THE BIOCHEMICAL GENETICS OF TWO PROTEINS INVOLVED IN WOUND HEALING AND BLOOD COAGULATION: FIBRONECTIN AND BLOOD COAGULATION FACTOR XIII

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ABSTRACT

1. This study investigates the biochemical genetics of two plasma proteins, fibronectin and blood coagulation factor XIII, which interact in the biological processes of blood coagulation and wound healing.
2. An electrophoretic technique for the rapid screening of blood samples for plasma fibronectin is described. This technique has been used to screen nearly 3,000 human plasma and serum samples as well as plasma samples from dogs, cats, pigs, goats, mice, donkeys, sheep, horses, camels, cattle and Highland ponies for genetic variants of plasma fibronectin. Only one possible variant from an Indonesian population was detected.
3. The electrophoretic technique described here should, in combination with somatic cell hybrids, be useful in determining the chromosomal location of the fibronectin structural gene(s). In addition it could prove useful as a diagnostic aid in disorders which may be a result of functionally abnormal fibronectin molecules.
4. The distribution of the three previously reported alleles, with normal products, at the FXIII-A structural locus, *FXIIIA*1*, *FXIIIA*2* and *FXIIIA*4* has been extended by the study of populations from the region extending from the Indonesian archipelago through Papua New Guinea, Australia and New Zealand to the Pacific Island regions of Micronesia, Melanesia and Polynesia. In addition a population from the Caspian Littoral of Iran and a population of S. American Indians were studied. The *FXIIIA*1* and *FXIIIA*2* alleles were polymorphic in all populations studied. The distribution of the *FXIIIA*4* allele suggests that it may be a

a Melanesian marker.

5. A new allele, *FXIIIA*3*, was found in a sample from New Zealand. The A subunits coded for by this allele are apparently unstable. The potential importance of this allele in congenital factor XIII deficiency is discussed. The Type 3 A subunits were partially biochemically characterised.
6. The Type 1 and Type 2 A subunits were purified and their substrate specificities, heat stability, pH range and optima were compared. There does not appear to be any physiologically important differences between them.
7. The literature concerning fibronectin and blood coagulation factor XIII is reviewed.

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EXPERIMENTAL AIMS

The basic objectives of this project can be summarised into three main points: a) attempt to elucidate the genetic relationship of plasma and cellular fibronectin; b) to further the knowledge of the biochemical genetics of coagulation factor XIII A subunits and c) to study the interaction between plasma fibronectin and the products of the two common alleles at the FXIII-A structural locus, *FXIIIA*1* and *FXIIIA*2*.

Fibronectin, a high molecular weight glycoprotein, is widely distributed in the body of most multicellular animals. The biological functions of fibronectin have not yet been clearly defined, but it has been proposed that fibronectin plays an integral role in the mediation of cell-cell and cell-substratum interaction, the maintenance of normal cell morphology and cell movement and tissue formation during embryogenesis as well as being involved in the wound healing process and the normal functioning of the mononuclear phagocytic system (see Chapter 1).

Although studies have attempted to determine the structure of the fibronectin molecule from different sources within the body and look at the relationship of these different forms of fibronectin, no studies have attempted to determine the genetic control of fibronectin. Therefore, because of the lack of knowledge concerning the genetics of fibronectin and the known interaction between factor XIII and fibronectin during the blood coagulation and wound healing processes, it was considered important to develop an electrophoretic system which would allow the biochemical genetic investigation of fibronectin (see Chapter 2).

Electrophoresis in a number of different media is a standard tool for the investigation of the genetic control of proteins. By utilising a suitable electrophoresis system to screen plasma fibronectin samples from a number of different human populations from several areas of the world it was hoped to determine the gene frequencies of any polymorphic or rare alleles at the structural locus for plasma fibronectin. In addition, it was hoped that the electrophoretic banding patterns produced by these alleles would allow a genetic model for plasma fibronectin to be determined together with the exact genetic relationship of plasma and cellular fibronectin. With the finding that plasma fibronectin from man was electrophoretically invariable, plasma samples from a number of domestic animals were also investigated in a further attempt to produce an explanation of the genetic control of fibronectin (see Chapter 2).

If plasma fibronectin was polymorphic, it was intended to look at the interaction of the purified products of the different alleles with the products of the polymorphic alleles, *FXIIIA*1* and *FXIIIA*2* at the factor XIII A subunit structural loci to determine if there were any differences which could be physiologically important. However, as plasma fibronectin proved to be electrophoretically invariable, this part of the study had to be restricted to the normal form of plasma fibronectin with the Type 1 and Type 2 A subunits of factor XIII (see Chapter 5).

The A subunits of coagulation factor XIII are polymorphic for two common alleles in all populations studied. In addition, a third allele, *FXIIIA*4*, has been reported in Melanesians from Fiji. Because of the potential importance of factor XIII A subunits as a genetic marker in population genetic studies, it was important to define the distribution of the alleles at the A subunit structural locus more

closely. Therefore, a number of populations in the region from the Indonesian archipelago, through Papua New Guinea and Australia to the Pacific Island regions of Melanesia, Micronesia and Polynesia as well as a population from the Caspian Littoral of Iran and of Pima Indians from South America, were investigated (see Chapter 4).

During the course of the population survey for factor XIII a new allele was found which codes for unstable A subunits. Because of the probable importance of this allele in the aetiology of congenital factor XIII deficiency it was considered that families with factor XIII deficiency should be investigated in order to determine the frequency of the occurrence of this allele in individuals with this disorder (see Chapter 4). In addition, the biochemical characteristics of these functionally abnormal A subunits were investigated to a limited extent (see Chapter 5).

The biochemical characteristics of the two common alleles of the factor XIII A subunit locus are largely unknown. Therefore, the Type 1 and Type 2 A subunits were purified and investigated in an attempt to determine if any physiologically important biochemical differences exist between them.

Chapter 1

I. HISTORICAL EVENTS (1948-76)

A. Nomenclature

The name Fibronectin is used to describe a family of structurally and immunologically related high molecular weight glycoproteins that are widely distributed in animal tissues and body fluids. The term fibronectin was suggested by Kuusela *et al.* (1976) to emphasise the tendancy of the protein to bind to fibrous proteins (*fibra*, fiber and *nectere*, to bind). Prior to the use of the term fibronectin the glycoprotein had been known by an abundance of names (Table 1.1).

B. History

In 1948, Morrison *et al.* reported the presence of cold insoluble globulin (CIg) in plasma cryoprecipitates. Unlike fibrinogen (another component of the cryoprecipitate) CIg was not thrombin coagulable. The report of Edsall *et al.* (1955) led to the suggestion that CIg was a modified dimer of fibrinogen. In 1957, Smith and Von Korff described a protein with similar properties to CIg in heparin-induced cold precipitates.

