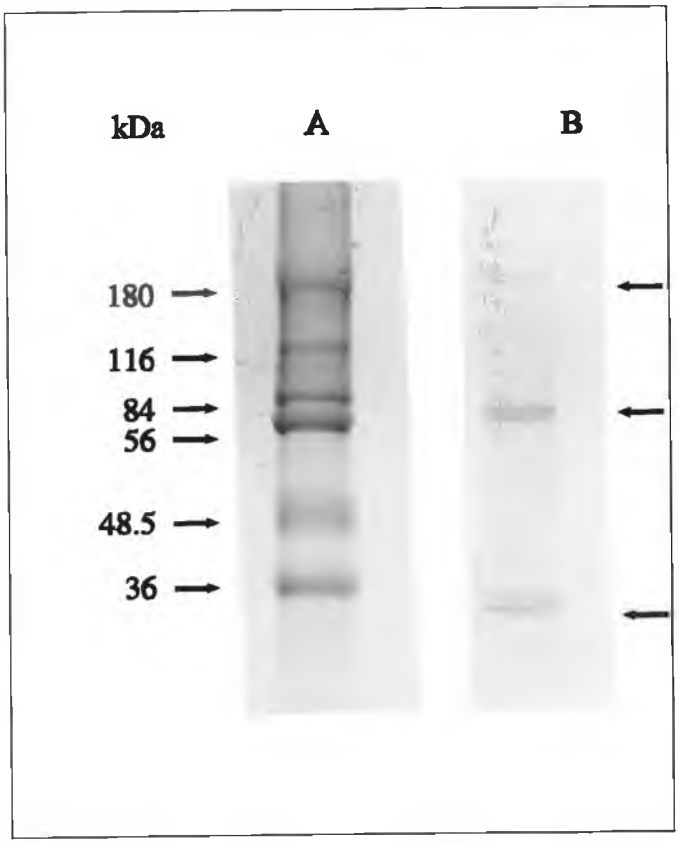


Figure 4.10 Assessment of the purity of M10 monoclonal antibody by SDS-PAGE. A sample of the purified antibody was run under reducing conditions in the presence of 2-mercaptoethanol (lane B), as well as molecular weight markers (lane A). In lane B, bands were visible corresponding to the characteristic bands for reduced IgM molecules - 80 kDa and 30 kDa. Another band was visible, corresponding to a molecular weight of approximately 180 kDa. This could be an IgG-like fragment of the IgM molecule which had not been fully reduced.

4.11 Western Blot Analysis

rFVIII was cleaved into its heavy and light chains by thrombin and separated by electrophoresis (Figure 4.11.1). These were then transferred onto nitrocellulose paper and probed as described in Section 2.19, using the monoclonal antibodies, to attempt to identify the binding sites of the antibodies. No sites could be identified, as the antibodies did not bind to the separated chains (Figure 4.11.2). A dot blot was carried out on rFVIII in sample buffer (Figure 4.11.3) and again the antibodies did not bind to the protein. The epitope, therefore, must be destroyed during unfolding of the protein in the presence of SDS in the sample buffer.

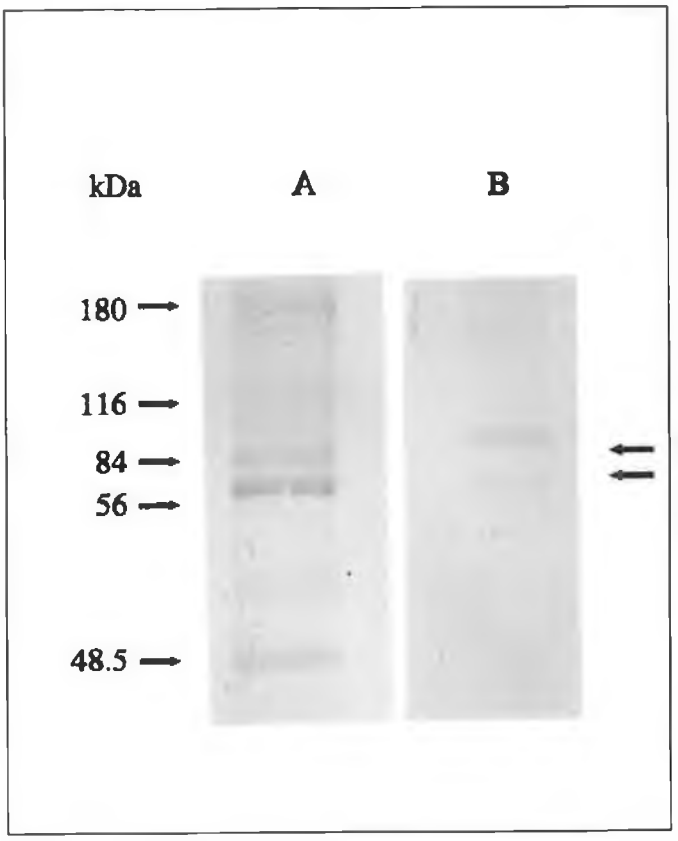


Figure 4.11.1 Identification of the heavy and light chains of FVIII formed by thrombin digestion. rFVIII was reacted with 1 IU/ml of thrombin and then subjected to SDS-PAGE (lane B). The bands correspond to the 90 kDa heavy chain, consisting of the A1-A2 domains, and the 80 kDa light chain comprised of the A3-C1-C2 domains. Molecular weight markers were also included (lane A).

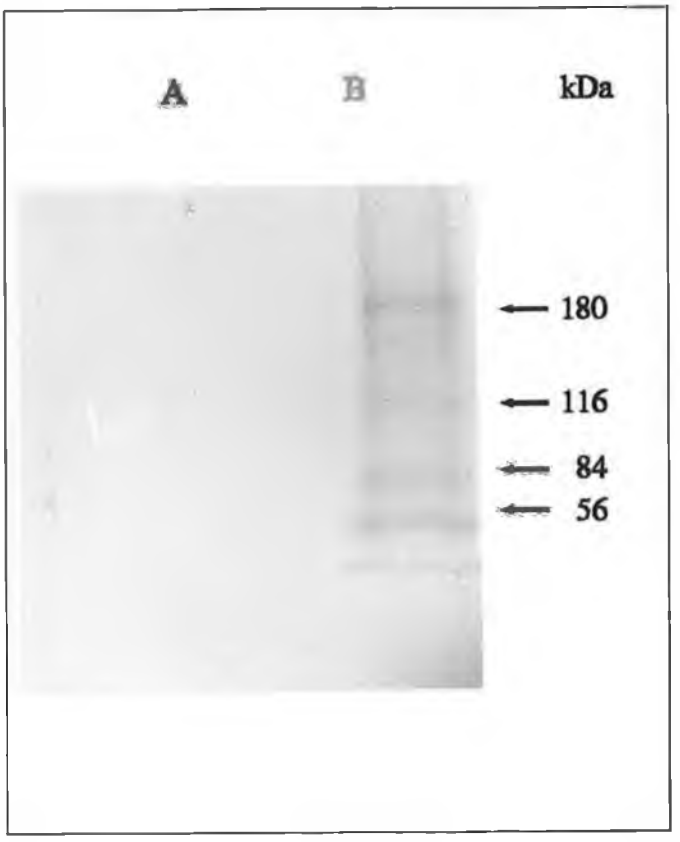
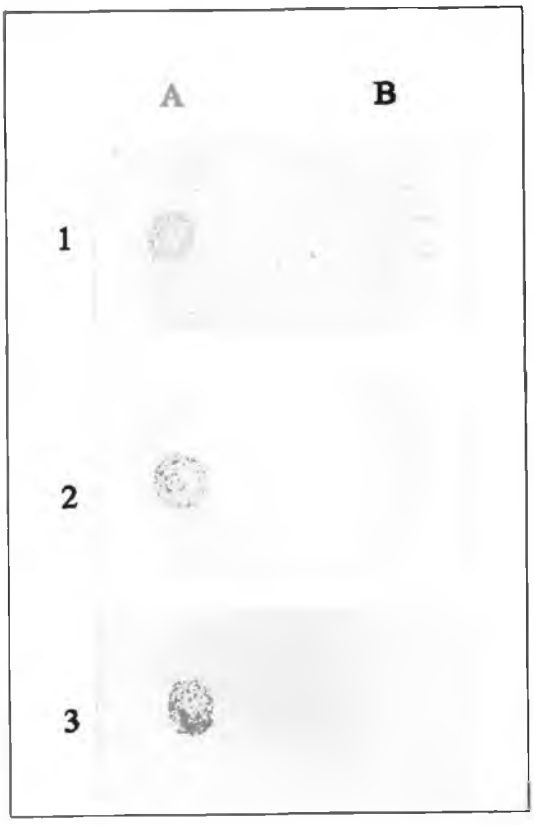


Figure 4.11.2 Western blot analysis of M1 monoclonal antibody.
rFVIII was separated into its heavy and light chains by thrombin digestion, and then subjected to SDS-PAGE (lane A), along with molecular weight markers (lane B). Separated bands were transferred onto nitrocellulose paper and probed using the monoclonal antibodies. No bands were identified by any of the three antibodies. The epitopes were most probably destroyed during some part of the experiment.



	A rFVIII DILUTED IN PBS	B rFVIII DILUTED IN SAMPLE BUFFER
1	M1	M1
2	M2	M2
3	M10	M10

Figure 4.11.3 Dot blots using three monoclonal antibodies on rFVIII before and after dilution in SDS-PAGE sample buffer.

rFVIII in 0.15M PBS, pH 7.2 (column A) and SDS-PAGE sample buffer (column B) was dotted onto nitrocellulose paper and probed with the three monoclonal antibodies (row 1: M1, row 2: M2, row 3: M10). When rFVIII was diluted in sample buffer (column B), none of the monoclonal antibodies bound to it, indicating that the binding epitopes of the antibodies were destroyed in the presence of SDS.

4.12 The Effects of Antibody binding on FVIII Procoagulant Activity

rFVIII was reacted with both the polyclonal and the monoclonal antibodies to determine whether such binding caused any change in the procoagulant activity.

Binding of the polyclonal antibodies did not cause any loss of procoagulant activity (Table 4.12.1), whereas the binding of all three monoclonals caused complete loss of coagulant activity (Table 4.12.2).

Concentration of antibody	ΔA/min ± S.D. at 405nm
Control (No antibody)	.171 ± .012
5 µg/ml	.132 ± .011
10 µg/ml	.142 ± .009
50 µg/ml	.153 ± .015
100 µg/ml	.149 ± .010

Table 4.12.1 The effects of binding of polyclonal antiserum on rFVIII procoagulant activity.

Increasing concentrations of antibody were reacted with rFVIII, and FVIII procoagulant activity was measured using the chromogenic assay. Here, activity was calculated as a function of the change in absorbance per minute (ΔA/min) at a defined wavelength. The binding of the antibodies did cause some reduction in procoagulant activity, especially at the lower antibody concentrations (5 and 10µg/ml), but approximately 90% of the control activity remained when the higher concentrations of antibody bound to rFVIII.

S.D.: Standard deviation where n=3.

Monoclonal Antibody (10 µg/ml)	ΔA/min at 405nm ± S.D.
Control (No antibody)	.155 ± .007
M1	.002 ± .000
M2	.006 ± .001
M10	.004 ± .001

Table 4.12.2 Effects of binding of monoclonal anti-rFVIII antibodies on rFVIII procoagulant activity.

10µg/ml of each monoclonal antibody was reacted with FVIII and FVIII activity measured by chromogenic assay. Here, activity was calculated as a function of the change in absorbance per minute (ΔA/min) at a defined wavelength.

The binding of all three monoclonals antibodies cause a complete loss of procoagulant activity.

S.D.: Standard deviation where n=3.

The polyclonal antibody/rFVIII complex was subjected to denaturation as described in Section 2.5 to investigate whether the antibody binding imparted additional thermostability on rFVIII (Figure 4.12.1). Although some slight improvement was observed, no significant increase in thermostability was obtained.

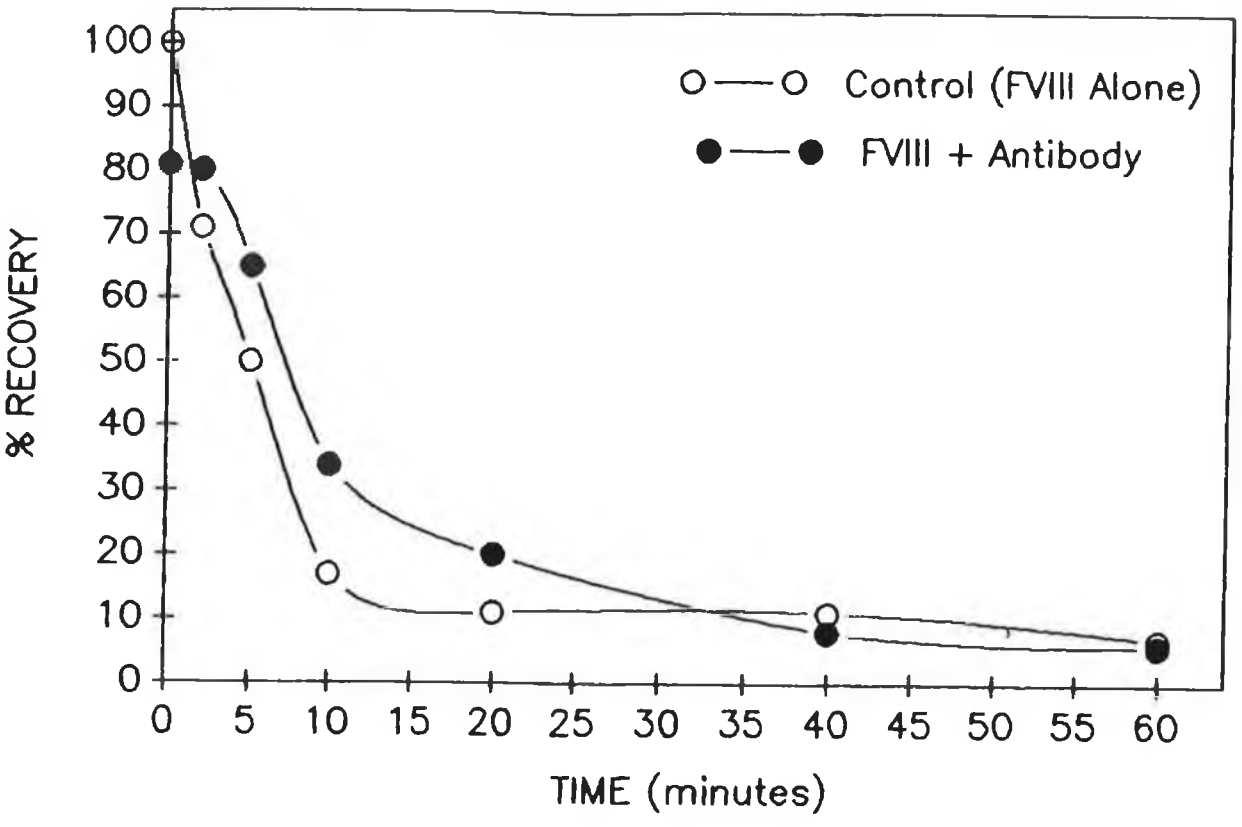


Figure 4.12.1 Thermodeactivation at 55°C of native rFVIII and rFVIII reacted with polyclonal anti-rFVIII antibody.
10µg/ml of anti-rFVIII antibody in 0.1M PBS, pH 7.2, was reacted with rFVIII in 0.1M Tris-HCl buffer, pH 7.4, for 1 hour at 37°C. Thermodeactivation was carried out to investigate whether the binding of the antibody would increase the thermostability of rFVIII. No significant increase was observed

4.13 Discussion

2 fusions were carried out between immunised mouse splenocytes and NSO murine myeloma cells. From these, a total of 263 hybridomas were obtained, yielding three monoclonals. These antibodies were identified by isotyping kits and by HPLC analysis as belonging to the IgM class.

IgM antibodies form the major proportion of the primary response to an antigen. In the secondary antigenic challenge, the antibody response is quicker, has a higher titre and consists mainly of IgG.

Immunization schedules are usually structured so as to ensure that the antibody response is in the secondary phase, and the antibodies are, therefore, most likely to be of the IgG class. The schedule followed for production of anti-rFVIII antibodies involved repeated immunization over a three month period. This would normally be sufficient for the production of IgG antibodies. In this case it appears that mainly IgM antibodies were produced. This would suggest that the animal was immune-compromised. Tests were carried out on the animals and they were diagnosed as being infected with mouse hepatitis virus (MHV). This is a highly infectious, single-stranded RNA virus of the genus *Coronovirus*. It infects many species of mammals and birds, causing respiratory or enteric infections. Many of the infections are subclinical.

The effects of this infection on the immune system of mice have been reported. Infected mice had a modified immune response to sheep red blood cells (Barthold, 1986). Immunodepression occurred during acute infections, if the mice were infected prior to antigen exposure (Barthold, 1986). Chronic immune-depression occurred in semi-susceptible mice with chronic infections (Virelizier, 1976). Infected mice show transient but significant functional disturbances in T and B lymphocytes (Barthold, 1976). It is not known whether the effects are due to direct or indirect action on the lymphoid tissue.

It appears therefore, that the infected mice were immunosuppressed. This accounts for the production of IgM rather than IgG antibodies.

A total of 1 x 10^8 spleen cells was fused with 2.75 x 10^7 myeloma cells (a ratio of approximately 4:1). From this, 263 hybridomas were produced - a frequency of approximately 1 hybridoma per 5 x 10^5 cells. The hybridisation frequency is difficult to predict, and is dependent on many factors, including the number of stimulated B lymphocytes and the ratio of cell types. Generally however, the hybridisation frequency for a murine system is within the range 1-100 clones per 10^7 cells (Campbell, 1984). The presence of the MHV therefore, does appear to have affected the hybridization frequency as it is up to 100-fold lower than normally expected.

The choice of method of purification for IgM antibodies is limited as compared with methods available for IgG purification. IgMs are easily denatured by reduction of the disulphide bonds between the monomers. Recommended methods of purification include an initial precipitation step with saturated ammonium sulphate, followed by gel or anion-exchange chromatography (Oppermann, 1993). Other methods have recently been suggested, including polyethylene glycol precipitation (Noeh, 1986; Tatum, 1993).
Here, three methods of purification were compared. Affinity chromatography, using rFVIII as a ligand, was an rapid and easy method of protein purification. As regards antibody purification, the availability of antigen in sufficient quantities may pose a problem. In this case, the quantity of rFVIII available was severely limited, allowing for only a small column to be prepared, and thus only small sample volumes to be applied. While the antibody eluted from the column was pure (Figure 4.8.1), the protein concentration was low and the final volume, small. Purification on a large scale would therefore be expensive and not always practical.

Protein A is a 42,000 molecular weight membrane protein isolated from *Staphylococcus aureus*. It exhibits a high binding capacity for the Fc portion of an IgG antibody (Tijssen, 1985). Polyclonal anti-rFVIII antibodies were successfully purified using this method. IgM, eluted from a Protein A column and analysed on HPLC, consisted of high molecular weight proteins (IgM) as well as proteins with molecular weight close to or slightly higher than that of an IgG molecule (Figure 4.9.4). These would appear to be fragments of the IgM molecule which have been denatured in the elution buffer, or by passage through the column.

The fractions containing these fragments retain anti-rFVIII activity, but this may also be due to the presence of intact IgM molecules.
Protein A chromatography is not, therefore, recommended for purification of IgM antibodies as the final product while being relatively pure, was not necessarily a homogenous preparation of antibody.

Gel filtration is the recommended method for the purification of IgM antibodies. It is a relatively rapid, one-step method, capable of handling large and small volume samples. It was found that in this method, using a Sepharose-6B column, the monoclonal antibody eluted in the void volume. The remaining proteins eluted in later fractions. When the anti-rFVIII containing fraction was analysed by SDS-PAGE, the whole molecule was too large to enter the gel. When the molecule was reduced, the characteristic bands of a reduced IgM molecule were observed (Figure 4.10).
Thus, of the three methods investigated, gel-filtration was found to be the method of choice for the purification of IgM antibodies. It does not involve the harsh conditions (low pH buffers) necessary in affinity chromatography, which can cause denaturation of the antibodies.
Polyclonal antibodies were successfully purified using Protein A affinity chromatography, after an initial ammonium sulphate precipitation.

The effects of the binding of both polyclonal and monoclonal antibodies on rFVIII procoagulant activity was investigated. Both sets of antibodies reacted with rFVIII in an ELISA system. Binding of all three of the monoclonals resulted in the complete loss of rFVIII procoagulant activity. Binding of the polyclonal antisera resulted in some loss of procoagulant activity, but at higher concentrations (50 and 100µg/ml), approximately 90% of the control activity was retained. The fact that the monoclonal antibodies were of the IgM class could account for this major loss of activity. In binding, the antibody may cause some sort of distortion within the rFVIII tertiary structure, and given rFVIII's instability this may lead to loss of procoagulant activity. Also, the antibodies may be blocking the active site of the cofactor. The actual epitopes to which the monoclonals were binding could not be determined, as they were destroyed by unfolding of the protein.

This suggests that the epitopes were in fact part of the tertiary structure. The subsequent loss of activity is probably, therefore, a combination of both of these factors.
Binding of the polyclonal antibodies did not cause any significant loss of procoagulant activity, especially at relatively high antibody concentrations (50 and 100µg/ml). These antibodies were of the IgG class, and therefore do not cause the same degree of strain when binding to an antigen. None of the antibodies appear to bind to the active site or adjacent to it. Antibody binding to rFVIII may give rise to additional, possibly thermostabilising intramolecular bridge(s). No increase in thermostability was observed in the presence of antibody, however.

The antibodies will be used in later chapters in the development of a number of methods, including an ELISA and an antibody-based immunosensor, for the detection and determination of rFVIII (and FVIII) and anti-rFVIII antibodies. This is a critical area for the diagnosis and treatment of haemophilia.

CHAPTER 5

DEVELOPMENT OF AN ELISA FOR THE DETECTION OF rFVIII AND ANTI-rFVIII ANTIBODIES

5.1 Introduction

FVIII can be detected by either its coagulant activity (FVIII:C) or immunologically (FVIIIC:Ag) (Section 1.12). FVIII coagulant activity can be measured by one-stage, two-stage or chromogenic assays (Section 1.12.1). Inhibitor antibodies or autoantibodies have traditionally been used to immunologically detect FVIII using a range of immunoassays, including radioimmunoassays and enzyme immunoassays (Section 1.12.2). In approximately 10% of cases of severe haemophilia, *i.e.* patients have less than 5% of normal FVIII coagulant activity, FVIII can be detected immunologically, even though there is no procoagulant activity (Section 1.9.1). It is important to be able to identify and diagnose these patients with different FVIII:C and FVIIIC:Ag patterns, as it is critical in the treatment of haemophilia.

The risks associated with the use of blood-derived products are well documented. Haemophiliacs are a particularly high-risk group, and it has been estimated that 60% of haemophiliacs have been infected with HIV from contaminated blood products (Fox, 1992). Thus, the risks associated with the use of inhibitor or autoantibodies from these patients are enormous. Recent advances in immunotechnology, especially the developments of monoclonal antibody production, has meant that antibodies with defined specifity and affinity can now be produced. These antibodies provide a safer alternative to inhibitor and autoantibodies for use in immunoassays. Recombinant FVIII is being increasingly used in the treatment of haemophilia (Section 1.9.2). It provides a contaminant-free alternative to human-derived FVIII, reducing the possibility of contamination from blood-borne viruses.

In this section the development of a number of ELISA systems is described using polyclonal and monoclonal anti-rFVIII antibodies generated previously (Chapter 4). The ELISAs provide a safe, rapid and reliable method for the detection of rFVIII and FVIII in normal human plasma. Anti-rFVIII antibodies can also be detected and determined.

5.2 Antigen-Capture ELISA

5.2.1 Binding of anti-rFVIII antibody to the solid phase

The binding of antibody to the solid phase in an ELISA is critically important as it provides the basis for the whole assay. The binding of five anti-FVIII antibodies (rabbit polyclonals, three monoclonal antibodies and a commercial polyclonal sheep anti-FVIII antibody) to a number of microtitre plates was examined. Three different binding buffers (50mM carbonate/bicarbonate, pH 9.6, 10mM Tris/HCl containing 100mM NaCl, pH 8.5 and 0.15M PBS, pH 7.2), in conjuction with several combinations of incubation times and temperatures, were examined. An IgG ELISA (Section 2.23.2) was used to investigate the binding properties of the antibodies to the plates under the different conditions. Antibody was bound to the plate and free binding sites blocked using a 1% (w/v) solution of BSA. Bound antibody was then detected using an alkaline phosphatase-labelled anti-rabbit antibody and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer. Absorbance was read at 620nm.

Nunc-Immuno module Maxisorb F8 strips gave the highest signals with minimum background "noise". Adsorption in carbonate/bicarbonate buffer with subsequent incubations at 37°C for 1 hour were the optimum incubation conditions. Rabbit polyclonal anti-rFVIII antibodies when bound to the Nunc F8 strips gave the highest signal when compared with a commercial anti-FVIII (obtained from Serotec) (Table 5.2.1).

	Absorbance ± S.D. at 620nm	
Antibody concentration (µg/ml)	Rabbit anti-rFVIII	Commercial anti-FVIII
1	.059 ± .001	.070 ± .002
2	.385 ± .033	.133 ± .002
5	.653 ± .052	.173 ± .005
10	.848 ± .028	.172 ± .011
15	.930 ± .073	.170 ± .001
20	1.174 ± .067	.167 ± .012
PBS	.000 ± .000	.000 ± .000

Table 5.2.1 The binding of two anti-FVIII antibodies, (a commercial sheep anti-FVIII, and a rabbit anti-rFVIII produced in the laboratory) to the surface of a Nunc-Immuno module Maxisorb microtitre well is shown. These antibodies were bound to the solid phase, and subsequently detected using alkaline phosphatase-labelled anti-rabbit or anti-sheep antibodies and 5-bromo-4-chloro-3-indolyl phosphate as substrate.
S.D. is the standard deviation where n = 3.

5.2.2 Blocking of free binding sites

The choice of blocking buffer is another important consideration. The use of an unrelated protein which exhibits no cross-reactivity with any of the components of the ELISA is essential. A number of different proteins including BSA, gelatin ovalbumin and human fibrinogen (a normal constituent of blood) were examined. As Table 5.2.2 shows, the rabbit polyclonal anti-rFVIII antibodies showed no cross-reactivity with any of the protein solutions. For this system, a 1% (w/v) solution of BSA was chosen. BSA is readily available, the antibodies did not cross-react with it and the 1% (w/v) solution is just as effective as the 5% (w/v) solution but is more cost effective. Enzyme-labelled antibodies were diluted in blocking buffer in order to reduced non-specific binding to the plate.

Blocking Buffer	Absorbance ± S.D. at 492nm 5µg/ml rFVIII	Absorbance ± S.D. at 492nm No rFVIII
PBS	.061 ± .002	.000 ± .000
1% (w/v) BSA	.471 ± .017	.057 ± .002
5% (w/v) BSA	.508 ± .021	.061 ± .004
0.25% (w/v) Gelatin	.517 ± .025	.070 ± .002
1% (w/v) Ovalbumin	.569 ± .024	.056 ± .007
1% (w/v) Fibrinogen	.407 ± .037	.061 ± .005

Table 5.2.2. 10µg/ml rFVIII was bound onto Nunc-Immuno module Maxisorb strips. Free binding sites were blocked using 1% and 5% (w/v) bovine serum albumin (BSA), 0.25 % (w/v) gelatin, 1% (w/v) ovalbumin and 1% (w/v) fibrinogen. Bound rFVIII was detected using horseradish peroxidase-labelled anti-rFVIII antibodies.
Controls in which no rFVIII was bound to the plate were included to test for cross-reactivity of the antibody with the blocking buffer.
S.D. is the standard deviation where n= 3.

5.2.3 Choice of Substrate

A variety of substrates for horseradish peroxidase, (HRP, the enzyme label for the secondary antibody) were examined. o-Phenylenediamine (OPD) is a commonly used substrate for HRP, with absorbance measured at 405nm or 492nm (if the reaction has been stopped by addition of 20% (v/v) H_2SO_4). An ELISA was carried out and after addition of OPD, absorbance was monitored at 5 minute intervals over a 60 minute period. From this, the optimum incubation time for the substrate was ascertained (Figure 5.2.3). OPD is a hazardous substance, and so an alternative, safer substrate (3,3′,5,5′-Tetramethylbenzidine, TMB) was examined. TMB, however, gave high background levels of absorbance (Table 5.2.3), and so OPD was retained as substrate for HRP. The minimum detection limit for rFVIII in the antigen-capture ELISA was 0.1µg/ml, with an optimum rFVIII concentration of 10µg/ml.

Antibody (10µg/ml)	Absorbance ± S.D. at 492nm (OPD)	Absorbance ± S.D. at 450nm (TMB)
Rabbit Polyclonal	.123 ± .010	.390 ± .006
M1 Monoclonal	.151 ± .017	.302 ± .016
M2 Monoclonal	.144 ± .020	.296 ± .001
M10 Monoclonal	.131 ± .003	.259 ± .006
PBS	.046 ± .001	.272 ± .006

Table 5.2.3 Comparison between o-phenylenediamine (OPD) and 3,3′,5,5′-tetramethylbenzidine (TMD) as substrates for horseradish peroxidase. rFVIII was bound to the solid phase and detected using horseradish peroxidase-labelled anti-rFVIII antibody. The substrates were then added and absorbance read at the respective wavelength.
S.D. is the standard deviation where n=3.

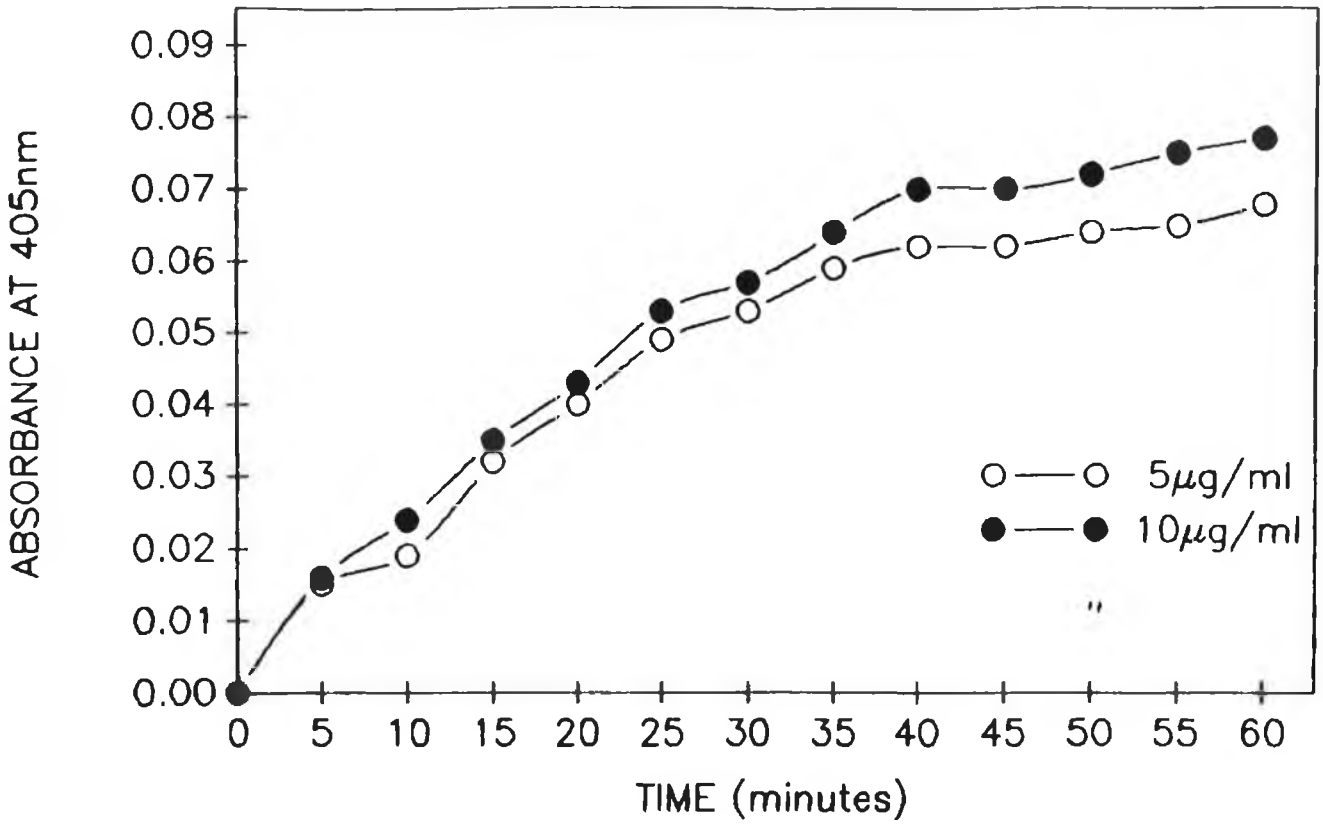


Figure 5.2.3 Substrate kinetics for o-phenylenediamine (OPD).
Anti-rFVIII antibody was bound to Nunc Maxisorb F8 strips and rFVIII added. Bound rFVIII was detected using horseradish peroxidase (HRP)-labelled anti-rFVIII antibodies and OPD. Absorbance at 405nm was read at 5 minute intervals over 60 minutes.

5.3 ELISA using a goat anti-rabbit antibody linking layer

In order to increase the sensitivity of the ELISA, a layer of goat anti-rabbit antibody was first coated, (in 50mM carbonate/bicarbonate buffer, pH 9.6) onto the bottom of the well. A range of concentrations were bound to the plate, and an optimum was found to be 10µg/ml (Figure 5.3.1). This immobilised anti-rabbit antibody captured and bound the rabbit anti-rFVIII antibody, which was in turn was detected using a goat anti-rabbit antibody labelled with alkaline phosphate (GAR-AP). Colour was developed with BCIP in AMP buffer. The absorbances obtained using this method were on average higher than those observed in the corresponding IgG ELISAs, where the anti-rFVIII antibodies were bound directly to the plate. The detection limit was improved by a factor of 50 allowing concentrations as low as 10ng of antibody to be detected (Figure 5.3.2).