Mosesson *et al.* (1968) demonstrated that CIg was immunochemically identical with a serum protein unrelated to fibrinogen during the investigation of a patient with a chronic intravascular coagulation syndrome. This led to the purification of CIg with the concomitant production of evidence that CIg was a unique and major plasma protein (Mosesson and Umfleet, 1970).

By the 1970's, a number of studies were focussed on the effect of transformation by oncogenic viruses on the cell surface proteins of fibroblasts. In 1975, Ruoslahti and Vaheri reported that CIg was

TABLE 1.1.

PREVIOUS NAMES OF FIBRONECTIN

Name	Abbreviation	Reference
Cold-Insoluble Globulin	CIg	Morrison *et al.* 1948
Large, External, Transformation Sensitive Protein	LETS	Hynes and Bye, 1974
Surface Fibroblast Antigen	SFA	Ruoslahti *et al.* 1973
Cell Surface Protein	CSP	Yamada and Weston, 1974
Galactoprotein A	Gap A	Gahmberg and Hakomuri, 1973
Anti-Gelatin Factor	AGF	Maurer, 1954
Cell Attachment Protein	c-CAP	Klebe, 1974
Opsonic Protein	-	Saba, 1970
Cell Adhesion Factor	CAF	Pearlstein, 1976
Zeta-Protein	Z	Blumberg and Robbins, 1975
Cell Spreading Factor	CSF	Grinnell, 1976
Band 1, LI Band, Band I	-	Hogg, 1974
Major Fibroblast Glycoprotein	-	Sear *et al.* 1976
Micro-Fibrillar Protein	-	Ross and Bornstein, 1969

antigenically identical to a large transformation-sensitive glycoprotein (LETS) released from the fibroblast cell surface. Thus, the uniqueness of fibronectin as a cell surface/matrix protein and as a blood protein was demonstrated. Subsequently, numerous studies of the various forms of fibronectin have been undertaken.

II. DISTRIBUTION

A. Cellular

Fibronectin is a major component of the connective tissue matrix. It is particularly abundant in basement and limiting membranes (Linder *et al.* 1975), however, Heathcote and Grant (1981) have suggested that fibronectin is a component of the cell surface-basement membrane interface rather than a subunit of the basement membrane matrix. Fibronectin has been located in the embryonic skin, trophoblast and alveolar basement membranes, the sinusoidal walls of the liver, the stroma of lymphatic tissue, exocrine glands, mucous membranes, sweat, sebaceous, mammary and parotid glands, the spleen and intestine (Mosesson and Amrani, 1980 and Pearlstein *et al.* 1980). Sanes and Cheney (1982) have shown that fibronectin is present synaptically and extrasynaptically in the basal membrane of muscle fibres as well as being present on the overlying reticular lamina, the surface of blood vessels, muscle spindle capsules, myelinated axons and the perineurium and diffusely throughout the endoperi- and epimysial connective tissue. The absence of fibronectin from glomerular basement membranes indicates that it is not invariably associated with basement membrane structures (Ruoslahti *et al.* 1981).

In culture fibronectin has been found to be synthesised by, or to be associated with, a variety of cells: fibroblasts, amniotic fluid cells, vascular and corneal endothelial cells, smooth muscle cells, astroglial cells, secondary Schwann cells, glomerular cells, intestinal, kidney and liver epithelial cells, hepatocytes, chondrocytes, myoblasts, some macrophages and early mesenchymal cells (Yamada and Olden, 1978; Mosesson and Amrani, 1980 and Pearlstein *et al.* 1980). Fibronectin has also been shown to be associated with cervical uterine epithelial cells (Alitalo *et al.* 1982), alveolar macrophages (Czop *et al.* 1982b), primor-

dial germ cells (Heasman *et al*. 1981) and platelets and megakaryocytes, where it is mainly found in the α-granules (Mosesson and Amrani, 1980 and Taketomi and Bevel, 1982), but it appears to be absent from liver parenchymal cells (Kojima *et al*. 1981) and neutrophils, eosinophils, lymphocytes, monocytes and red blood cells (Grinnell and Feld, 1981).

The fibronectin on the cell surface and in the extracellular matrix is usually located in fibrillar arrays, although on some cells the distribution may be punctate in appearance (Yamada and Olden, 1978; Mosesson and Amrani, 1980 and Pearlstein *et al*. 1980). Lyubimov and Vasiliev (1982) found that, on mouse fibroblasts, large fibronectin fibrils are preferentially located on the surface of the endoplasm, while smaller structures are only found on the lammeloplasm. It is proposed that the centripetal movement of receptor-attached fibronectin, a characteristic of other receptor-attached proteins (Vasiliev and Gelfand, 1981), on the cell surface promotes this distribution pattern. Intracellular fibronectin has been located on the cisternae of rough endoplasmic reticulum and in the large vacuoles of the Golgi area in a granular perinuclear distribution (Yamada, 1978 and Vaheri *et al*. 1980). The distribution of fibronectin is usually determined by immunolocalisation studies with anti-fibronectin antibodies.

B. Body fluids

Fibronectin is also found in plasma and serum (Morrison *et al*. 1948), amniotic fluid (Chen *et al*. 1978), synovial fluid (Vartio *et al*. 1981), the cerebrospinal fluid (Kuusela *et al*. 1978, the bronchial fluid (Rennard and Crystal, 1982) and human milk (Zardi *et al*. 1982). The source of fibronectin in body fluids is uncertain. The results of Sodium Dodecyl Sulphate gel electrophoresis (Birdwell *et al*. 1980) suggest that plasma fibronectin does not originate in vascular endothelial cells as has been proposed (Mosesson and Amrani, 1980). At

least in plasma, where fibronectin is one of the major proteins (325±76 µg/ml, Stathakis *et al.* 1981) it is likely that fibronectin is secreted by a variety of cells. Oh *et al.* (1981) suggested that plasma fibronectin is in equilibrium with tissue fibronectin and that they act as pools for each other. The concentration of plasma fibronectin increases exponentially with age (Labat-Robert *et al.* 1981b). It is thought that this may be related to the fact that fibronectin from senescent fibroblasts does not bind to the cell surface in the normal manner (Vogel *et al.* 1981).