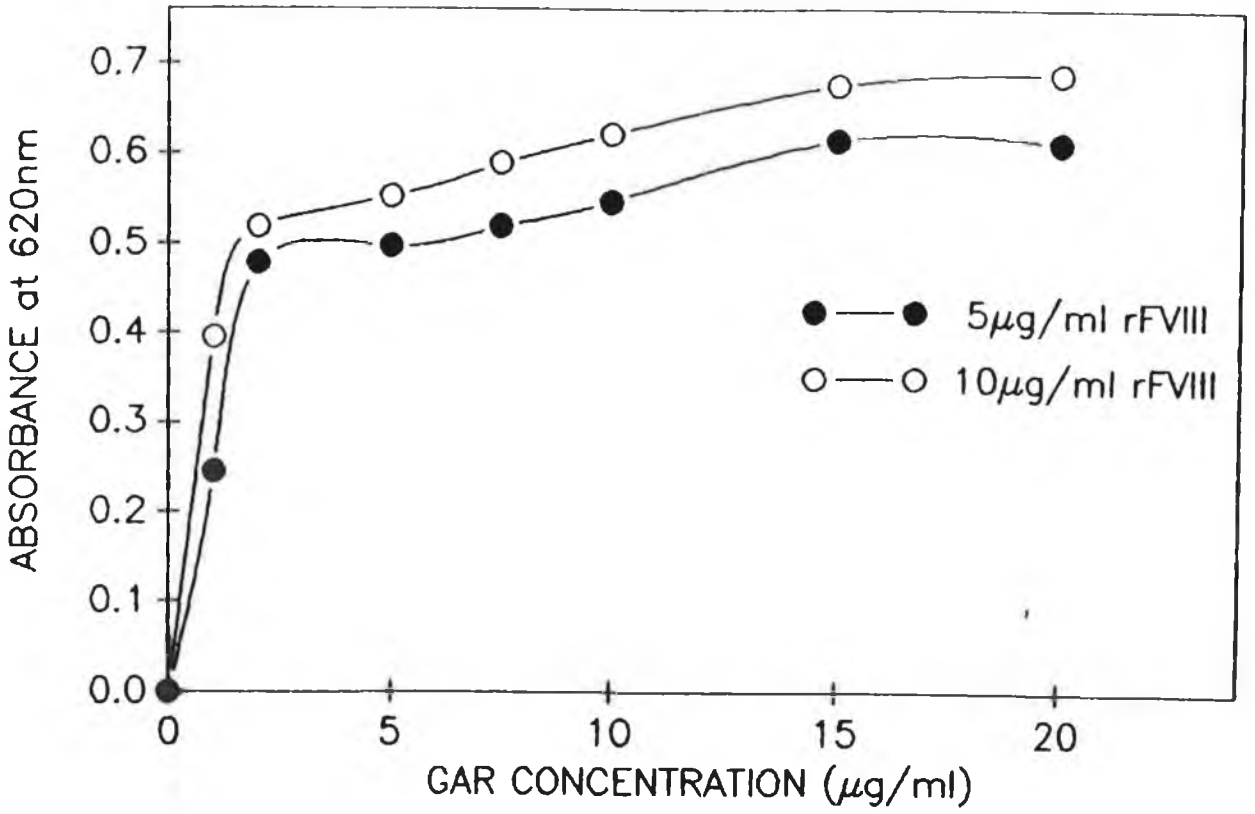


Figure 5.3.1 Optimisation of the binding of goat anti-rabbit antibody (GAR) onto Nunc Maxisorb F8 strips. A range of concentrations of GAR were bound to the solid phase and detected using an alkaline phosphatase-labelled anti-goat antibody and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer.

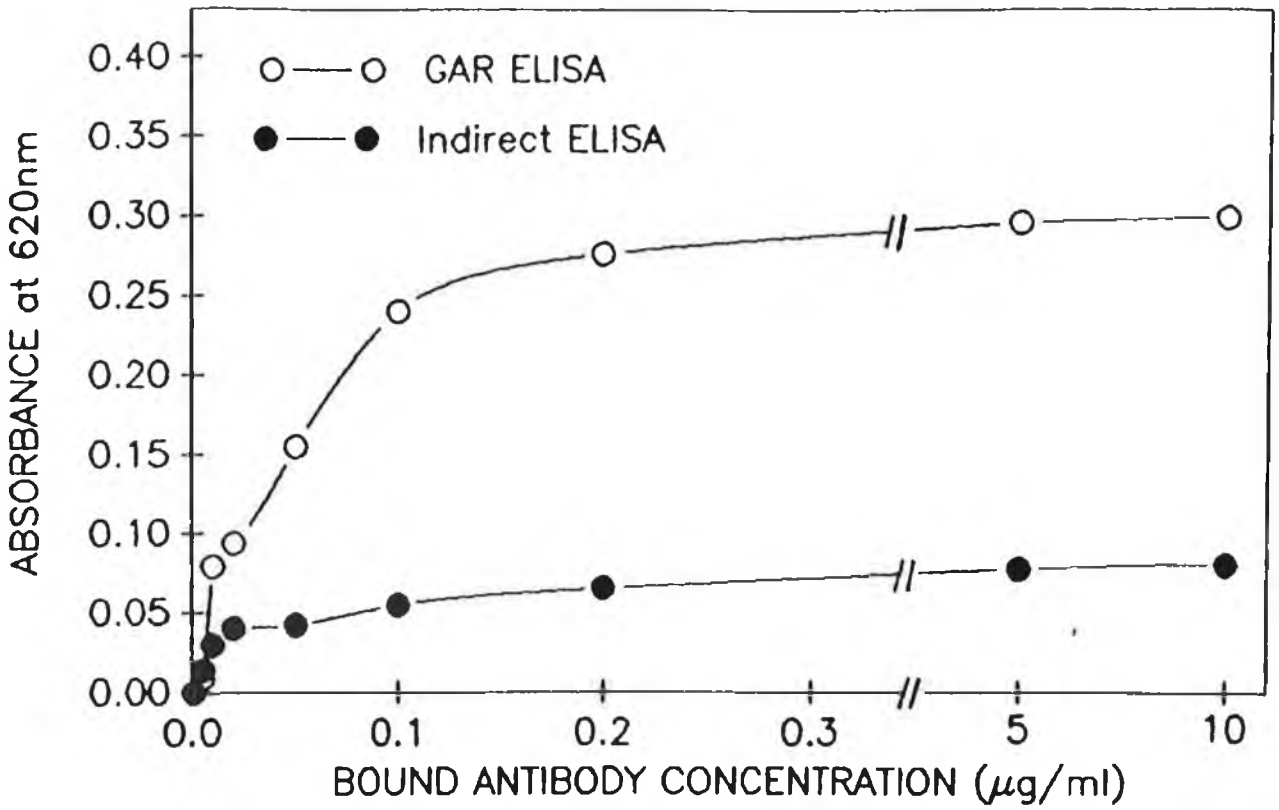


Figure 5.3.2 Comparison between the goat anti-rabbit (GAR) and direct ELISA for the detection of rFVIII.

In the GAR ELISA, a layer of GAR antibody was used to capture rabbit anti-rFVIII antibodies. These were detected with alkaline phosphatase-labelled anti-rabbit antibody and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer.

In the Indirect ELISA, the anti-rFVIII antibodies were bound directly to the plate and detected in the same way.

5.4 Indirect ELISA

Using the results obtained in the antigen-capture ELISA, an indirect ELISA was developed, where rFVIII was bound directly to the solid phase. The solid phase, coating buffer, incubation conditions and blocking buffer were all the same as those used above (Sections 5.2.1 and 5.2.2). After coating and blocking, anti-rFVIII antibody in PBS (0.15M, pH 7.2) was added. Any bound IgG was detected using GAR-AP in blocking buffer. Colour was developed using BCIP in AMP buffer, and absorbance was read at 620nm. A range of concentrations of the rFVIII antigen was bound to the plate (0.1-20µg/ml) and an optimal concentration found to be 5µg/ml. (Figure 5.4.1).

The four antibodies were all tested in this system, and similar binding patterns to those found in the capture ELISA were obtained. The rabbit polyclonal antibodies again proved to be more reactive than the monoclonal antibodies (Table 5.4). The optimal concentration for the rabbit polyclonal antibodies was found to be 10µg/ml (Figure 5.4.2) and the lowest concentration at which rFVIII (which was bound to the plate) could be determined was 0.2µg/ml.

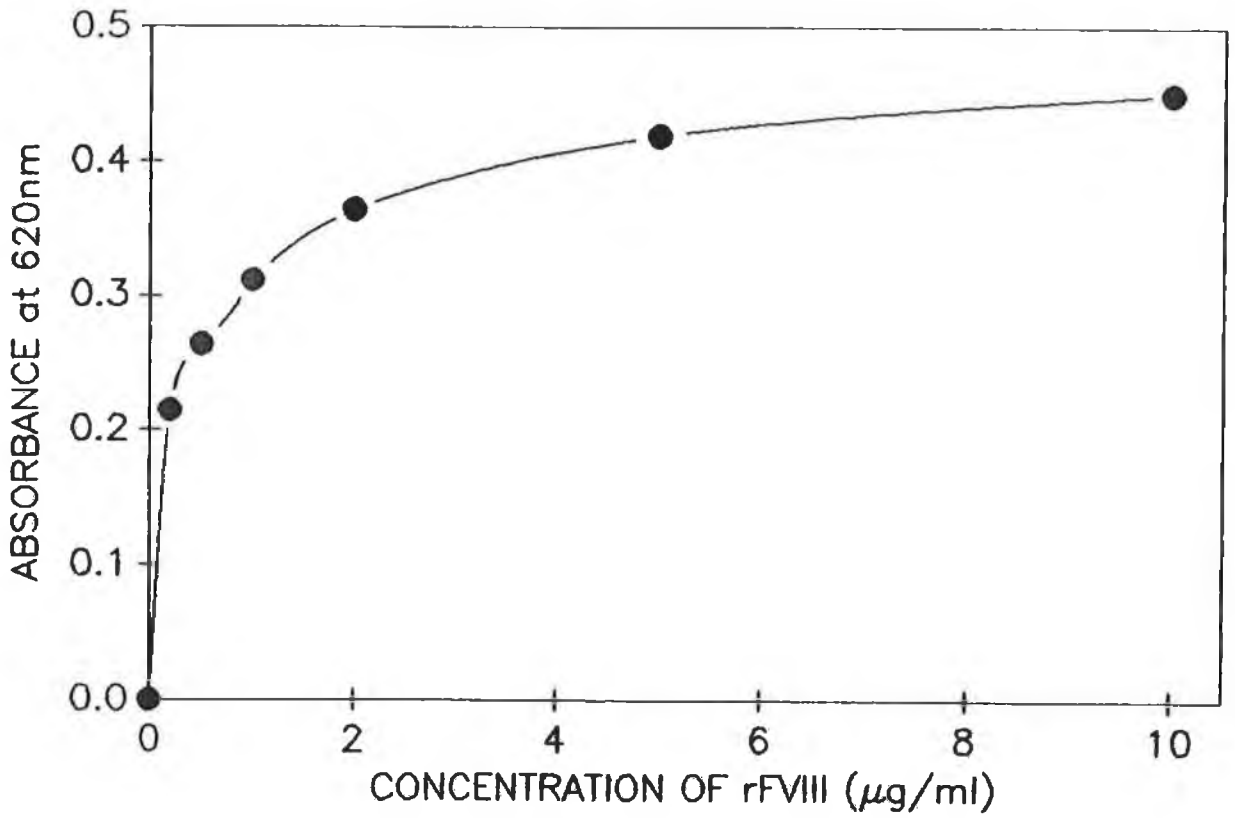


Figure 5.4.1 Binding of rFVIII to the solid phase for use in an indirect ELISA. A range of concentrations of rFVIII were bound to Nunc Maxisorb F8 strips. Anti-rFVIII antibodies were added and bound antibody detected using alkaline phosphate-labelled goat anti-rabbit antibody and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer.

	Absorbance ± S.D. at 620nm		
Antibody concentration (μg/ml)	M1 Monoclonal	M2 Monoclonal	M10 Monoclonal
1	.070 ± .001	.057 ± .003	.061 ± .005
2	.088 ± .002	.062 ± .006	.067 ± .007
5	.089 ± .005	.071 ± .002	.073 ± .002
10	.080 ± .007	.091 ± .008	.095 ± .007
PBS	.000 ± .000	.000 ± .000	.000 ± .000

Table 5.4 The reaction of four antibodies with different concentrations of rFVIII in an indirect ELISA. rFVIII was bound to the solid phase, and the anti-rFVIII antibodies added. Bound antibody was detected using alkaline phosphatase-labelled antibodies and 5-bromo-4-chloro-3-indoyl phosphate as substrate.
S.D. is the standard deviation where n = 3.

The substrate characteristics were also examined, with absorbance at 620nm being monitored over 60 minutes. The optimal incubation period was taken to be 30 minutes (Figure 5.4.3).

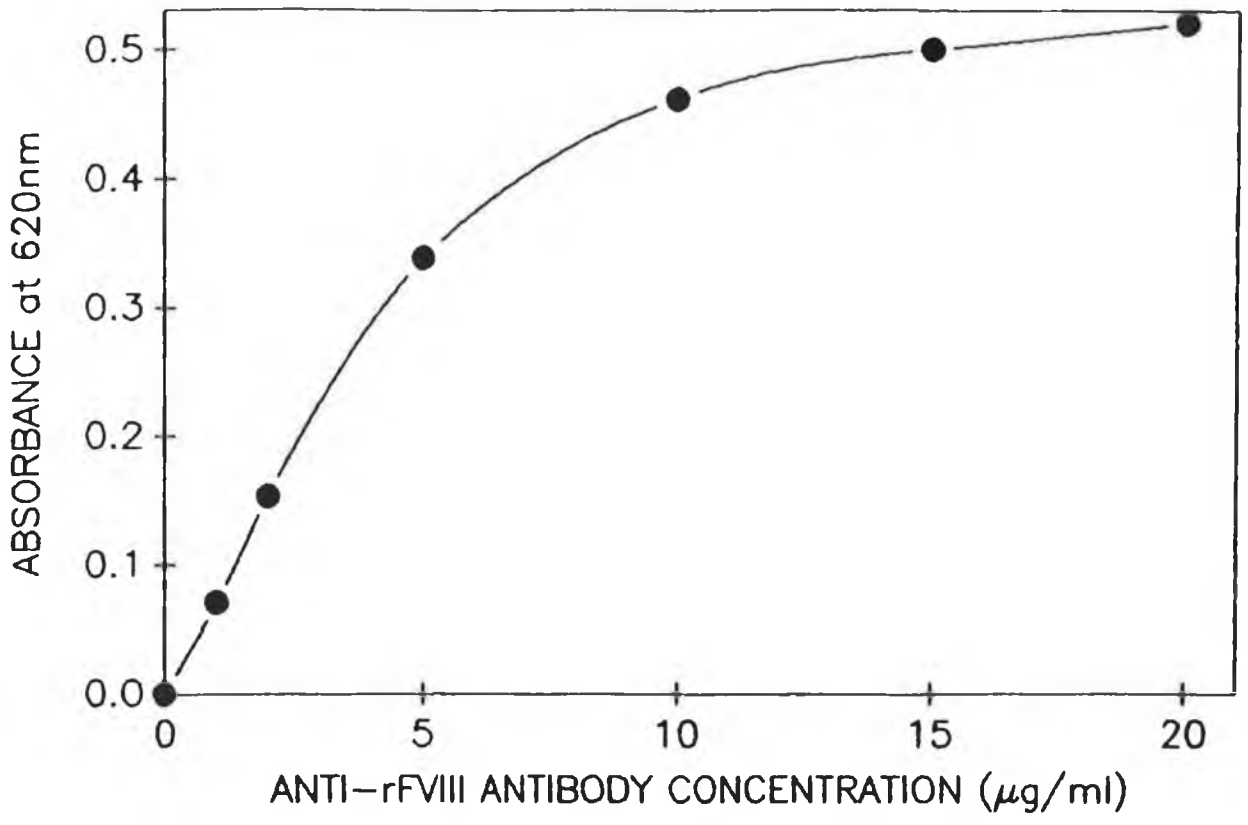


Figure 5.4.2 Concentration of primary anti-rFVIII antibody in an indirect ELISA. 5μg/ml rFVIII was bound onto the solid phase, and a range of concentrations of anti-rFVIII ploYclonal antibody added. Bound antibody was detected with alkaline phosphatase-labelled anti-rabbit antibody and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer.

The optimal primary antibody concentration chosen for use was 10μg/ml.

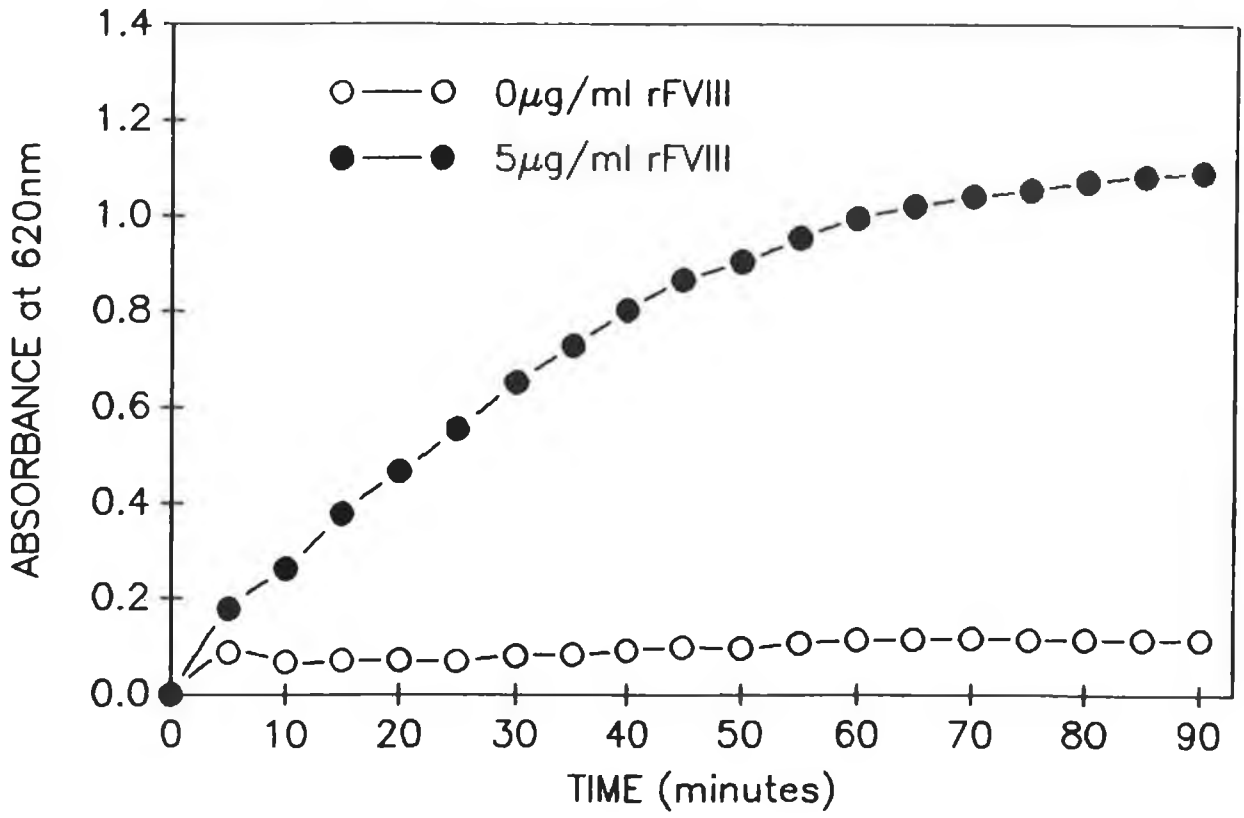


Figure 5.4.3 Substrate kinetics for 5-bromo-4-chloro-3-indoyl phosphate (BCIP). rFVIII was bound onto the solid phase and anti-rFVIII antibodies added. Bound antibody was detected with alkaline phosphatase-labelled anti-rabbit antibody, and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer. The absorbance at 620nm was read at 5 minute intervals over 60 minutes.

5.5 ELISA using a Luminescent Substrate

The method used was as described for the capture and indirect ELISAs with the exception that Dynatech MicroFLUOR plates were used. In the capture ELISA a range of concentrations of anti-rFVIII antibodies (monoclonal and polyclonal) were bound to these plates, and the effectiveness of blocking free binding sites was examined. It was found that there was minimal difference in signals between the blocked and unblocked wells (Figure 5.5.1). Despite using the optimised parameters from the antigen-capture ELISA (Section 5.2), no luminescent signal was obtained. This could have been caused by the different binding properties of the opaque plates required for use with a luminescent substrate. ELISA plates are manufactured and processed to maximise protein binding. The MicroFLUOR plates are not manufactured with the same specifity as the ELISA plates and, thus, do not exhibit the same binding characteristics. This may have contribituted to the failure to obtain a signal using the antigen-capture ELISA format.

An indirect ELISA format was used, based on that optimised in Section 5.4. The range of rFVIII which bound to the plates was greater than that found in Section 5.4, and the optimum binding concentration was higher, 10µg/ml (a two-fold increase) (Figure 5.5.2). Anti-rFVIII antibodies samples were added, and bound antibodies were detected using goat anti-rabbit IgG linked to HRP (GAR-HRP). The signal was developed using ECL reagent and luminescence was monitored on an Amersham Amerlite luminometer.

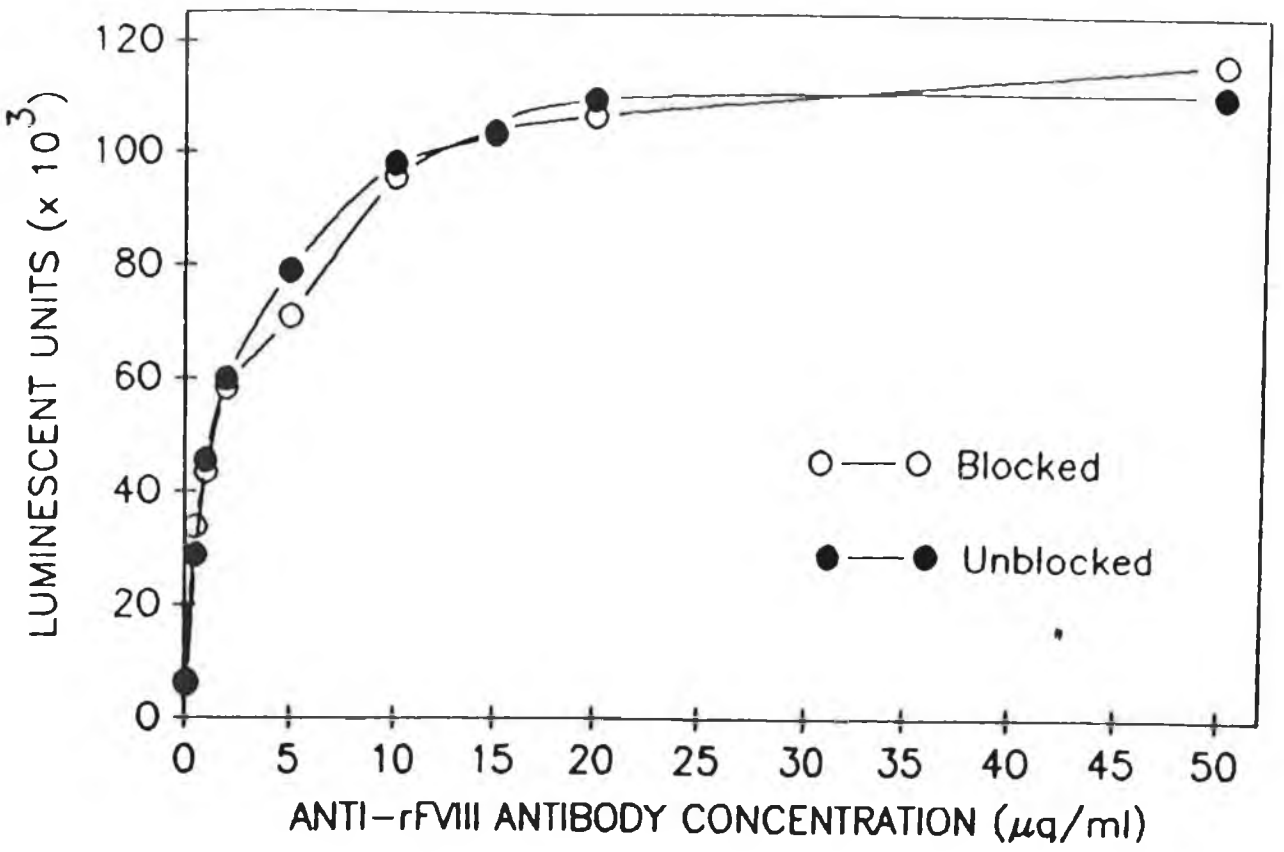


Figure 5.5.1 The effectiveness of blocking free binding sites on opaque plates designed for use with a luminescent substrate.

5µg/ml of rFVIII was bound to wells of Dynatech MicroFLUOR plates and the free binding sites in wells of one half of the plate were blocked, and the remaining half left unblocked. Dilutions of anti-rFVIII antibody were added and bound antibody probed using a horseradish peroxidase-labelled anti-rabbit antibody and a luminescent substrate (ECL). Luminescence was monitored using an Amersham Amerlite luminometer.

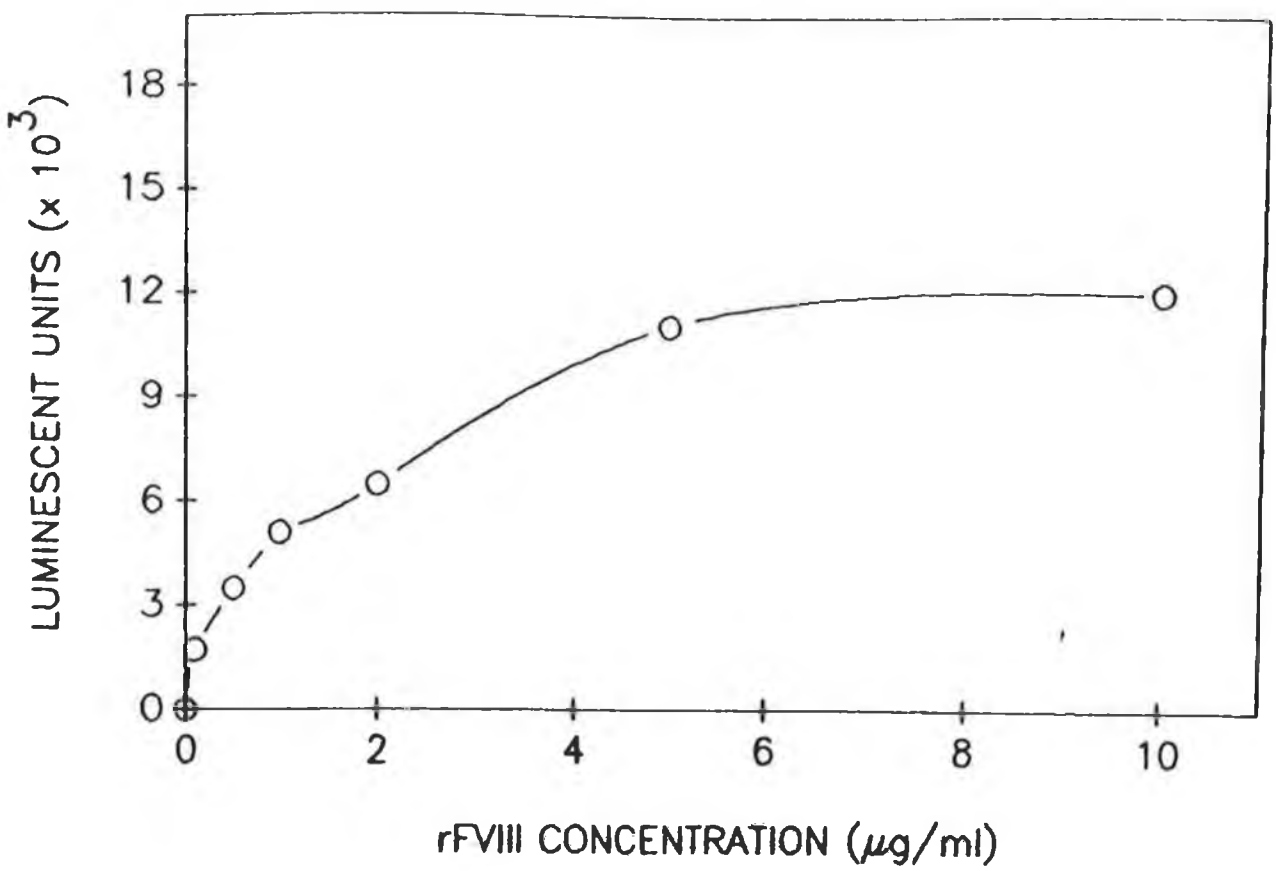


Figure 5.5.2 Binding of rFVIII to the surface of Dynatech MicroFLOUR microtitre plates for use in an indirect luminescent ELISA.
A range of concentrations of rFVIII were bound to the solid phase and detected using a horseradish peroxidase-labelled anti-rFVIII antibody and a luminescent substrate (ECL).

5.6 Detection of FVIII in Plasma

Normal human plasma, Coag Cal N calibration plasma and FVIII-deficient plasma spiked with rFVIII were all used in an attempt to detect FVIII in "real samples". Using the antigen-capture ELISA format, FVIII could not be detected in whole Coag Cal N samples or in samples in which FVIII had been separated from vWF by high ionic strength buffers (Section 2.27) (Table 5.6.1). The lower detection limit was 1.5 times the background value (the background value was determined in a well containing all the reagents minus the sample (rFVIII)). FVIII was activated by thrombin (Section 2.25), but a clot formed before the FVIII sample could be added to the ELISA. Two anti-coagulants, heparin and 0.11M sodium citrate, were used to prevent clot formation. In both cases, no FVIII could be detected in Coag Cal N by any of the four antibodies (Table 5.6.2).

FVIII in normal human plasma could be detected once the sample had been partially purified, and the FVIII/vWF complex separated (Section 2.27). Absorbances obtained were comparable with controls using a similar concentration of rFVIII (Table 5.6.3). The antibodies did not cross-react with any component in FVIII-deficient plasma. Therefore, FVIII-deficient plasma was spiked with a range of concentrations of FVIII which were detected in an antigen-capture ELISA (Table 5.6.4).

FVIII Sample	Absorbance ± S.D. at 492nm
Coag Cal N	
Undiluted	.055 ± .002
1:10 Dilution	.054 ± .004
FVIII-deficient Plasma	
Undiluted	.051 ± .001
1:10 Dilution	.052 ± .003
rFVIII Control	
5µg/ml	.073 ± .000
10µg/ml	.105 ± .001
PBS	0.000 ± .002

Table 5.6.1 The reaction of Coag Cal N calibration plasma and FVIII-deficient plasma in an antigen-capture ELISA. Anti-rFVIII antibody was bound to the solid phase and samples of Coag Cal N and FVIII-deficient plasma added. Bound FVIII was detected using horseradish peroxidase-labelled anti-rFVIII antibodies and o-phenylenediamine as substrate. The anti-rFVIII antibodies did not react with FVIII in Coag Cal N in any way. Controls consisting of 5µg/ml and 10 µg/ml rFVIII were included. S.D. is the standard deviation where n=3.

	Absorbance ± S.D. at 492nm			
Anti-rFVIII Antibody	rFVIII (10µg/ml)	Coag Cal N	Citrated Plasma	FVIII-deficient Plasma
Rabbit Polyclonal	.190 ± .002	.051 ± .005	.010 ± .001	.040 ± .007
M1 Monoclonal	.210 ± .007	.055 ± .001	.059 ± .003	.061 ± .005
M2 Monoclonal	.121 ± .011	.057 ± .002	.095 ± .010	.063 ± .007
M10 Monoclonal	.200 ± .021	.062 ± .007	.061 ± .001	.060 ± .001
PBS	.000 ± .000	.000 ± .000	.000 ± .000	.000 ± .000

Table 5.6.2 The reaction of four anti-rFVIII antibodies with different sources of FVIII in an antigen-capture ELISA. Anti-rFVIII antibody was bound to the solid phase, and the above samples of FVIII added. Bound FVIII was detected using horseradish peroxidase-labelled anti-rFVIII antibodies and o-phenylenediamine as substrate.

Citrated plasma consisted of thrombin-activated Coag Cal N, containing 0.11M sodium citrate as an anti-coagulant. Coag Cal N and FVIII-deficient plasma are commercial calibration plasmas.

S.D. is the standard deviation where n = 3.

FVIII Source	Absorbance ± S.D. at 492nm
Coag Cal N Calibration Plasma	.055 ± .001
Purified Plasma (neat)	.330 ± .007
Purified Plasma (1:5 dilution)	.216 ± .005
Purified Plasma (1:10 dilution)	.160 ± .008
Control (5µg/ml rFVIII)	.131 ± .010
PBS	.000 ± .000

Table 5.6.3 Detection of FVIII in normal human plasma using an antigen-capture ELISA. Anti-rFVIII antibody was bound to the solid phase and partially-purified plasma added. Bound FVIII was detected using horseradish peroxidase-labelled anti-rFVIII antibodies and o-phenylenediamine as substrate. The plasma was purified by gel filtration chromatography through a Sepharose 6B column.
S.D. is the standard deviation where n = 3.

FVIII source	Absorbance ± S.D. at 492nm
FVIII-deficient Plasma	.056 ± .000
FVIII-deficient Plasma + 5µg/ml rFVIII	.087 ± .002
FVIII-deficient Plasma + 10µg/ml rFVIII	.112 ± .010
Control (10µg/ml rFVIII)	.111 ± .012
PBS	.000 ± .000

Table 5.6.4 Detection of rFVIII in spiked samples of FVIII-deficient Plasma using an antigen capture ELISA. Anti-rFVIII antibody was bound to the solid phase and spiked and unspiked samples of FVIII-deficient plasma added. Bound FVIII was detected using horseradish peroxidase-labelled anti-rFVIII antibodies and o-phenylenediamine as substrate.
S.D. is the standard deviation where n = 3.

5.7 Competitive ELISA

Competitive ELISAs using a number of different formats were developed to detect rFVIII and antibodies to rFVIII.

In the first format, the antigen (rFVIII) was immobilised onto the solid phase. This then competes with free rFVIII in the sample (or standard) for enzyme-labelled specific anti-rFVIII antibody in solution. The amount of product formed was inversely proportional to the concentration of free rFVIII in solution. Concentration of rFVIII as low as 0.5µg/ml could be detected using this system.

In the second format, both enzyme-labelled and free antibody competed for binding rFVIII which had been immobilised onto the surface of the microtitre plate. In this case, the amount of colour obtained was inversely proportional to the level of free antibody present.

In this system, concentrations of 0.5µg/ml of anti-rFVIII antibody could be detected.

When absorbance was plotted against antigen (rFVIII) or antibody concentration, the graphs show second order characteristics (Figures 5.7.1 and 5.7.2). For rFVIII measurement $k_1 = 2.35 \pm 0.1$ and $k_2 = 0.042 \pm 0.001$, while for antibody measurement, $k_1 = 3.78 \pm 0.2$ and $k_2 = 0.007 \pm 0.001$.

These ELISAs could be useful in the determination of FVIII, rFVIII or anti-rFVIII antibodies in plasma. When samples of normal human plasma, and Coag Cal calibration plasma were tested in this system, no signal was obtained for any of the samples. This may be caused by non-specific binding of components of plasma to the surface of the well, blocking the antibodies, and preventing binding of rFVIII. Alternatively, the labelled antibodies may be cross-reacting with some other constituant of plasma. This is likely, because the labelled antibodies are polyclonal, and thus contain antibodies against a wide range of determinants, which may not be unique to rFVIII.

"Real samples" of anti-FVIII antibodies were not tested, as the risks of contamination from blood-borne infectious agents (such as HIV and hepatitis) are high, especially from haemophiliacs receiving replacement FVIII therapy.