III. FUNCTIONS

A. The cellular matrix

Fibronectin has been shown to interact with two components of the extracellular matrix, collagen and glycosaminoglycans. These interactions have been discussed in detail elsewhere (Mosesson and Amrani, 1980; Pearlstein *et al.* 1980; Ruoslahti *et al.* 1980a and 1980b; Yamada *et al.* 1980; Mosher and Furcht, 1981 and Vuento *et al.* 1982). The type of matrix formed by a cell appears to differ in a manner which is dependent on the type of adhesion exhibited by the cell, for example, the typical fibrillar matrix is only shown by non-motile cells (Couchman *et al.* 1982). The extracellular matrix structure is also one of the components that determines the growth potential of a cell (Couchman *et al.* 1982).

Fibronectin binds to all the genetic types of collagen. The interstitial collagens (Type I, II and III) interact more readily than the basement membrane collagens (Type IV and V) (Engvall *et al.* 1978). This is probably due to the conformation of the collagen molecules. Silver *et al.* (1981) have suggested that cells use fibronectin to bind collagen in the matrix but, as will be discussed below, the glycosaminoglycan composition of the matrix is important in the fibronectin-collagen interaction. Mosher *et al.* (1980) demonstrated that fibronectin could be cross-linked to collagen by the plasma transglutaminase, factor XIIIa. The *in vivo* significance of this reaction is uncertain, but it could lead to the stabilisation of the matrix. The collagen binding site is distinct from the site which is susceptible to factor XIII catalysed cross-linking.

The use of double staining fluorescent antibody techniques has shown that both in connective tissues and the extracellular matrix fibronectin and collagen are distributed together (Linder *et al.* 1975

and Bornstein and Ash, 1977). However, some cells form a matrix without any collagen (Culp and Bensusan, 1978) so the interaction cannot be a necessity in all cell matrices.

Fibronectin binds to the following glycosaminoglycans: the more heavily sulphated forms of heparan sulphate, hyaluronic acid and chondroitin sulphate - containing proteoglycans. Perkins *et al.* (1979) showed that fibronectin near the cell surface is closely associated with proteoglycans and suggested that the association may be important in cell-extracellular matrix interactions. The role of glycosaminoglycans in determining the organisation of cell surface fibronectin and the composition and quantity of the extra-cellular matrix deposited by a cell has recently been more closely defined (Laterra and Culp, 1982 and Oldberg and Ruoslahti, 1982). Cell surface fibronectin is insolubilised by the presence of both collagen and proteoglycans (Oldberg and Ruoslahti, 1982). Only endogenous fibronectin binds to hyaluronate and then only if it is in multimer aggregates (Laterra and Culp, 1982 and Rosso *et al.* 1982). As hyaluronate inhibits the formation of fibronectin-collagen complexes (Rosso *et al.* 1982) this may represent an important difference between endogenous and exogenous fibronectins. The binding of fibronectin to heparin is of three types: a) Ca^{2+} only sensitive; b) non-specific divalent cation sensitive; and c) divalent cation insensitive. Each type of binding occurs at a unique site on the fibronectin molecule (see Chapter I.V.C) (Hayashi and Yamada, 1982).

The *in vivo* formation of cross-linked multimers of fibronectin by factor XIIIa has been demonstrated (Mosher, 1975 and Keski-Oja 1976). Similar cross-linked multimers have been located *in vivo* (Mosher and McKeown-Longo, 1982) and may, therefore, be important in the irreversible incorporation of fibronectin into the matrix.

However, the reduction of disulphide bonds in the fibronectin molecule may be a prerequisite for cross-linking to occur (Williams *et al.* 1982).

It is probable that the formation and structure of the extracellular matrix is dependent on the complex interactions between the cell, fibronectin, collagen, glycosaminoglycans and a transglutaminase, with fibronectin possibly acting as a central organiser.

B. The cellular cytoskeleton

Immunofluorescence studies have shown that extracellular fibronectin fibrils run parallel to the long axis of the cell in a similar manner to intracellular microfilament bundles (Wartiovaara *et al.* 1974 and Hynes and Destree, 1978) although they do not always coincide Yamada and Olden, 1978). As fibronectin has been shown to bind to actin *in vitro* (Pearlstein *et al.* 1980) a transmembrane link has been postulated between the two proteins in areas where their distribution coincides. Singer (1979) used electron microscopy to show that, in the area of cell-substratum interaction actin cables and fibronectin fibres are co-linear with a maximum separation of 8-22 nm.

Fibronectin is particularly abundant in the area of cell-substratum contact (Culp, 1976). Transformed fibroblasts have no detectable fibronectin and an abnormal morphology. If exogenous fibronectin is added, the fibroblasts return to a normal morphology and form normal actin containing microfilaments (Yamada *et al.* 1976a and 1976b). This is possibly due to the area in contact with the substratum being used to anchor the microfilaments.

It is uncertain whether fibronectin controls the distribution of microfilaments or if microfilaments control the distribution of cell associated fibronectin (Pearlstein *et al.* 1980). However, Virtanen *et al.* (1982) have shown that *in vitro* fibroblasts which lack fibronectin do not form the characteristic bundles of microfilaments and

plaques of vinculin at adhesion sites.

C. Cell adhesion and spreading

From the interactions discussed in the previous two sections and the interaction of fibronectin with fibrin (Ruoslahti and Vaheri, 1975) it is likely that one of the main functions of fibronectin *in vivo* is to mediate cell-cell and cell-substratum interactions. Although the demonstration that cellular fibronectin agglutinates formalinised sheep red blood cells (Yamada *et al.* 1975) indicated that fibronectin played a role in cell-cell interactions, most studies have concentrated on cell-substratum interactions in *in vitro* models.

The normal cell is anchorage dependent for growth in culture. It is possible that this is caused by the presence of fibronectin, as cells with intercellular and surface fibrillar fibronectin do not grow under anchorage-independent conditions (Rajaraman and Lonergan, 1982). The attachment of cells to the substratum requires divalent cations and the expenditure of cellular metabolic energy (Klebe, 1974; Grinnell, 1976 and Pearlstein, 1976). The presence of fibronectin appears to be essential for most cell types to adhere to a substratum in culture (Pearlstein, 1976; Pearlstein and Gold, 1978; Grinnell and Hays, 1978 and Couchman *et al.* 1982). However, some cell types can adhere in the absence of fibronectin (Federgreen and Stenn, 1980). Virtanen *et al.* (1982) have demonstrated that cultured human fibroblasts do not require fibronectin for adhesion and spreading, although focal adhesion sites are not formed in the absence of fibronectin. It is suggested that there are cell-dependent and cell-independent phases in cell-substratum adhesion and that the deposition of endogenous pericellular fibronectin is only required in the former phase.

Investigators in the early 1970's described the presence of cellular attachment plaques, areas of the cell in contact with the sub-

stratum and other cells. Fibronectin is found in high concentrations in attachment plaques (Abercrombie *et al.* 1971; Harris, 1973 and Shields and Pollock, 1974) and in several cell types it is concentrated in the area where the cell is in contact with the basement membrane (Pearlstein *et al.* 1980). In addition to fibronectin, a 140,000 molecular weight protein is also abundant in cell attachment plaques and is thought to act in a cooperative manner with fibronectin in cell attachment (Carter, 1982 and Rieber and Rieber, 1982).

All these observations suggest that fibronectin forms a bridge between cell surfaces and between the cell surface and the substratum. The proof that the cell binding site is in a separate domain of the fibronectin molecule to the collagen binding site is important in this regard (McDonald *et al.* 1981). If this model is correct then there must be receptors for fibronectin on the cell surface. Yamada *et al.* (1981) have shown that these receptors consist of or contain gangliosides or other negatively charged lipids. Glycoprotein receptors which contain N-acetyl-glycosamine residues have been isolated from platelet membranes (Hansen and Clemmensen 1981 and 1982). As some cells only adhere to fibronectin complexed with collagen (Pearlstein, 1978) the receptors of these appear to only recognise the complex. The stability of the cell-cell and cell-substratum links is probably enhanced by the interaction of fibronectin which heparan sulphate and the fact that fibronectin molecules can be cross-linked by intermolecular disulphide bonds and by a transglutaminase. The binding polysaccharide for fibronectin on neural tumour cells is a heavily N-sulphated heparan sulphate (Culp and Domen, 1982). Grinnell and Feld (1982) proposed that *in vivo* heparan sulphate is as important as fibronectin in cell spreading.

The behaviour of fibronectin in the cell cycle is consistent with a role in the maintenance of cell morphology and cellular attachment (Hynes and Bye, 1974).

Culp *et al.* (1979) suggested that hyaluronic acid and chondroitin may interact with fibronectin to loosen the adhesion for cell movement to occur as these two molecules are deposited in "footprints" left behind by cells when they move over a substrate. Fibronectin has also been shown to stimulate the directed migration or chemotaxis of cells (Vaheri *et al.* 1980 and Baron-Van Evercooren *et al.* 1982).

D. Embryogenesis

If fibronectin mediates cell adhesion and spreading through a variety of molecular interactions as suggested above, it is probable that the levels of fibronectin in embryonic tissues will play a role in development. Linder *et al.* (1975) showed that the amount of fibronectin in embryonic tissues is developmentally regulated with the report that the differentiation of myoblasts to form muscles is accompanied by a loss of fibronectin. Hassel *et al.* (1979) suggested that the loss is due to the cells no longer being able to bind fibronectin rather than through a reduction of fibronectin synthesis. Support for this hypothesis comes from the results of Chiquet *et al.* (1981).

Studies of embryogenesis in several diverse organisms, sea urchins (Spiegal *et al.* 1980), *Xenopus laevis* (Heasman *et al.* 1981), avian species (Hanke and Sawyer, 1982; Mitrani and Faberov, 1982 and Tomasek *et al.* 1982) and mice (Brownell *et al.* 1981 and Silver *et al.* 1981) indicate that the deposition of fibronectin on the cell surface is important as early as the blastula and gastrula stages as well as in tissue and organ morphogenesis. It is probable that fibronectin is essential for directed cell migration and the maintenance of the cohesion and orientation of cells during the cleavage stages of embryogenesis (Spiegal *et al.* 1980 and Mitrani and Faberov, 1982).

E. Blood coagulation and wound healing

Fibronectin has been shown to interact with fibrinogen and fibrin during clot formation (Ruoslahti and Vaheri, 1975). The concentration of fibronectin in human serum is between 20-50% lower than in plasma and a normal clot contains 94-95% fibrin, 3-5% fibronectin and 0-1% α_2-plasmin inhibitor (Chen *et al.* 1977 and Mosher and Furcht, 1981). *In vitro* fibronectin can bind covalently or non-covalently with fibrin. The covalent association results from γ-glutamyl-ε-lysyl cross-linkages catalysed by plasma factor XIIIa (Mosher, 1975). In patients with factor XIIIa deficiency the fibronectin concentration is the same in the serum after clot formation as that in the plasma (Mosher, 1976a). It is therefore probable that *in vivo* fibronectin is cross-linked to the clot. McDonagh (1981) has suggested that a direct interaction between factor XIII and fibronectin could be part of a control mechanism that ensures that a sufficiently high level of enzyme concentration exists for normal clot formation to occur.

Fibronectin is also associated with platelets (Mosesson and Umfleet, 1970). Most of the fibronectin is found in the intracellular α-granules. After the platelet release reaction induced by thrombin about 50% is released into the medium, the rest is bound to the platelet plasma membrane (Zucker *et al.* 1979 and Ginsberg and Plow, 1981). Plow and Ginsberg (1981) have shown that thrombin-stimulated platelets specifically bind plasma fibronectin. Both these reactions will result in fibronectin concentrations being high at the site of clot formation. The presence of fibronectin on the platelet surface should enable the platelets to be cross-linked into the clot.

The presence of fibronectin in the clot only subtly alters the properties of the clot. In fine clots the elastic modulus is reduced by half while, in coarse clots, the elastic modulus is doubled (Mosher, 1975).

At the site of the wound fibronectin forms a coat on the fibrin clot (Grinnell and Feld, 1981) which may play a dual role. If the fibronectin is cross-linked to the surrounding collagen substratum, the clot would be anchored and stabilised. It could also act as a matrix for collagen deposition (Mosher and Furcht, 1981).

Grinnell *et al.* (1980) have shown that fibronectin is required for fibroblasts to interact with fibrin *in vitro*. The implication that the presence of fibronectin in the clot is required for normal healing is supported by studies on factor XIII deficient patients (Duckert *et al.* 1960 and Beck *et al.* 1961), where the abnormal processes of wound healing are probably associated with the failure of fibronectin to be covalently incorporated into the clot and that the migration of fibroblasts into the clot is dependent on the chemotactic activity of fibronectin (Seppa *et al.* 1981). The observation that exogenous fibronectin can promote the premature entry into the growth phase by motile fibroblasts may also be important in the wound healing process (Couchman *et al.* 1982). Fibronectin may also play a role in clot lysis as the presence of fibronectin enhances the initial rate of activation of plasminogen by urokinase (Iwanaga *et al.* 1978).

The occurrence of fibronectin in the necrotic area of fibrotic human livers (Hahn *et al.* 1980 and Kojina *et al.* 1981) and in arthritic joints (Scott *et al.* 1982) suggests that the presence of fibronectin is important in the early stages of fibrosis. At least in the liver, it appears that the deposition of fibronectin precedes that of collagen at the locus of tissue repair (Kojima *et al.* 1981).