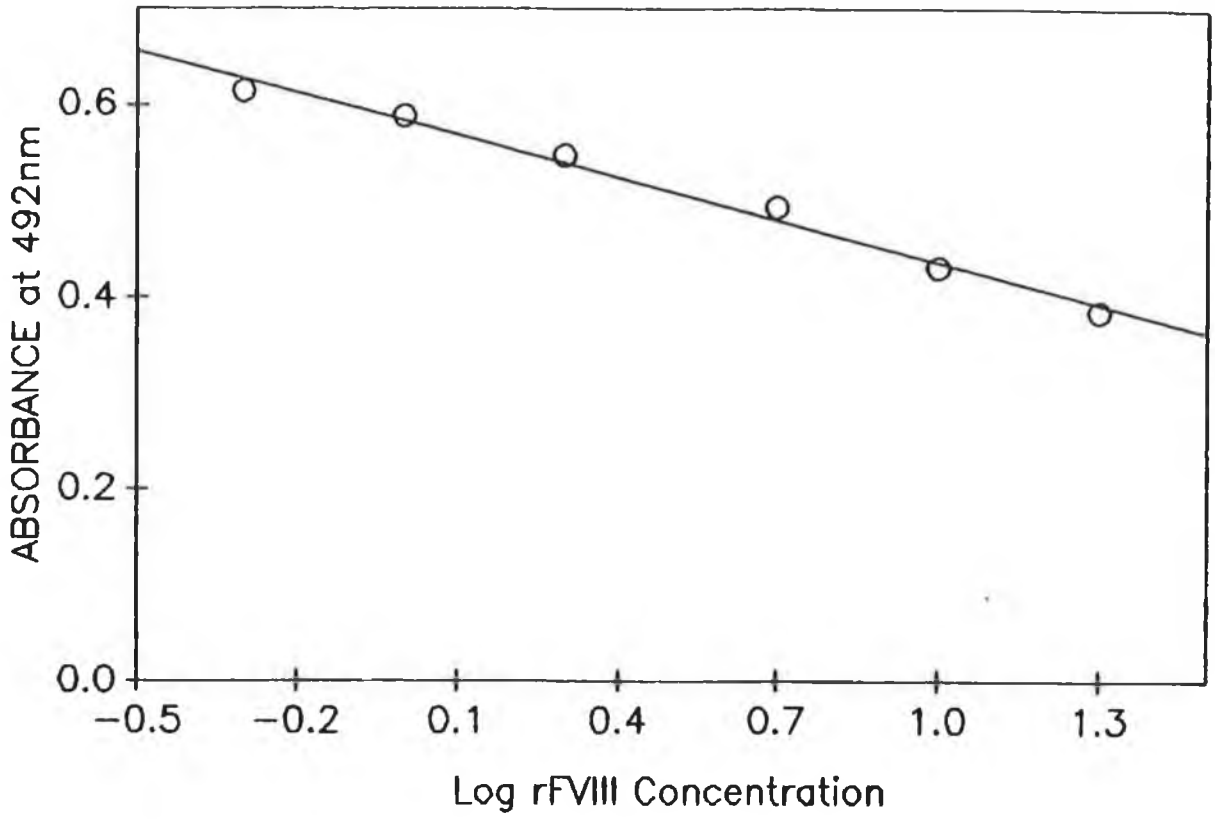


Figure 5.7.1 Competitive ELISA for the detection of rFVIII.

rFVIII was bound to the solid phase and equal volumes of horseradish peroxide (HRP)-labelled anti-rFVIII antibodies and free rFVIII were added. o-Phenylenediamine (OPD) was added and absorbance measured at 492nm. The absorbance was inversely proportional to the concentration of free rFVIII in solution.

The graph follows second order characteristics with k_1= 2.35 and k_2= 0.042. r = 0.99, where r is the correlation coefficient.

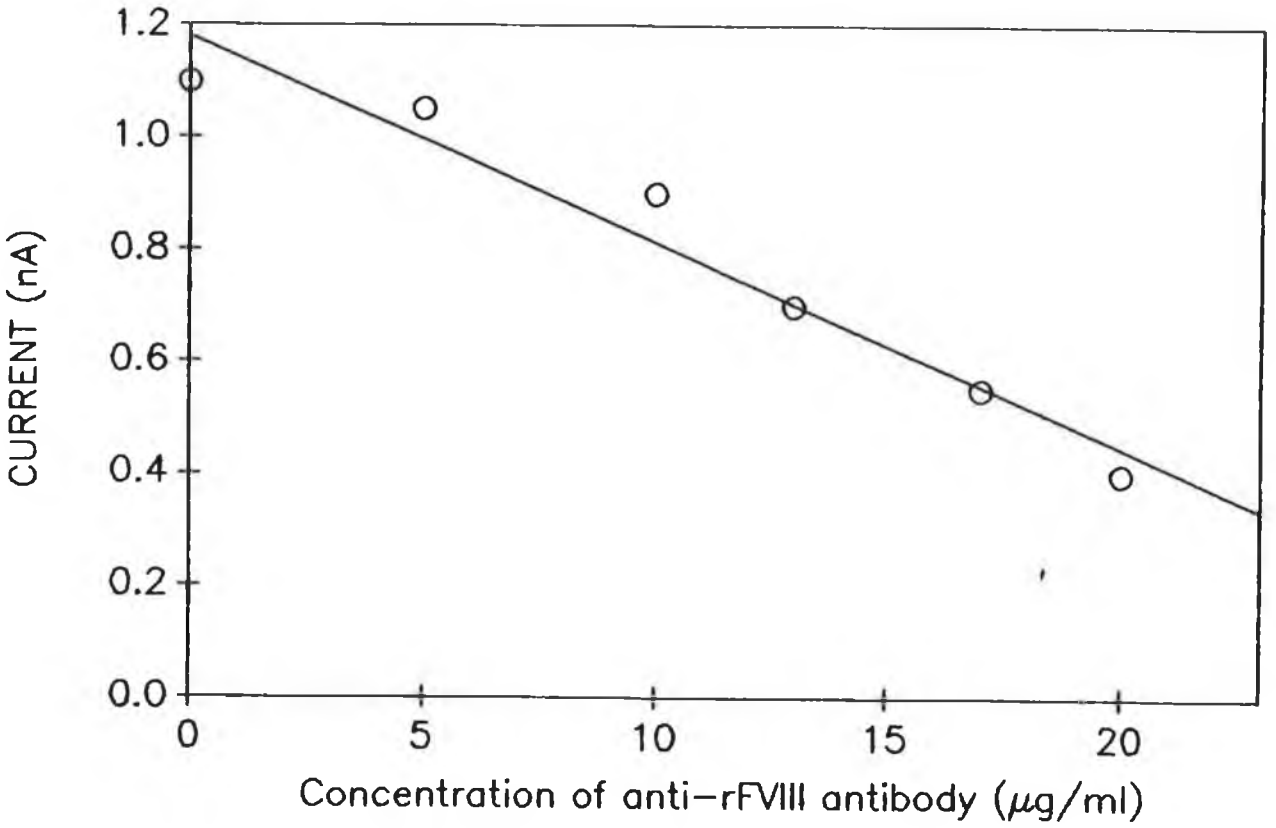


Figure 5.7.2 Competitive ELISA for the detection of anti-rFVIII antibodies. rFVIII was bound to the solid phase and equal volumes of horseradish peroxide (HRP)-labelled anti-rFVIII antibodies and unlabelled anti-rFVIII antibodies were added. o-Phenylenediamine (OPD) was added and absorbance measured at 492nm. The absorbance was inversely proportional to the concentration of unlabelled anti-rFVIII antibodies in solution. The graph follows second order characteristics with k_1= 3.78, and k_2= 0.007. r = 0.97, where r is the correlation coefficient.

5.8 Discussion

The choice of solid phase is one of the fundamental factors in the development of an ELISA system. Carriers can be classified into two categories - high and low capacity binders. High capacity materials include nitrocellulose, cellulose and cyanogen bromide-activated agarose. These are stable and can be stored for long periods of time. They are particularly useful for relatively impure antigen preparations, viral, or bacterial antigens (Kemeny, 1991). The disadvantages of these are that they are difficult to wash, and give high background binding. Low-capacity carriers, such as plastic and PVC, are easier to wash, and give low background binding. However, they are more difficult to coat effectively (Kemeny, 1992).

The capacity of the solid phase is limited (1µg/cm^2 for IgG) (Porstmann *et al.*, 1992) and therefore it is important to optimise the concentration of the bound protein (O'Kennedy, 1989). If the concentration is too high, multiple layers can form consisting of proteins binding to each other. These protein-protein interactions are weaker than the protein-solid phase ones, and so are more easily disrupted. Dissociation or leaching can occur, and the dissociated proteins can interfere with the assay. Generally, the concentration should be within the range 1-10µg/ml (Kemeny, 1992).

Charge and hydrophobic interactions are thought to be important in the binding of the proteins to the solid phase (Kemeny, 1991). The charge on a protein is influenced by the pH of the buffer. It was found that a buffer with a high pH (*i.e.*, pH 9.6), gave the highest degree of binding.

The need to block free binding sites, once the antigen or antibody has been immobilised, is the subject of conflicting reports (Schønheyder and Andersen, 1984; Mohammed and Esen, 1990). Sites were blocked using a 1% (w/v) solution of BSA. It has been proposed that the size of the protein molecule could be an important factor in the choice of blocking buffer, with small protein molecules being more effective than larger ones (Kemeny, 1992). The need for a blocking step in the ELISA using a luminescent substrate was eliminated. Different plates were used in this system (Dynatech MicroFLUOR plates), which perhaps did not have the same binding characteristics as the specially treated Nunc Maxisorb strips. The Dynatech plates did bind a greater range of rFVIII concentrations, suggesting that the plates could be coated more effectively, and the risk of non-specific binding reduced.

The binding of an antibody to the solid phase can compromise the integrity of the molecule, resulting in loss of activity, or the alteration or loss of some antigenic sites in the case of an antigen. A layer of linking agent can be used to prevent this happening. For antigens, cross-linking reagents such as glutaraldehyde and carbodiimide (Place and Schroeder, 1982; Rotmans and Scheven, 1984) have been successfully used. Anti-mouse or rabbit IgG is generally used as the linking agent for antibodies. This method proved useful for the detection of the anti-rFVIII antibodies: the limit of detection was improved 50 fold to allow concentrations of antibody as low as 10ng/ml to be detected. Compared with this, the limit of detection of the IgG ELISA was 500ng/ml. This goat anti-rabbit ELISA, however, involves an additional step resulting in a longer assay time, as well as the additional expense of another antibody layer. The goat anti-rabbit antibody linker could not be used in the full capture ELISA because the secondary enzyme-labelled antibody was also a rabbit antibody, and would cross-react with the linking agent.

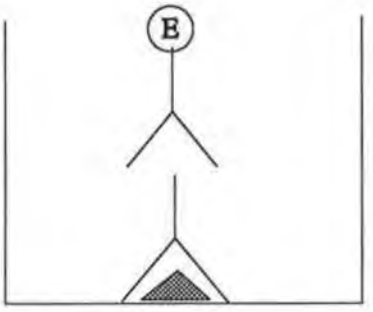
The absorbanses recorded with the monoclonal antibody-based ELISAs are generally lower than those of the polyclonal antibody-based assays. The lower avidity and small fraction of high-affinity antibodies (which are dominant in polyclonal sera) are reasons for lower detectability of monoclonal antibodies in enzyme-immunoassay (EIA) when compared with polyclonal antibodies, despite lower concentration of antibody in the latter (Tijssen, 1985). Affinity (the strength of a single antigen-antibody bond) and avidity (the overall binding strength of an antibody for an antigen, it is dependant on the affinity and the valency of the antibody) are significant. Avidity is an important characteristic of polyclonal antisera, since they generally contain antibodies against all determinants of a given antigen, which contribute to the avidity. Monoclonal antibodies on the other hand, react with a single unique epitope with a defined specifity and affinity and, therefore, generally do not exhibit the same levels of avidity as polyclonal antisera (Tijssen, 1985). It is therefore, essential that the monoclonal antibodies have high affinity for their determinants. The monoclonal antibodies used in the ELISAs described here were of the IgM class. IgM is a pentameric molecule, but because of lack of flexibility in the molecule leading to steric hindrance, it has a reduced valency of 5 (Roitt, 1980). These antibodies tend to have lower affinity than IgG antibodies, but higher avidity because of their valency.

There are other problems associated with the use of monoclonal antibodies in EIA. When an antigen binds to the solid phase, the single epitope recognised by the antibody may be hidden or changed in some way, and this may prevent antibody binding. Polyclonal preparations, containing a mixture of antibodies, are capable of recognising a number of different epitopes. Therefore, some epitopes are generally available for binding under most conditions. The monoclonal antibodies were not used as a capture layer in the antigen-capture ELISA. They recognise only single epitopes and therefore, the level of antigen which would be captured and bound would be severely restricted. If the binding site was altered in any way by the binding of the antibody to the solid phase, no binding at all could take place. Polyclonal antiserum binds to a range of epitopes, and thus provides an ideal capture layer, as many alternative sites will be available.

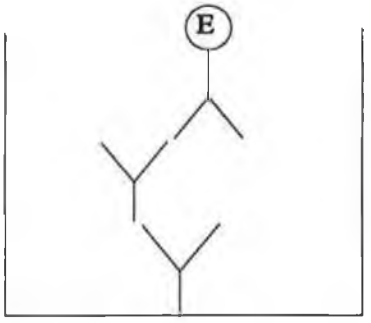
von Willebrand Factor (vWF) comprises approximately 99% of the mass of the vWF/FVIII complex (Manning *et al.*, 1993), and so it has proved very difficult to detect FVIII immunologically in normal plasma. Attempts to isolate and immunodetect FVIII from normal human calibration plasma (Coag Cal N) proved unsuccessful. This was the case regardless of whether or not FVIII had been separated from the FVIII/vWF complex using high ionic strength buffers (Hoyer, 1981). However, FVIII was successfully detected in normal human plasma after separation of the complex and in FVIII-deficient plasma spiked with the recombinant protein. It was discovered that the antibodies were targeted against conformational epitopes on the rFVIII molecule (Section 4.11). These epitopes could have been destroyed during the lyophilisation process in the preparation of Coag Cal N without affecting the functional properties of the FVIII molecule.

A number of ELISAs have been developed using several formats (Figure 5.8.1).

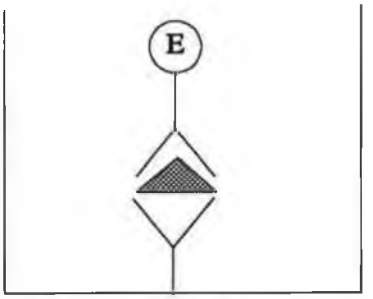
The antigen-capture ELISA is primarily used to quantitate antigens in a test solution or for the titration of antisera (Tijssen, 1985). The ELISA described here provides a simple and reliable method for the detection of rFVIII or its antibody. Its main advantage over the direct ELISA is that the antigen does not have to be bound directly to the plate, avoiding problems of poor epitope orientation or structural change on binding. Modifications can be made to speed up the assay, including overnight blocking, or use of a luminescent substrate where a signal can be read after 5 minutes.



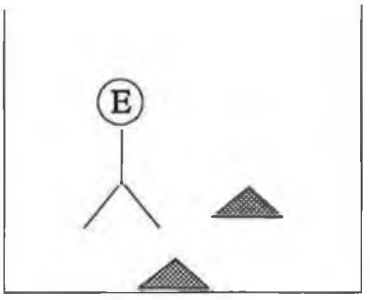
A. INDIRECT ELISA

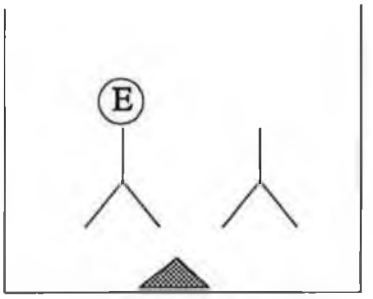


B. GOAT ANTI-RABBIT ANTIBODY LINKER ELISA



C. ANTIGEN-CAPTURE ELISA





E. COMPETITIVE ELISA

Enzyme-labelled secondary antibody

Antigen (rFVIII)

Primary antibody

Figure 5.8.1 Formats used in the development of an ELISA for the detection of rFVIII and anti-rFVIII antibodies

The indirect ELISA on the other hand, is widely used for the determination of antibody activity (Tijssen, 1985). Anti-rFVIII antibodies were successfully detected and quantified using this method. rFVIII can be readily adsorbed onto the surface of wells, as compared with the normal FVIII molecule, because of its reduced size. It can, therefore, provide an alternative to normal FVIII for this purpose. Inhibitor antibodies, which develop in haemophliacs receiving FVIII replacement therapy, could be detected using this assay.

Competitive assays allow for the quantitation of a particular antigen or antibody, even when they cannot be isolated from the medium in which they are found (Kemeny, 1991). Antibodies to disease-causing organisms have been successfully detected and quantified using ELISAs (Tijssen, 1985). In this section, competitive ELISAs have been developed for the quantitation of rFVIII and anti-rFVIII antibodies. FVIII in normal human samples could not be detected, as other components of plasma possibly interfered with the assay. A partial purification of the plasma still did not eliminate the interfering substance. Blood and plasma are complex matrices, with a wide variety of constituants. The antibodies used in the ELISA were polyclonal, and thus were directed against a large number of antigenic determinants, some of which were not necessarily unique to rFVIII. There was, therefore, a possibility of cross-reactivity with other components of plasma. A labelled monoclonal antibody antibody would reduce the chances of cross-reactivity, but for reasons outlined earlier, the antibody would have to be highly specific and have high affinity for its antigenic determinant.

The competitive ELISA for the detection of anti-rFVIII antibodies could be useful in the monitoring of inhibitor antibodies. Levels of inhibitor antibodies are an important consideration in the treatment of haemophilia (Section 1.11.2).

Inhibitor antibodies (or antibody fragments) have been used as a capture layer coated onto the solid phase (Dinesen and Feddersen, 1983; Nordfang *et al.*, 1985). In these assays, the sample was added and bound FVIII detected using either labelled inhibitor antibodies or an anti-FVIII monoclonal antibody. These was subsequently detected using a HRP-labelled goat anti-mouse antibody. In the system described in this chapter, monoclonal and polyclonal anti-rFVIII antibodies were employed, providing a safe and reliable alternative to human-derived inhibitor antibodies. A number of different formats have been developed allowing for alterations in the methodology. Using these, rFVIII itself or antibodies against it can be successfully detected and quantitated.

CHAPTER 6

DEVELOPMENT OF AN ANTIBODY-BASED BIOSENSOR FOR THE DETECTION OF rFVIII AND ANTI-rFVIII ANTIBODIES

6.1 Introduction

In the system described here, rFVIII is immobilised onto the surface of an electrode. Horseradish peroxidase (HRP)-labelled anti-rFVIII antibodies bind to the antigen and a signal is produced via a mediator (hydroquinone) (Figure 6.1.1). HRP catalyses the reaction between hydrogen peroxide (H_2O_2) and hydroquinone (HQ) forming benzoquinone (BQ). HQ is regenerated by reduction of BQ at the electrode surface at an applied potential of -0.03V, generating a measurable signal:

$$H_2O_2 + HQ \xrightarrow{\text{HRP}} 2H_2O + BQ$$

$$BQ + 2H^+ + 2e^- \xrightarrow[\text{Applied Pot. -0.03V}]{} HQ$$

Figure 6.1.2 shows the current-time response for the modified electrode.

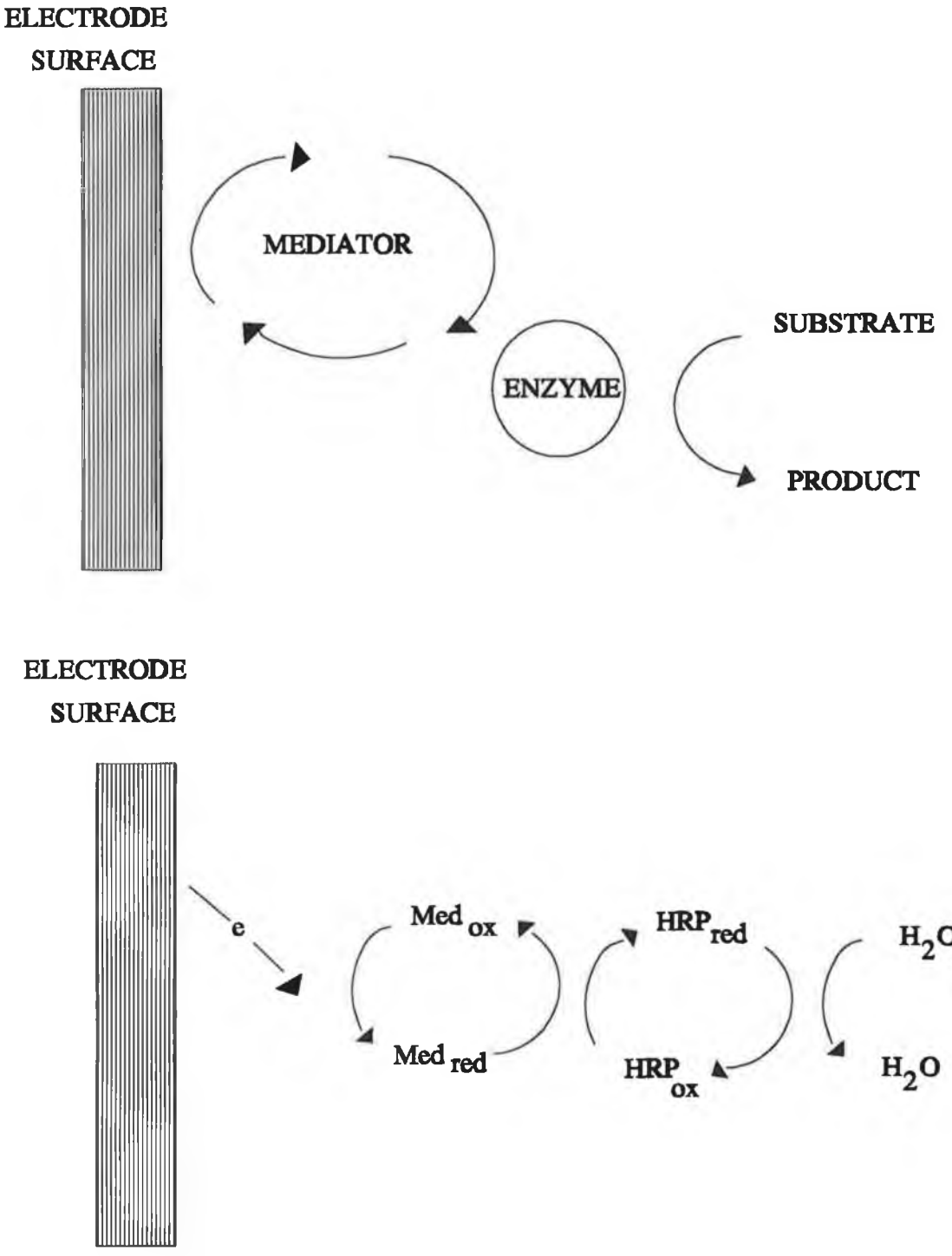


Figure 6.1.1 Generation of a signal from the reduction of of a substrate by an enzyme, via a mediator. The substrate in this system is hydrogen peroxide (H_2O_2). The mediator, hydroquinone, catalyses the reduction of H_2O_2 by the enzyme horseradish peroxidase. The mediator is regenerated by reduction at the electrode surface, generating a signal.

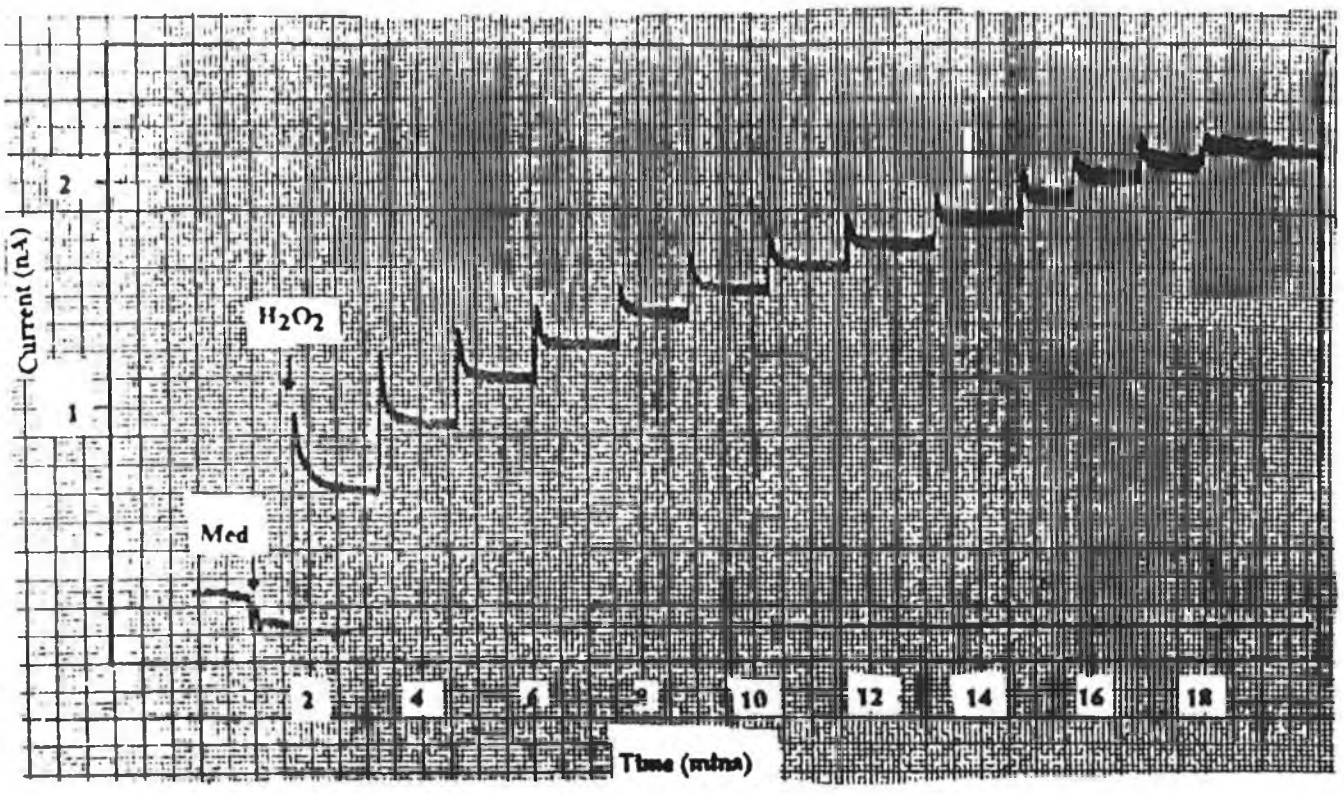


Figure 6.1.2 Current-time responses for successive 1mM injections of hydrogen peroxide at rFVIII-modified electrodes. The operating potential was -0.03V with a stirring rate of 450rpm. The electrolyte was 0.1M phosphate buffer, pH 7.2 and contained 2mM hydroquinone. The modified electrode contained 20µg/ml rFVIII, and had been incubated in a 33.3 µg/ml solution of HRP-labelled anti-rFVIII antibody for 1 hour at 37°C.

6.2 Preparation of the Modified Electrode

A modified electrode was prepared by immobilising rFVIII onto the surface of a glassy carbon electrode using a perfluorinated, ion-exchange polymer (Nafion). Both the Nafion and rFVIII concentrations were optimised.

6.2.1 Nafion Concentration

Nafion was used to immobilise rFVIII onto the surface of a glassy carbon electrode. A concentration 5% (v/v) Nafion was used to coat 10µl of 30µg/ml rFVIII onto the electrode surface, forming a stable film. It was found that at concentrations below this, the film was unstable and cracked, exposing the electrode surface. This allowed labelled antibody to bind non-specifically to the exposed electrode surface creating false positive signals.

6.2.2 Concentration of rFVIII on the electrode surface

The concentration of rFVIII on the surface of the electrode is critically important. It is important to have sufficient antigen incorporated into the membrane to allow for antibody recognition while, at the same time, allowing access to the electrode surface by the flow of electrons within the cell. Overloading the electrode surface can lead to the generation of a blanket-like structure, which the electrons cannot penetrate easily, resulting in a slow signal.

A range of rFVIII concentrations were examined (Figure 6.2.2). The current increased up to a concentration of 50µg/ml, but decreased at 75µg/ml. At concentrations higher than this, the film became unstable and cracked.

A concentration of 30µg/ml rFVIII was chosen for future work.

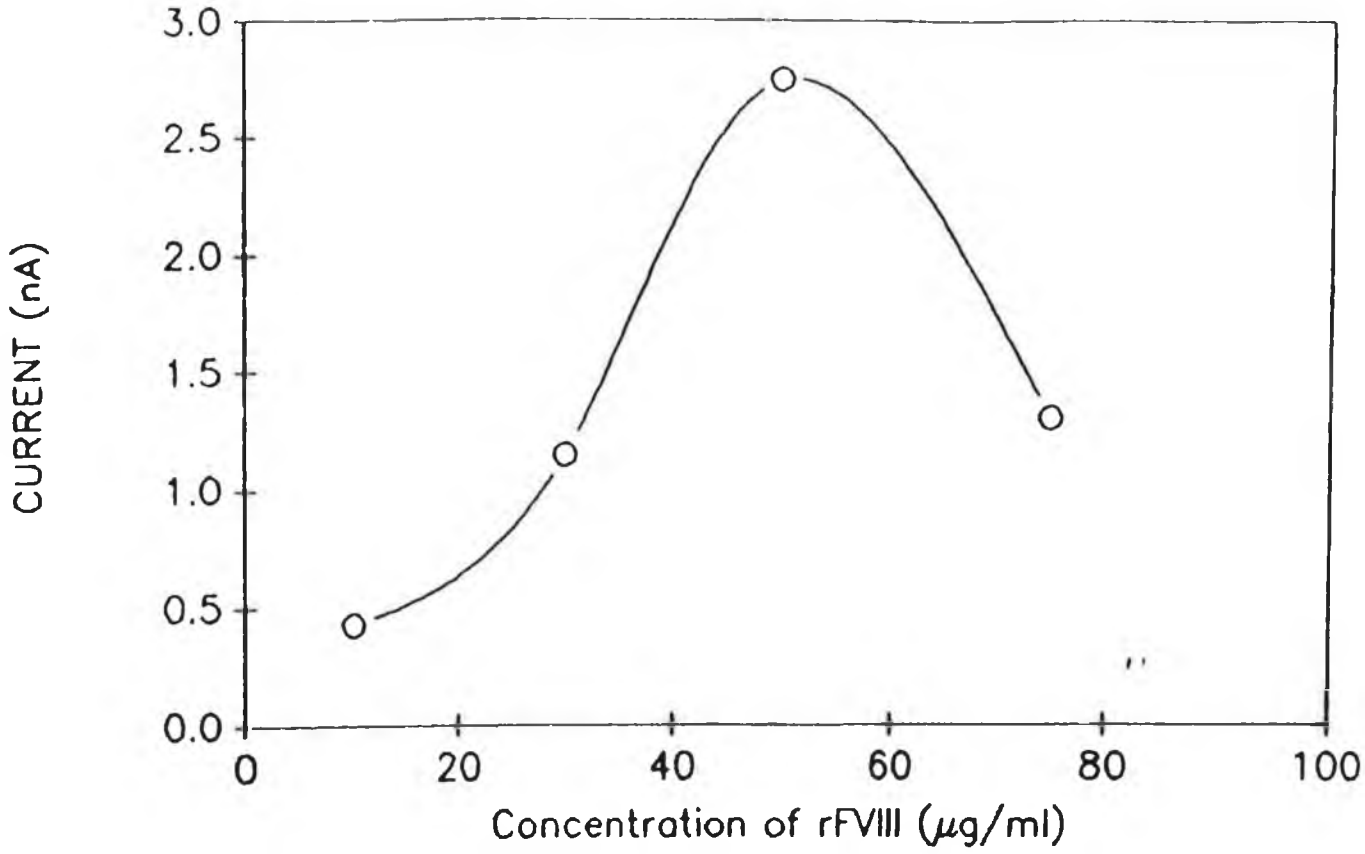


Figure 6.2.2 Determination of the optimum concentration of rFVIII immobilised onto the surface of a glassy carbon electrode.

10μl of rFVIII in 5% (v/v) Nafion was pipetted onto the electrode surface. The organic solvent evaporated leaving the rFVIII immobilised onto the surface. The electrode was incubated with labelled anti-rFVIII antibody and introduced into the electrochemical cell. Mediator (hydroquinone) was added to a final concentration of 2mM, followed by 1mM substrate (H_2O_2) and the signal measured.

6.3 Labelled Antibody Concentration

The coated electrode was incubated for 1 hour in a range of concentrations of anti-rFVIII antibody labelled with HRP (prepared as in Section 2.21), in 0.1M phosphate buffer, pH 7.1 (Figure 6.3).

The concentration of the enzyme-labelled antibody was considerably lower than that used in an ELISA (Section 5.2). In that system, rFVIII bound to an anti-rFVIII antibody which had been immobilised onto surface of a microtitre plate. rFVIII was subsequently detected using a HRP-labelled anti-rFVIII antibody. The same batch of labelled antibody was used but in the ELISA, the dilution used was 1:1000 (1µg/ml) whereas, for electrochemical detection, at least a 1:100 dilution (10µg/ml) was necessary. This represents a tenfold increase in concentration. This was also despite the fact that only threefold more protein was immobilised onto the electrode (30µg/ml as opposed to 10µg/ml on a well of a microtitre plate). The generation of a signal in the electrochemical assay involved the flow of electrons (current) produced by the reduction of a product at the electrode surface. In an ELISA, a signal was produced by the reduction of a substrate forming a coloured product which absorbes light at a defined wavelength. The difference between the two methods of generating a signal could account for the difference in labelled antibody concentration.

It was important to use the same batch of conjugate when developing the system, owing to batch to batch variability in the production of antibody-enzyme conjugates. In order to ensure this, a 1:30 dilution of labelled antibody was chosen despite the fact that a 1:15 dilution produced higher signals.

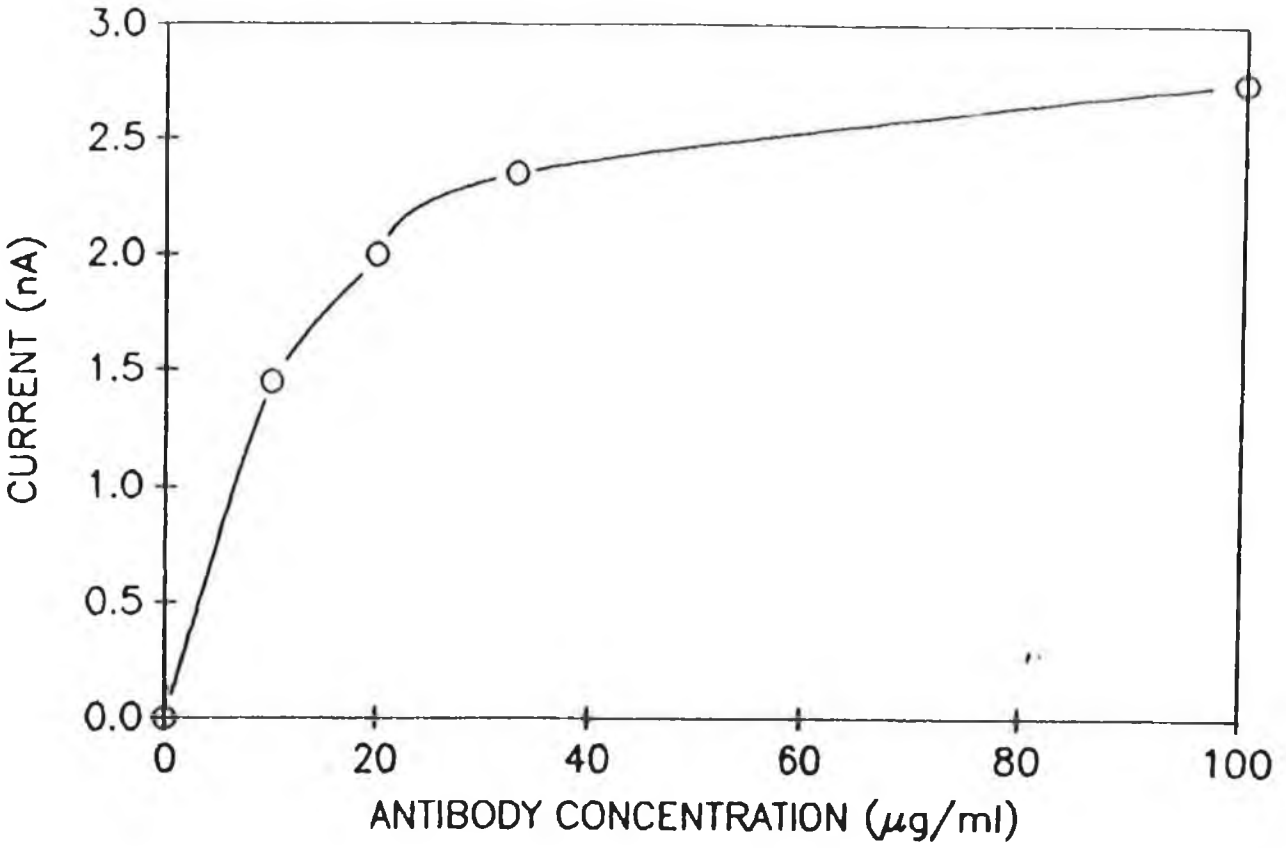


Figure 6.3 Incubation of the modified electrode with dilutions of horseradish peroxidase (HRP)-labelled anti-rFVIII antibody. 30 µg/ml of rFVIII in 5% (v/v) Nafion was pipetted onto the electrode surface. The organic solvent evaporated leaving the rFVIII immobilised onto the surface. The electrode was incubated with different dilutions of HRP-labelled anti-rFVIII antibody and introduced into the electrochemical cell. Mediator (hydroquinone) was added to a final concentration of 2mM, followed by 1mM substrate (H_2O_2) and the signal measured.