F. <u>The mononuclear phagocytic system (The reticuloendothelial system)</u>

The mononuclear phagocytic cells (macrophages) are distributed throughout the body, although they are mainly associated with the spleen, liver and bone marrow. Macrophages function by recognising and removing

(via phagocytosis) a variety of particulate matter from the vascular system, including bacteria, immune complexes, injured platelets, fibrin aggregates and tumour cells (Saba, 1970; Mosesson and Amrani, 1980 and Pearlstein *et al.*1980). The uptake of particulate matter is, at least partly, controlled by the presence of opsonic signals on the surface of the targets. The best studied opsonins are immunoglobulin G (IgG) and C3b, a component of the complement pathway. Opsonisation means the promotion of particle ingestion by phagocytic cells. Generally opsonins mediate particle recognition and initiate the mechanisms necessary for ingestion.

The exact role that fibronectin has in phagocytosis is uncertain. *In vitro* studies have shown that fibronectin mediates the attachment of certain particulate matter to macrophages and monocytes (Molnar 1979a; Mosesson and Amrani, 1980 and Marquette *et al.* 1981). The ability to maintain normal phagocytic activity *in vivo* has been correlated with the level of plasma fibronectin (Saba, 1970 and Blumenstock *et al.* 1978). However, no causal relationship has been proven for the two factors (Lundsgaard-Hansen, 1982 and Rubli *et al.* 1982). The opsonin-independent phagocytosis of particles that activate the alternative complement pathway by alveolar macrophages is enhanced by fibronectin (Czop *et al.* 1982b). Also an 160,000 M.W. protein thought to be derived from fibronectin promotes the phagocytosis of the above particulate activators by peripheral blood monocytes (Czop *et al.* 1982a). This observation is important as other studies have shown that the complete fibronectin molecule does not promote phagocytosis by the mononuclear phagocytic system (Molnar *et al.* 1979a; Mosesson and Amrani, 1980 and Marquette *et al.* 1981).

Fibronectin binds and can be cross-linked to *Staphylococcus aureus* but not to *Escherichia coli* or *Mycobacterium butyricum* (Mosher and

Procter, 1980 and Mosher and Furcht, 1981). Verbrugh *et al.* (1981) have shown that fibronectin binds to a staphylococcal surface protein shared by strains of *S. aureus* and *Staphylococcus epidermidis*, but that compared to IgG and C3b fibronectin has a relatively minor role as an opsonin for staphylococci. Fibronectin also mediates the attachment of *S. aureus* to neutrophils (Procter *et al.* 1982), *Streptococcus pyogenes* to neutrophils (Simpson *et al.* 1981) and *Pseudomonas aeniginosa* to buccal epithelial cells (Woods *et al.* 1979). Although *S. aureus* is not ingested, the number of colony forming units is reduced, therefore this attachment may play an important role in limiting acute infection prior to the development of specific immunity (Procter *et al.* 1982). Fibronectin also specifically promotes fibrin attachment to monocytes (Jilek and Hörmann, 1978), stimulates the alternative complement pathway (Czop *et al.* 1981), binds to Clq, a component of the complement pathway (Menzel *et al.* 1981 and Bing *et al.* 1982) and binds deoxyribonucleic acid (DNA) (Mosher and Furcht, 1981).

It is therefore apparent that fibronectin is involved in the mononuclear phagocytic system, but whether it acts as an opsonin, as a stimulator of the alternate complement pathway, as a molecule that permits macrophages to attach to their targets or all three *in vivo* is unclear at the present.

G. Summary

Fibronectin, through its primary interactions with collagen, fibrin (ogen), glycosaminoglycans and cells, has been shown to have a range of biological activities (Table 1.2). As a result of these activities, fibronectin has been postulated to play an important role in the maintenance of cellular morphology, tissue morphogenesis, wound healing, blood coagulation and the mononuclear phagocytic system.

Fibronectins from the cell surface and plasma do not exhibit

identical biological activities (Yamada and Kennedy, 1979). The biological activities in the mediation of cell attachment to collagen and in cell spreading in serum-free medium are the same, but they differ markedly (plasma fibronectin shows very little activity) in the capacity to haemaglutinate or to restore a normal morphology and social behaviour to transformed fibroblasts. These results suggest that plasma fibronectin is more important for platelet attachment or cell spreading, while the cellular form has a more general role.

TABLE 1.2.

THE PROPOSED BIOLOGICAL ACTIVITIES

OF FIBRONECTIN

The deposition of the extracellular matrix

The mediation of cell-cell aggregation

The mediation of cell-substrate interaction

The control of cell spreading

The Mediation of fibrin(ogen) attachment to macrophages

The control of clot lysis

The mediation of fibrin clot retraction by fibroblasts

The control of platelet binding to clots

A chemotactic activity

A regulatory role in the cell cycle

A role in cell differentiation and morphogenesis

IV. FIBRONECTIN IN DISEASE

A. Fibronectin and malignant transformation

Hynes and Bye (1974) reported the presence of a large, external transformation sensitive protein (fibronectin) on cell surfaces. The changes in the distribution of cell surface fibronectin on transformed cells and the possible importance of these changes have been the subject of a large number of studies since that report. Several comprehensive reviews of the role of fibronectin in malignant transformation have been published (Vaheri and Mosher, 1978; Yamada and Olden, 1978; Chen *et al.* 1979; Hynes *et al.* 1979; Pearlstein *et al.* 1980; Ruoslahti *et al.* 1980a and Yamada, 1980) therefore only the current views are summarised here.

The amount of cell surface fibronectin is usually decreased after cell lines have been transformed by oncogenic viruses (Vaheri and Mosher, 1978). The correlation between fibronectin loss and transformation is best illustrated by temperature sensitive oncogenic virus mutants. At the permissive temperature fibronectin is lost, but if the temperature is reduced to a non-permissive level fibronectin is bound to the cell surface (Yamada and Olden, 1978). The level of surface fibronectin is also reduced in some chemically and spontaneously transformed cell lines and some cultured tumour cells. However, there are exceptions where the surface bound fibronectin is unaltered, especially among spontaneously transformed cell lines (Ruoslahti *et al.* 1980a). Stanbridge *et al.* (1982) have utilised human cell hybrids between fibroblasts and HeLa cells to show that both nontumourigenic and tumourigenic hybrids express reduced levels of fibronectin relative to the fibroblast parent cell. However, the distribution of fibronectin on the cell surface differs. The nontumourigenic hybrids exhibit an incomplete branched fibrillar network over areas of cell contact, while

tumourigenic hybrids show a short stitchlike pattern exclusively at cell-cell junctions.