6.4 Incubation Temperature

The incubation temperature for antibody-antigen interactions in ELISA is generally taken as 37°C. This is not always the most suitable, and therefore, it was important to optimize the temperature at which a particular antibody reacts with its antigen in a certain environment in order to maximise assay specificity.

The temperature at which anti-rFVII-labelled antibody reacts with rFVIII immobilised onto the electrode surface was investigated. A range of temperatures were studied (20°C, 30°C, 37°C and 45°C) and from these it was seen that when incubated at 37°C the antibody-antigen interactions were maximised (Figure 6.4).

Thus, 37°C was chosen as the temperature for all further incubations.

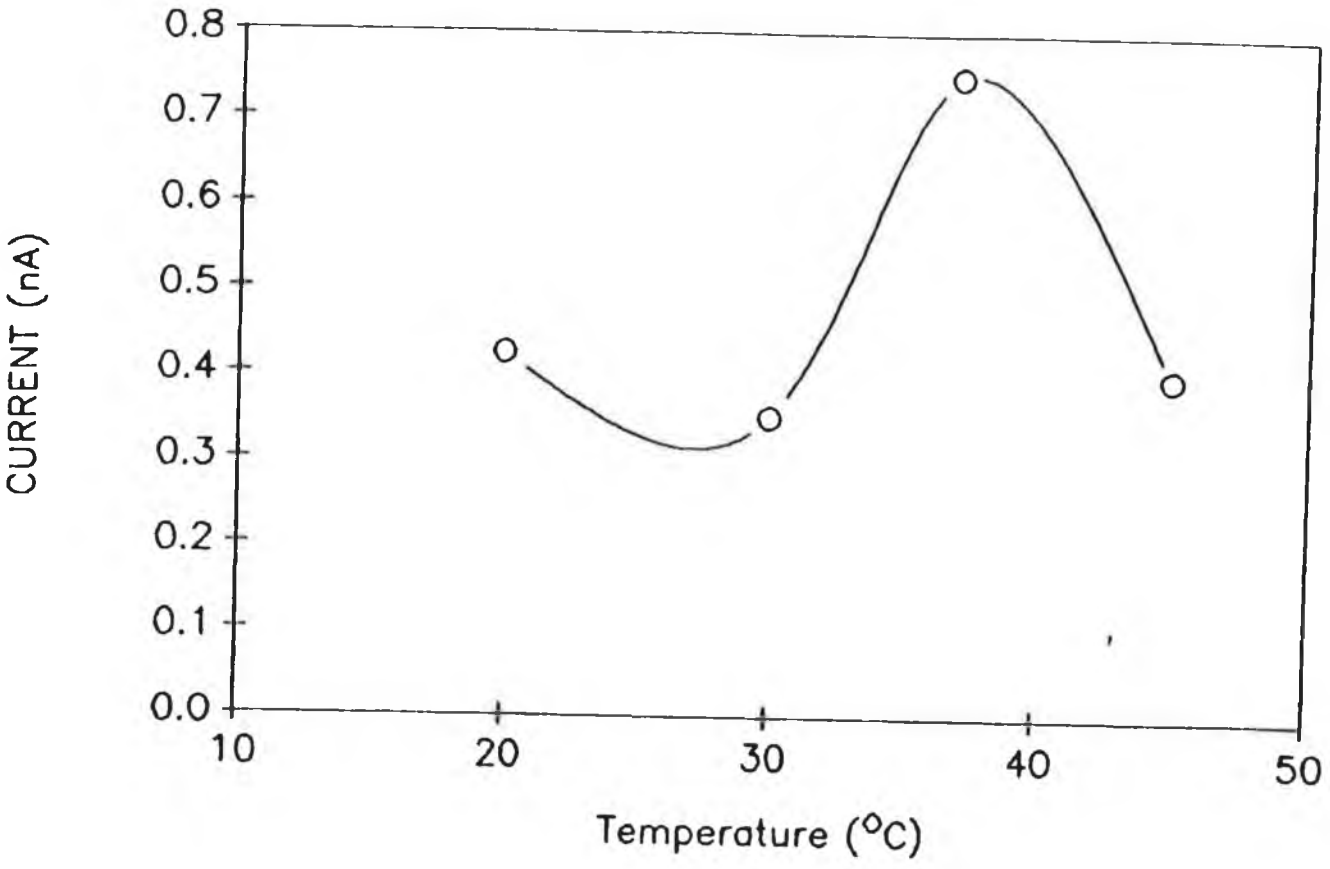


Figure 6.4 Determination of the optimal incubation temperature for the rFVIII/horseradish peroxidase (HRP)-labelled anti-rFVIII antibody interaction. 30µg/ml of rFVIII in 5% (v/v) Nafion was pipetted onto the electrode surface. The organic solvent evaporated leaving the rFVIII immobilised onto the surface. The electrode was incubated in HRP-labelled anti-rFVIII antibody at different temperatures, and then introduced into the electrochemical cell. Mediator (hydroquinone) was added to a final concentration of 2mM, followed by 1mM substrate (H_2O_2) and the signal measured.

6.5 Competitive Immunoassay

A competitive immunoassay was developed for the detection of rFVIII, and FVIII in normal plasma. The system was based on the competition between rFVIII immobilised onto the electrode surface, and free rFVIII (or FVIII in plasma), for binding to HRP-labelled anti-rFVIII antibodies (Figure 6.5.1). The signal generated is inversely proportional to the concentration of free rFVIII (or FVIII) in solution. Using the optimized experimental parameters, increasing concentrations of rFVIII were incubated with an equal volume of labelled antibody and the signal measured. A calibration curve was prepared using these signals, and is shown in Figure 6.5.2. Concentrations as low as 0.1µg/ml could be detected using this method.

Samples of normal human plasma were also tested. When undiluted plasma was used, only a very small signal could be detected. The sample was, therefore, partially purified by gel filtration (Section 2.27). FVIII, because of its size (molecular weight approx. 335 kDa), eluted just after the void volume, and its presence could be determined by FVIII activity assay. FVIII activity was identified in fraction 3 (Table 6.5.1). The protein concentration of the purified sample was 50- to 100-fold less than that of whole plasma. When a sample of this fraction was tested, a signal was obtained and the concentration of FVIII in the plasma determined (Table 6.5.2).

Commercial calibration plasma (Coag Cal N) was also tested and similar results to those in Section 5.6 (where Coag Cal N was analysed by ELISA) were obtained. No signal was produced by samples of whole unpurified Coag Cal N. The plasma was partially purified by gel filtration on a size exclusion column with a fractionation range of Mr 10,000 to 4,000,000 Da., and FVIII activity identified in fraction 4. The protein concentration of the purified sample was 50-fold less than that of whole plasma (Table 6.5.1). The FVIII-containing fraction was analysed using the electro-immunoassay, and a signal was obtained, from which the concentration of FVIII in the sample was calculated (Table 6.5.2).

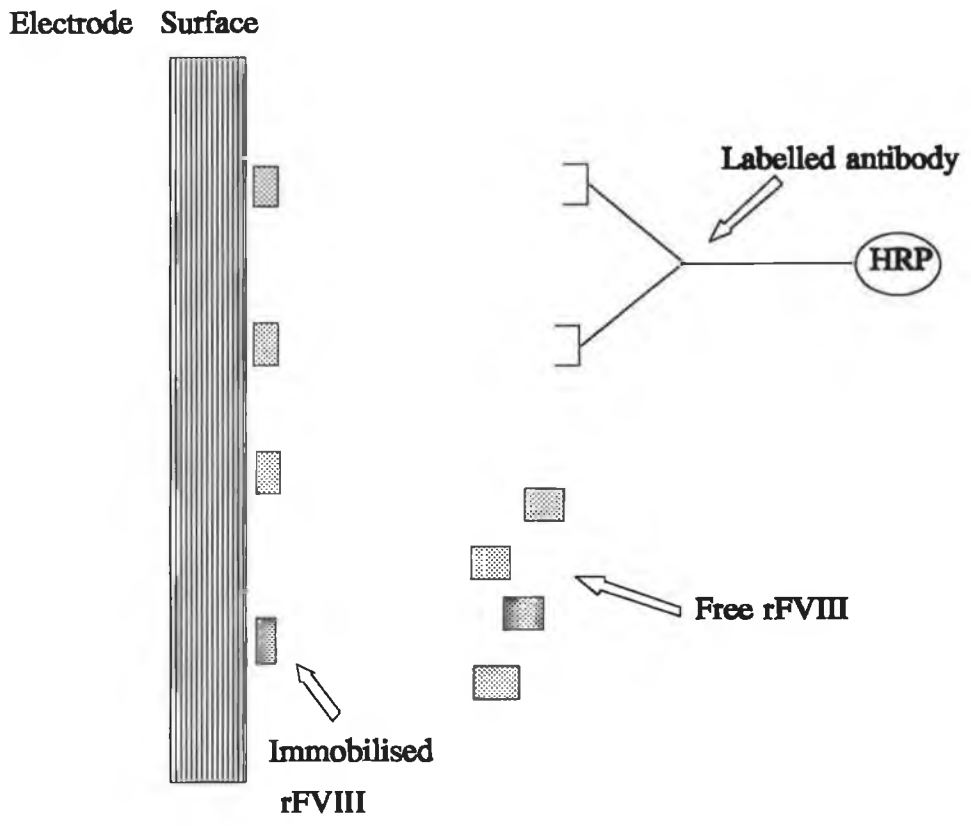


Figure 6.5.1 Competitive immunoassay for the detection of rFVIII in solution. rFVIII was immobilised onto the surface of the electrode and incubated in a solution containing equal volumes of free rFVIII and horseradish peroxidase (HRP)-labelled anti-rFVIII antibody. Both free and bound rFVIII compete for binding of the labelled antibody. The signal is inversely proportional to the concentration of the free rFVIII in solution.

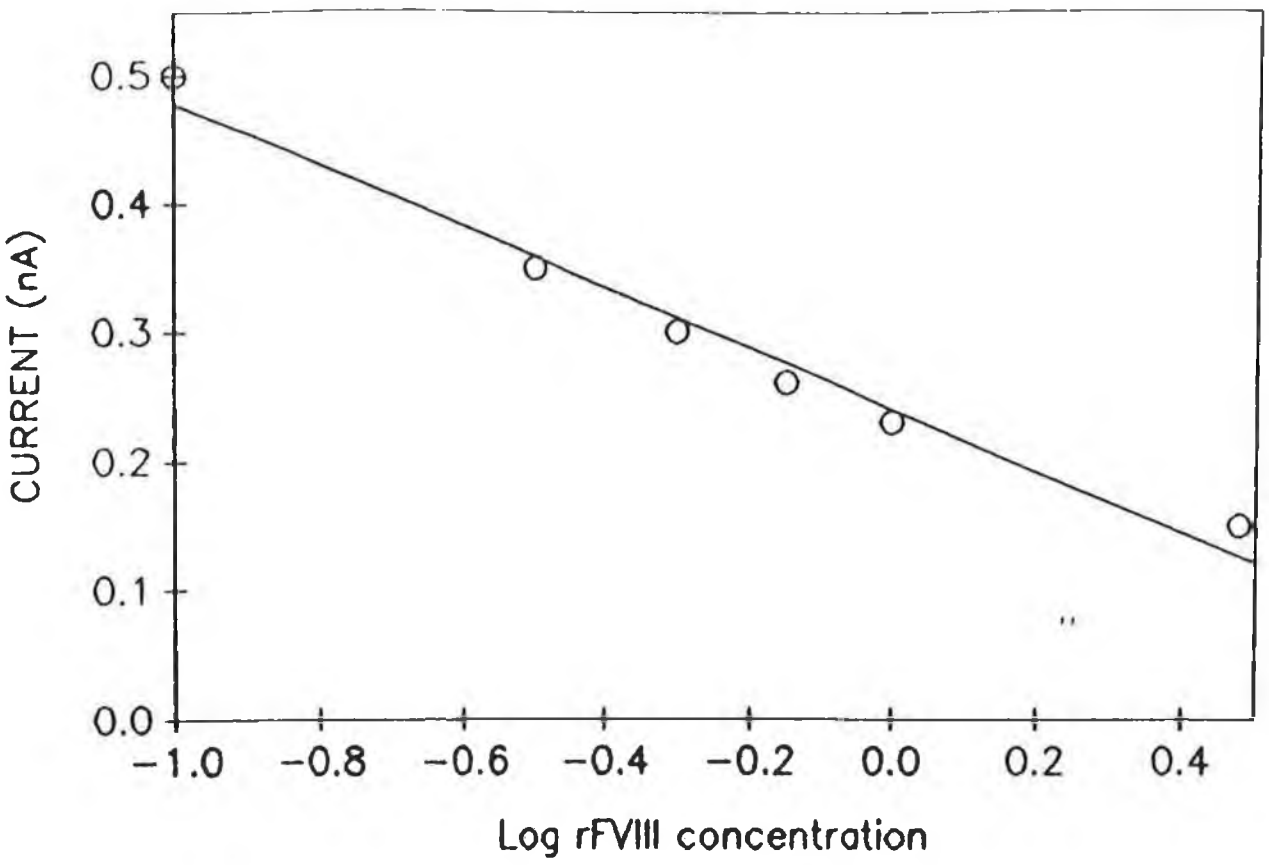


Figure 6.5.2 Calibration curve for the determination of rFVIII by electrochemical immunoassay. 20µg/ml of rFVIII in 5% (v/v) Nafion was immobilised onto the electrode surface. The electrode was incubated at 37°C with equal volumes of rFVIII and HRP-labelled anti- rFVIII antibody (33.3 µg/ml). r = 0.98, where r is the correlation coefficient.

Fraction	FVIII Activity (IU/ml)	Protein Concentration (mg/ml)
Plasma Sample 1		
1	-	-
2	-	-
3	1.10	1.8
4	0.03	> 3.0
5	-	> 3.0
6	-	> 3.0
Plasma Sample 2		
1	-	-
2	-	-
3	0.95	1.0
4	0.20	> 3.0
5	-	> 3.0
6	-	> 3.0
Coag Cal N Calibration Plasma		
1	-	0.20
2	-	0.17
3	-	1.80
4	1.34	> 3.00
5	0.40	0.60
6	-	0.40
7	-	

Table 6.5.1 Partial purification of whole normal plasma and calibration plasma. Plasma was purified by gel filtration on a Sepharose 6B column. This was a size exclusion column with a fractionation range of Mr 10 to 4,000 kDa. The protein concentration and FVIII activity of all fractions was monitored. FVIII-containing fractions were subsequently analysed by immunosensor. The spectrophotometer was capable only of reading absorbances up to approximately 3.5 absorbance units which corresponded to a protein concentration of 3 mg/ml. Above this value, only a tiny percentage of light was transmitted.

Sample	Signal (nA)	FVIII concentration (µg/ml)
Plasma Sample 1	0.25	3.0
Plasma Sample 2	0.20	7.5
Coag Cal N Calibration Plasma	0.50	0.5

Table 6.5.2 Determination of the concentration of FVIII in normal human plasma and commercial calibration plasma using a competitive electro-immunoassay. The plasmas were partially-purified by gel-filtration on a Sepharose 6B column.

A 1:5 dilution of the FVIII-containing fraction in PBS (0.15M, pH 7.2) was incubate with a modified glassy carbon electrode (with rFVIII immobilised onto the surface), and an equal volume of horseradish peroxidase-labelled anti-rFVIII antibodies. A signal was recorded after addition of mediator (hydroquinone) and substrate (H_2O_2).

Taking into consideration the sensitivity of the recorder, the signal obtained was converted into current, where:

1 cm on the graph = 0.5nA

The difference in values reflects the considerable variation in normal FVIII concentrations.

A similar competitive immunoassay to that for the detection of rFVIII was developed to detect anti-rFVIII antibodies in solution. Labelled and un-labelled anti-rFVIII antibody in solution compete for binding to the rFVIII immobilised onto the electrode surface (Figure 6.5.3). A calibration graph was prepared (Figure 6.5.4). Anti-FVIII antibodies develop in haemophiliac patients receiving FVIII replacement therapy. "Real" samples therefore, were not tested because of the risks of contamination with blood-borne viruses, especially hepatitis and human immunodeficiency virus (HIV).

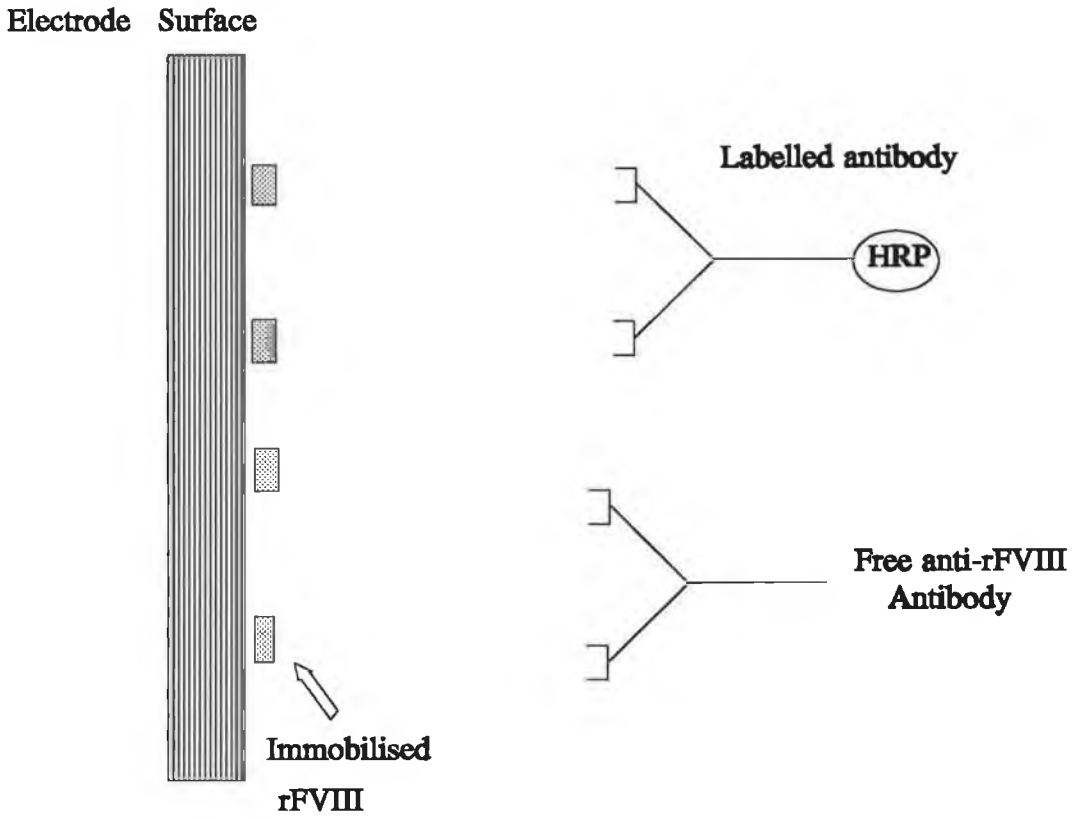


Figure 6.5.3 Competitive immunoassay for the detection of anti-rFVIII antibodies in solution.

rFVIII was immobilised onto the surface of the electrode and incubated in a solution containing equal volumes of horseradish peroxidase (HRP)-labelled and unlabelled anti-rFVIII antibody. Both labelled and unlabelled antibodies compete for binding to the immobilised rFVIII on the electrode surface. The signal is inversely proportional to the concentration of the unlabelled anti-rFVIII antibodies in solution.

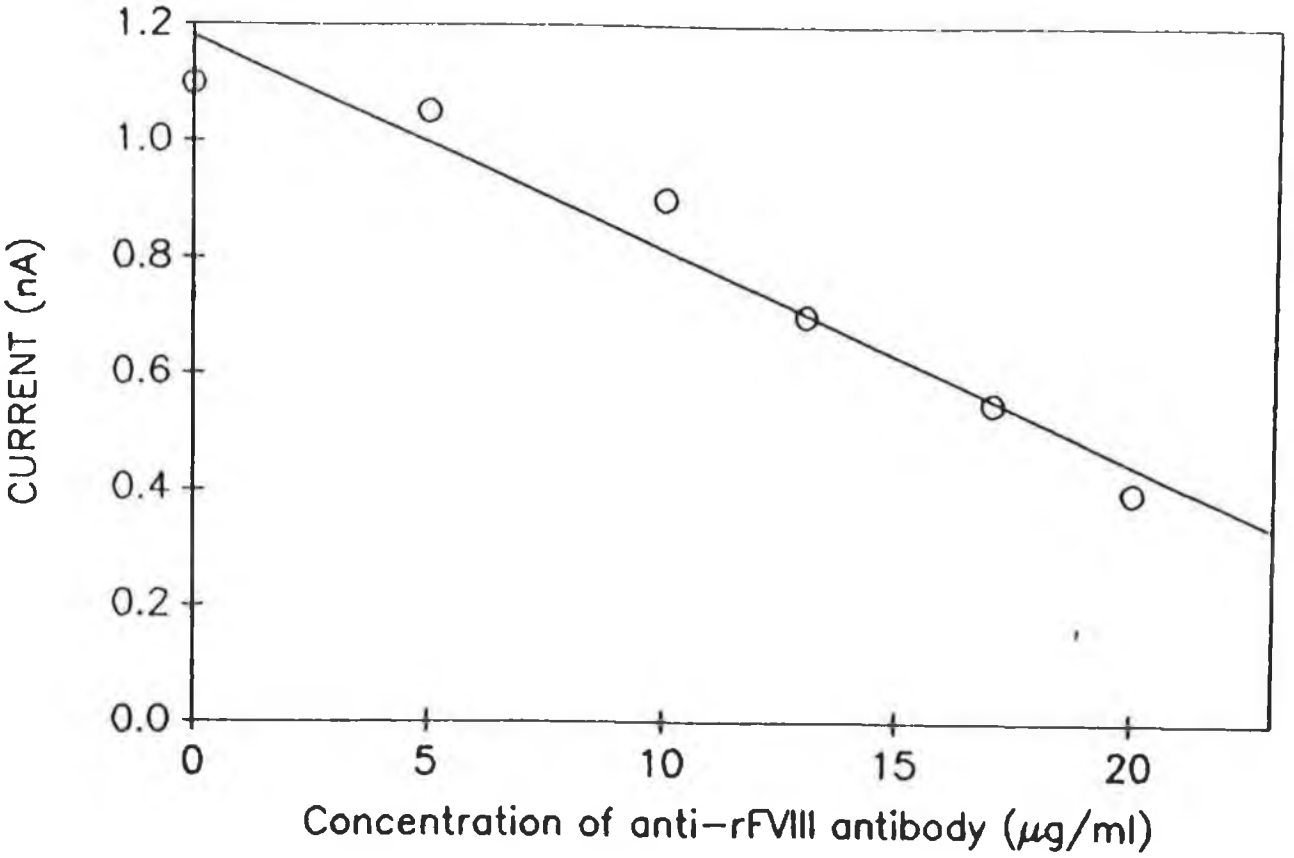


Figure 6.5.4 Calibration curve for the determination of anti-rFVIII antibodies by electrochemical immunoassay. 20μg/ml of rFVIII in 5% (v/v) Nafion was immobilised onto the electrode surface. The electrode was incubated at 37°C with equal volumes of anti-rFVIII antibody and HRP-labelled anti-rFVIII antibody (33.3 μg/ml).

r = 0.975, where r is the correlation coefficient.

6.6 Discussion

The immobilisation of rFVIII onto the surface of a glassy carbon electrode was accomplished using Nafion, a perfluorosulphonic acid polymer. It consists of a hydrophobic fluorocarbon backbone with anionic sulphonated side chains (Bindra and Wilson, 1989) and is, therefore, an anionic ion-exchange material. It particularly suitable for the immobilisation of proteins onto electrode surfaces because of its cationic selectivity, and exclusion of anionic species.

Nafion is soluble only in organic solvents, and so it is important to select the appropriate dilution and volume of polymer required. 10µl of FVIII in 5% (v/v) Nafion proved to give stable and reproducible films. Any dilution of Nafion lower than 5% (v/v) caused the FVIII film to crack, exposing the electrode surface.

The cationic selectivity of Nafion means that the coated electrode is sometimes prone to fouling. Other cationic species in the medium could become attracted to the electrode and interfere with the assay. This is particularly relevant in biosensors, as the analyte is generally present in a complex matrix (plasma, for example), where there is an abundance of cationic species and other possible interfering substance.

The incubation conditions for the labelled antibody are similar to those used in an ELISA. The concentration of the labelled-antibody used in this system was, however, at least 10-fold higher than that used in ELISA. This may be as a result of the different ways in which the signal is generated. In the electrochemical system the signal was generated by the flow of electrons (current) via a mediator. In an ELISA, the signal was generated by the oxidation of the substrate by the enzyme. The level of reducing agent (*i.e.*, the enzyme HRP) necessary to generate each signal differed, provided the substrate was in excess. More HRP was required to provide enough electrons to generate current, while the oxidation of substrate in the ELISA was a single event, and, therefore, required less enzyme. The orientation of the antigen (rFVIII) on the electrode surface could also have been a factor. rFVIII is immobilised onto the electrode surface in an ion-exchange membrane. Some of the epitopes may be hidden or altered in some way, preventing some of the antibodies from binding. As a result, it may not have been

possible for some of the high-affinity antibodies to bind to the antigen necessitating a higher overall concentration of the labelled-antibody.
It is important to determine the optimal incubation temperature for the antigen-antibody interaction, as the optimal conditions for the reaction may be different in different systems. The optimal conditions for an ELISA may not always be optimal for other systems. Generally speaking, the temperature at which binding is maximised is 37°C (Tijssen, 1985). This was found to be the case in this immunoassay.

The protein concentration of FVIII in normal serum is about 5-10µg/ml (Hoyer, 1981) out of a total protein concentration of around 100 mg/ml. It circulates in normal plasma as part of a complex with vWF. The two components can be separated using high ionic strength buffers (Section 1.7). In order to separate FVIII from vWF and to reduce the protein concentration (and thus possible interfering substances), plasma was partially purified on a size exclusion column. This facilitated recognition of FVIII by the specific antibodies in the immunosensor. Samples of whole and calibration plasma (lyophilised normal human plasma) did not produce a signal prior to the clean-up step, probably because of electrode fouling by contaminating proteins. After separation of the complex and sample clean-up a signal was obtained.

FVIII concentration in normal human plasma is usually within the range 5-10 µg/ml, but the levels are prone to daily physiological fluctuations. FVIII levels are raised during pregnancy, stress, after exercise, in inflammatory and neoplastic diseases and in patients with current thromboses (Spaethe, 1984). FVIII is, therefore, usually quantitated by activity, where 1 unit is defined as the amount of FVIII activity in 1 ml of normal plasma. In the determination of FVIII activity, there is a broad reference range from 50-200% (100% corresponds to the activity in a normal reference sample; reference range is the range covering 95% of all examined samples showing normal clotting function).

The FVIII concentrations of the normal plasma samples as determined by the FVIII immunosensor appear to be within the reference range (approximately 2.5-20 µg/ml). Competitive immunoassays for the detection of rFVIII could also detect FVIII in normal human plasma, and in Coag Cal N calibration plasma.

No FVIII could be detected in Coag Cal N samples assayed by antigen-capture ELISA (Section 5.2) or in normal human plasma assayed by competitive ELISA (Section 5.7). In the antigen-capture ELISA anti-rFVIII antibodies immobilised onto the surface of a microtitre plate captured and bound FVIII. The bound FVIII antibodies were subsequently detected using a labelled anti-rFVIII antibody and a chromogenic substrate (OPD). In the immunosensor, FVIII was measured by competitive assay. The use of an ion-selective membrane reduced the possibility of non-specific binding which may have contributed to the failure to obtain a signal in the competitive ELISA. The membrane selectively excluded anionic species, many of which are present in plasma (Gunasingham and Tan, 1989). This in turn minimised the risks of electrode fouling which would reduce the sensitivity of the assay. The reactants also remain in solution in the immunoassay, and so the chances of alteration of the binding sites when the antibodies are bound to the solid phase are eliminated.

A competitive immunosensor was developed for the quantification of anti-rFVIII antibodies. "Real samples" were not used because of the risks of contamination from blood-borne infectious agents (such as HIV or hepatitis), which are especially high in samples from haemophiliacs receiving FVIII replacememt therapy. The immunosensor provides an excellent means of quantitating rFVIII, FVIII and anti-rFVIII antibodies. The assay is simple to use, no expensive equipment is required and the assay time is considerably shorter than that of an ELISA. It was also more sensitive, allowing concentrations as low as 0.1µg/ml rFVIII to be detected, as compared with 0.5µg/ml of rFVIII which could be detected by competitive ELISA (Section 5.7). The assay time could be reduced even further with the use of a reusable modified electrode. rFVIII (or any antigen) could be immobilised onto the surface of the electrode, and incubated with the labelled-antibody. After the signal had been read, the antibody would be removed without disrupting the electrode surface. This is a critical point, as the electrode must be standardised for use throughout the complete assay, and not be subject to degeneration. A low pH glycine buffer (0.1M, pH 2.5) was used for this purpose, but it was found that the time required for complete antibody dissociation disrupted the film on the electrode surface, leading to variations in the signal. The development of reusable modified electrodes, however, will be an important step in biosensor technology.

In conclusion, the system described in this chapter provides a novel way of monitoring antibody-antigen interactions. The electrochemical immunoassay has proved to be a simple, highly selective and sensitive method for the determination of rFVIII and anti-rFVIII antibodies. The assay time was considerably shorter than that of an ELISA, with only three steps (two incubation periods, and the measurement of signal), and a response time of aproximately 1 minute. This is characteristic of most bioprobes (Bardeletti *et al.*, 1991). The assay time could be reduced further, by shortening the immobilisation step, and decreasing the antigen-antibody incubation step, or by use of a reusable modified electrode. The electroimmunoassay is safer than EIA, as it does not require the use of hazardous chemicals which are often used as enzyme substrates in ELISAs.

With the current developments in biosensor technology, electrochemical immunoassays are set to become a wide-spread alternative to the more traditional methods such as EIA.

CHAPTER 7

CONCLUSIONS

FVIII is inherently unstable in the absence of vWF. One of the aims of this project was to investigate the effects of chemical cross-linkers and protein modifying reagents on recombinant FVIII procoagulant activity, and determine whether any modification caused by these reagents resulted in an increase in the thermostability of rFVIII. A range of amino- and thiol-specific reagents were employed. It was discovered that the reaction of amino-specific cross-linking reagents caused a complete loss of FVIII procoagulant activity. In contrast, the thiol-specific reagents did not lead to rFVIII inactivation. This suggests that some free amino groups play an integral role in the expression of FVIII procoagulant activity. At least some of the free thiol groups can be modified without adverse effects on FVIII activity. However, any changes introduced into the molecule did not result in any increase in thermostability.

Polyclonal and monoclonal antibodies were raised against rFVIII. The effects of the binding of these antibodies on FVIII procoagulant activity and thermostability were investigated. The monoclonal antibodies were of the IgM class and binding of these antibodies caused complete loss of FVIII activity. The binding epitopes could not be identified by Western blot analysis. This suggested that the antibodies were directed against some part of the tertiary structure of the molecule, which was destroyed during the course of the experiment (possibly by SDS). The antigenic determinants could have been located at or adjacent to the thrombin cleavage sites, preventing access to the cleavage sites by steric hindrance. Alternatively, the binding of the pentameric IgM molecule may cause some degree of strain within rFVIII, distorting the molecule and destroying procoagulant activity.

The polyclonal antibodies bound to rFVIII without causing the same degree of inactivation. These antibodies were of the IgG class and, therefore, would not induce the same degree of strain or steric hindrance in the antigen. As the preparation was polyclonal it was composed of a variety of antibodies with different specificities and affinities, which may not have been targeted near the active portion of rFVIII. The binding of these did result in a small increase in thermostability. Monoclonal IgG antibodies directed against sites near to the inactivation sites may prove to increase the half-life of active FVIII, by preventing access to the cleavage sites.

Alternatively, antibodies that link the heterotrimer (which constitutes the active form of FVIII) together may stabilise the protein by preventing dissociation of the subunits.

Another objective of these studies was to develop analytical methods for the detection and quantitation of FVIII. It is extremely important to be able to quantify FVIII levels in the diagnosis and treatment of haemophilia, the deficiency disease of FVIII. Current methods rely on the ability of FVIII to correct the prolonged bleeding time of FVIII-deficient plasma. These methods are difficult to standardise and are prone to error, as they are dependant on the operator. rFVIII is being increasingly used in replacement therapy for the treatment of haemophilia. It provides a safer alternative to human-derived FVIII, and should be less immunogenic than animal derived FVIII. The development of an ELISA for the detection of rFVIII and anti-rFVIII antibodies allows for the detection and quantitation of rFVIII and anti-rFVIII antibodies which may develop in patients. It is a simple, reliable and easy to use method. A number of modifications can be made which allow for different specifities: different incubation conditions for blocking free binding sites, a 50-fold increase in sensitivity with the use of a linker layer, shorter assay times with the use of a luminescent substrate. The use of polyclonal and monoclonal antibodies in this system provides a safer alternative to the human-derived antibodies traditionally used. The risk of infection from blood-borne viruses is eliminated.

Biosensors provide an ideal analytical device for the modern era. A biosensor was developed which incorporated rFVIII onto the surface of an electrode, and was capable of detecting FVIII in normal human plasma, as well as rFVIII in solution. The assay is simple to use, does not involve the use of hazardous chemicals, is sensitive with an assay time of only hours. This may be reduced even further, with the development of a reusable modified electrode. This could prove to be an exciting development, as future commercial applications may be important. The possibility of a home test to monitor FVIII and anti-FVIII antibodies levels could reduce the cost of treatment, and revolutionise the treatment of disorders such as haemophilia.

CHAPTER 8

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