Reports which correlated the loss of cell surface fibronectin with tumourigenicity or the propensity of tumours to metastasise have been questioned because of the methodology used (Vaheri and Mosher, 1978) and the results of *in vivo* studies of human rectal carcinomas (Niemczuk *et al.* 1982). However, Rajaraman and Lonergan (1982) have demonstrated a correlation between the loss of cell surface fibrillar fibronectin and the ability of cells to grow in anchorage independent conditions (a property usually considered to be the best *in vitro* correlate of *in vivo* tumourigenicity). The proteolysis of fibronectin may be involved in cell transformation as De Petro *et al,* (1981) have shown that the transformation of fibroblasts infected with temperature sensitive mutants of Rous sarcoma virus is enhanced by defined proteolytic fragments of plasma fibronectin.

The addition of fibronectin to the culture medium of transformed cells can partly restore the normal morphology of the cells (Yamada and Olden, 1978). The changes seen can all be accounted for by the restoration of normal cell-substratum adhesion (Vaheri and Mosher, 1978) and intracellular microfilament structure (Rieber and Rieber, 1982); both of which involve fibronectin (Chapter 1.III.B and C).

The cellular matrix of transformed cells show a loss of collagen and an alteration in quantitative proportions of glycosaminoglycans as well as the loss of fibronectin (Hayman *et al.* 1982). This indicates that there is a general disorganisation of the cell matrix around transformed cells which removes controls on cell multiplication and locomotion (Vaheri and Mosher, 1978). The fact that this can occur without fibronectin loss suggests that it is the alteration of a number of the cell matrix components rather than just one that is important.

It is unlikely that these changes are the mechanisms which cause cells to metastasise or be tumourigenic, but rather that they are one of the changes that allow these two processes to occur when the cell responds to some, as yet unknown, signal (Vaheri and Mosher, 1978 and Pearlstein *et al.* 1980).

The mechanisms which cause the loss of fibrillar fibronectin from the cell surface are uncertain (Vaheri and Mosher, 1978). Some cells show a decreased synthesis of fibronectin but in hepatocytes synthesis is increased by transformation (Hahn *et al.* 1982). Ihara *et al.* (1982) have demonstrated that the human cytomegalovirus, a suspected oncogen, can specifically suppress fibronectin synthesis in some cell types. Transformed cells exhibit an altered microfilament structure but, as discussed above (Chapter 1.III.B), fibronectin is only one factor involved in the formation of actin bundles. If the model of Lyubimov and Vasiliev (1982) for the cell surface distribution of fibronectin is correct (Chapter 1.II.A), then the loss of fibrillar fibronectin may involve an intracellular change which effects the movement of cell surface receptors for fibronectin. The possibility that the increased proteolytic activity associated with transformed cells results in the proteolytic cleavage of fibronectin is doubtful as the fibronectin released in culture has the same molecular weight as the intact molecule (Vaheri and Mosher, 1978). However, a fragment of fibronectin (MAD-2) which binds DNA, has an elevated concentration in the serum of patients with malignant disease (Parsons *et al.* 1979). A major alteraction in the structure of fibronectin does not occur after transformation (Vaheri and Mosher, 1978), although fibronectin from malignant cells migrates at a slower rate on sodium dodecyl sulphate gels. Embryonic fibronectin migrates at the same rate as fibronectin from malignant cells (Vaheri *et al.* 1980).

It has recently become apparent that abnormal protein phosphorylation has an important role in some virally mediated cancers (Cohen, 1982). Therefore, the finding of an increased phosphorylation of fibronectin from transformed cells (Ali and Hunter, 1981) may be of special significance.

The level of plasma fibronectin in most cancer patients does not differ from that of controls, 348 µg/ml compared to 350 µg/ml (Stathakis *et al.* 1981; Klingemann *et al.* 1982 and Suzuki and Abe, 1982). However, in extensive liver metastases the level is lowered to 259.8 µg/ml (Stathakis *et al.* 1981) which suggests that the liver either synthesises some of the plasma fibronectin or that its catabolism is increased. The levels of plasma fibronectin have also been reported to drop to 50% of normal in acute leukaemia patients in poor clinical condition (Brodin *et al.* 1982), while in chronic lymphatic leukaemia a reduction in serum opsonic activity, which is not correlated with IgG, IgA, IgM, C3 or C4 (Kansu *et al.* 1982), may be correlated with fibronectin levels. Two complications of cancer also alter the plasma levels: in obstructive jaundice, due to carcinoma of the pancreas, the level is raised to 444.6 µg/ml while, if cryofibrinogenaemia is a complication, the level drops to 282.2 µg/ml (Stathakis *et al.* 1981). Klingemann *et al.* (1982) have reported the presence of a slower fibronectin component (FN:C) in the plasma of acute myelocytic and acute lymphocytic leukaemia patients after two-dimensional immunoelectrophoresis. The levels of FN:C are correlated with the clinical state of the patients with the lowest levels occurring during remission (Klingemann *et al.* 1982). Suzuki and Abe (1982) have found a similar component in the plasma of patients with gastric carcinomas.

B. Other diseases

1. Liver and kidney disease

The liver is thought to act in the turnover of plasma fibronectin in at least two ways: a) fibronectin synthesis by hepatocytes and b) consumption by Kupffer cells (Höfelar *et al.* 1982). In severe liver disease, either due to neoplastic infiltration (Stathakis *et al.* 1981 and Matsuda *et al.* 1982) or due to cirrhotic lesions (Höfelar *et al.* 1982), the plasma concentration is reduced to as low as 50% of normal. The degree of reduction is dependent on the severity of the lesion (Höfelar *et al.* 1982). The reduction of plasma fibronectin levels could be due either to an increase in catabolism mediated by a raised fibrinolytic activity (Matsuda *et al.* 1982) or to a reduced hepatic synthesis (Höfelar *et al.* 1982). In some less severe liver and kidney disorders the plasma level of fibronectin is raised (Stathakis *et al.* 1981 and Matsuda *et al.* 1982); possibly due to an increased synthesis of fibronectin for tissue repair.

Höfelar *et al.* (1982) have found a second slower migrating fraction of fibronectin after two-dimensional electrophoresis in plasma from cirrhotic patients, similar to that found in some cancer patients (Klingemann *et al.* 1982 and Suzuki and Abe, 1982) (Chapter 1.IV.A), which increases with the severity of the disease. It is possible that it is fibronectin bound to phagocytable biological substances, which are not eliminated from the circulation because of the impaired clearance function of the liver mononuclear phagocytic system (Höfelar *et al.* 1982). It is probable that a similar model could explain the FN:C band in some cancer patients.

2. Interstitial lung diseases

The interstitial lung diseases are a heterogenous group characterised by the abnormal accummulation of inflammatory cells and connective

tissue elements in the pulmonary interstitium together with changes in parenchymal cell populations and organisation. They often lead to fibrosis with derangements of interstitial collagen (Rennard and Crystal, 1982).

Fibronectin may play a role in the mononuclear phagocytic system (Chapter 1.III.F). Therefore the knowledge that alveolar macrophages are activated in interstitial lung diseases and that there are increased levels of fibronectin in the lower respiratory tract of patients with interstitial lung diseases (Rennard and Crystal, 1981) suggests that the two may be involved in the alteration of alveolar structures associated with these diseases.

Rennard *et al.* (1981a) have shown that alveolar macrophages are probably the only inflammatory and immune effector cells in the human lower respiratory tract that produce significant quantities of fibronectin and that in patients with interstitial lung disease alveolar macrophages produce an increased level of fibronectin (Table 1.3). The increased synthesis of fibronectin by alveolar macrophages is probably responsible for the reported increased fibronectin levels in the bronchial lavage fluid (Rennard and Crystal, 1982) (Table 1.3). The fibronectin produced is chemotactic for fibroblasts. Rennard *et al.* (1981a) suggest that alveolar macrophages play a role in the reorganisation of alveolar structures in interstitial lung diseases through the increased secretion of fibronectin, which may function in a number of ways, which include fibroblast recruitment, the induction of fibroblast division and collagen opsonisation, all of which lead to the development of fibrosis.

3. Scleroderma and systemic lupus erythematosus

In normal skin fibronectin is found in the papillary layer and

TABLE 1.3.

FIBRONECTIN PRODUCTION IN INTERSTITIAL LUNG DISEASES

(from Rennard *et al.* 1981a; Rennard and Crystal, 1982)

Patients	Fibronectin Production mg/10^6 cells/hr	Fibronectin Concentration of the Bronchial Lavage Fluid µg/mg albumin
Normal	0.49±0.23	1.6±0.4
Idiopathic Pulmonary Fibrosis	10.5±1.7*	4.3±0.9
Sarcoidosis	5.4±2.4*	5.4±0.1
Others	3.5±1.3	4.3±0.8

* Significant $P<0.02$

within collagen bundles in the reticular dermis. Subcutaneous tissue is negative for its presence as judged by immunofluorescence.

Scleroderma (progressive systemic sclerosis) is a generalised connective tissue disorder characterised by the deposition of collagen in the skin and internal organs. In this disorder there is an increased deposition of fibronectin in the involved deep dermis which parallels the accummulation of collagen in the involved reticular dermis. Fibronectin is also found in the subcutaneous tissue, but there is only slight change in the subepidermal papillary region (Cooper *et al.* 1979 and Fleischmajer *et al.* 1980). Fibronectin binds to collagen in the region of the collagenase cleavage site. It is, therefore, possible that collagenase may be inhibited by steric hindrance. A decrease in collagenase activity has been demonstrated in scleroderma and is thought to be responsible for collagen accummulation (Brady, 1975).

Changes in the skin distribution of fibronectin have also been reported in patients with systemic lupus erythematosus (Fyrand, 1980). There are fibronectin negative gaps and slit formation in the dermal/epidermal junction combined with the presence of fibronectin positive globular bodies and the transport of fibronectin into the epidermis. Also fibronectin is found in cryoprecipitates of the synovial fluid of systemic lupus erythematosus patients (Beaulieu *et al.* 1981).

4. Inflammatory bowel disease (Scott *et al.* 1981)

In the normal rectal mucosa fibronectin is found in epithelial cells, on basement membranes and as a loose cribriform network in the extracellular matrix of the lamina propria, which codistributed with reticulin (a matrix bound to immature collagen). Immunoelectron-microscope studies show fibronectin on collagen fibres, on smooth muscle cells and within and between columnar epithelial cells.

Patients with active ulcerative colitis, or active Crohn's colitis, with marked inflammatory changes show a different distribution. Fibronectin is thickened and more prominent on the basal membranes and is seen as sparse strands between inflammatory cells that have infiltrated the lamina propria. In patients with longstanding ulcerative colitis, which is no longer active, there is a diffuse increase in fibronectin which is densely and uniformly present throughout the lamina propria. The fibronectin distribution of the lamina propria appears to go through a sequence of changes in inflammatory bowel disease. At first the cribriform distribution is unaltered, but a coarse reticular pattern develops which leads to an eventual diffuse distribution.

As fibrosis is not typical of these diseases the deposition of fibronectin is apparently involved in a different function. It is thought that the changes in fibronectin distribution may be a useful means of indirectly assessing the disease process in rectal biopsies.

5. Rheumatoid arthritis

The synovial cavity of joints is an extension of the interstitial space and lacks a basement membrane. The composition of the synovial fluid is dependent on the permeability of the adjacent blood vessels and on the local synthesis of proteins by cells of the synovial membrane. Under normal conditions the amount of synovial fluid is too small to permit samples to be taken. However, in the inflammed joint, characteristic of rheumatoid arthritis, the joint volume and protein content increase to levels that allow the aspiration of samples.

Vartio *et al.* (1981) reported that the average synovial fluid fibronectin level in arthritis patients was 445±103 µg/ml while the plasma levels were in the normal range, a result confirmed by Clemmensen and Anderson (1982) and Scott *et al.* (1982). There was also a prominent increase of fibronectin in the proliferating con-

nective tissue in rheumatoid arthritis as compared to the normal membrane. These results suggest that the level of fibronectin is due to an increased local synthesis in the synovial cavity (Vartio *et al.* 1981 and Scott *et al.* 1982). Cryoprecipitates are found in the synovial fluid and the plasma of rheumatoid arthritis patients (also in ankylosing spondylitis and mixed essential cryoglobinaemia patients). Fibronectin is present in and facilitates the formation of all mixed cryoglobulins, but is absent from polyclonal cryoglobulins (Levo, 1980). It is therefore not responsible for the formation of the cryoprecipitate antigen-antibody complexes (Beaulieu *et al.* 1981).

Tissue breakdown and repair is a continuous process in most inflammatory diseases and it is possible that the increased local production of fibronectin is a general marker for the formation of new connective tissue rather than a specific marker for disease. The fact that fibronectin is generally at a higher level in differentiating tissues lends support to this possibility. However, Scott *et al.* (1982) suggest that fibronectin levels in the synovial fluid could serve as an indicator of tissue response to rheumatoid arthritis.

Clemmensen (1981) has suggested that the proteolytic products of fibronectin may play a role in the inflammatory process. However, the evidence for fibronectin proteolysis in the synovial fluid is contradictory (Vartio *et al.* 1981 and Clemmensen and Anderson, 1982).

6. Shock, trauma and septicaemia

A reduction in the level of plasma fibronectin is a common feature of patients with sepsis after surgery, in trauma (burn and haemorrhage) patients and patients with intravascular coagulation syndromes (Mosesson *et al.* 1975; Mosher and Williams, 1978; Saba *et al.* 1978 and Pott *et al.* 1982). Part of the reason for this is the formation of fibrinogen-fibrin complexes in which fibronectin

appears to act as the nuclear molecule (Mosher and Furcht, 1981 and Clemmensen, 1982). The other major reason for the reduction in fibronectin levels is thought to be the increased utilisation of fibronectin in the opsonisation of autologous tissue and colloidal matter in the circulatory system (Saba *et al.* 1978 and Clemmensen, 1982).

The reduction in fibronectin levels appears to be important in the depression of the mononuclear phagocytic system in the above patients (Dillon and Saba, 1982 and Dillon *et al.* 1982). The injection of cryoprecipitate or purified fibronectin intravenously has been shown to restore the level of plasma fibronectin and improve septicaemia, pulmonary insufficiency and the duration of recovery (Saba *et al.* 1978). The mononuclear phagocytic systems activity is also raised (Saba *et al.* 1978). The depression of the mononuclear phagocytic system in disseminated intravascular coagulation is partially mediated by plasmin breakdown products of plasma fibronectin (Ehrlich *et al.* 1981). Despite this the infusion of cryoprecipitate in septic injured patients offers a simple method for the prevention of multiple organ failure (Saba *et al.* 1978).

The concentration of plasma fibronectin required for normal function of the mononuclear phagocytic system is not known, but patients with less than 50% of the normal concentration (less than 150 μg/ml) have a higher mortality rate (Mosher and Furcht, 1981). Despite this uncertainty, Saba *et al.* (1978) have suggested that the measurement of plasma fibronectin levels during severe sepsis which follows trauma may be an accurate non-intrusive measure of the activity of the mononuclear phagocytic system. It is also possible that low plasma fibronectin concentrations may define a group of patients at high risk for developing disseminated intravascular coagulation (Clemmensen, 1982).

Fibronectin levels are also reduced in some infectious diseases, for example, Rocky Mountain Spotted Fever (Mosher, 1976). In septicaemia the fibronectin pattern on isoelectric focussing gels is altered; possibly due to the action of proteases and glycosidases which show increased levels in patients (Pott, 1982).

7. Ehlers-Danlos syndrome

The Ehlers-Danlos syndromes (EDS) are a heterogenous group of heritable disorders of the connective tissue characterised by skin and joint hyperextensibility. Bleeding manifestations are common and, occasionally, a defect in platelet aggregation or an abnormality in blood coagulation have been reported. In some kindreds a biochemical defect in collagen synthesis has been shown (Hollister, 1978) but in the rest the underlying defect has not been determined.

A new form of EDS, designated EDSIX, characterised by hypermobile joints, defective wound healing, hyperdistensible skin and defective platelet aggregation has been recently reported (Furcht *et al.* 1979 and Arneson *et al.* 1980). It is a mild recessively inherited variant of EDS in which all the abnormalities cosegregate. The defective platelet function was shown to result from a consistent resistance to collagen induced aggregation. This defect could be partially corrected *in vitro* by the addition of cryoprecipitate or purified fibronectin, from normal individuals, to the patients platelet rich plasma. The fact that only incomplete correction of platelet aggregation was achieved, is possibly explained by the fibronectin of the platelet α-granule having a role in aggregation.

As fibronectin plays an adhesive role in platelet aggregation (Furcht *et al.* 1979) and binds to collagen and other components of the extracellular matrix in connective tissue, it is possible that EDSIX results from an alteration of the collagen binding site of fibronectin,

presumably caused by a point mutation.

8. Glanzmann's thrombasthenia

Glanzmann's thrombasthenias are probably a heterogeneous group of rare inherited disorders. The normal mode of inheritance is recessive, but a dominant form has been reported (McKusick, 1978). Glanzmann's thrombasthenia is characterised by a bleeding diathesis, deficient clot retraction and abnormal platelet morhpology *in vivo* and reduced platelet aggregation and adhesivenss *in vitro*. A patient with the typical laboratory characteristics, but with an additional wound healing abnormality has been reported (Donati *et al.* 1977 and Remuzzi *et al.* 1977). Additionally fibroblasts from the skin of this patient are unable to induce fibrin-clot retraction. The presence of fibronectin has been shown to be essential for fibrin-clot retraction (Grinnell *et al.* 1980).

As the levels of plasma fibronectin are normal in thrombasthenic patients (Pearlstein *et al.* 1981) it has been claimed that the fibrin binding site of fibronectin is deficient (Clemmenson, 1981). However, there is no direct evidence to support this claim. An abnormal platelet membrane glycoprotein (152,000 M.W.) and the loss of a platelet membrane glycoprotein (M.W. 120,000-132,000) have been reported in some thrombasthenic patients (Nurden and Caen, 1974 and Degos *et al.* 1975) which is compatible with the recent observation that there is either a deficient membrane binding site for Ca^{2+} or a deficient membrane glycoprotein which anchors the major platelet antigen to the membrane in Glanzmann's thrombasthenia (Howard *et al.* 1982). This, combined with the fact that platelets from thrombasthenic patients do not bind fibronectin (Ginsberg *et al.* 1981), suggests that the platelet membrane receptors for fibronectin, rather than the fibronectin molecule, are abnormal in some cases of Glanzmann's thrombasthenia.

However, as the disease appears to be heterogeneous (Moser *et al.* 1968 and Hathaway, 1972), a fibronectin with an abnormal fibrin-binding site could be the cause of some cases.

C. Summary

Although changes in fibronectin distribution and concentration are associated with a variety of disease states in man, no disease has been proven to result from an alteration of the structure or function of the fibronectin molecule. While it seems certain that the loss of cell surface fibronectin is important in the oncogenic process, it is doubtful that it is a causal mechanism. The increased levels of fibronectin in diseases that are associated with fibrosis or tissue repair (Chapter 1.IV.B 1-5) probably reflects the role of fibronectin in these processes (Cooper *et al.* 1979; Fleischmajer *et al.* 1980 and Kojima *et al.* 1981). The exact relationship between plasma fibronectin depletion and the reduced function of the mononuclear phagocytic system in several groups of patients is uncertain, but is the subject of continued study.

V. THE MOLECULAR STRUCTURE OF FIBRONECTIN

Fibronectin from several species of mammals has been examined in an attempt to determine the molecular structure of fibronectin with nearly identical results. Therefore, results from these studies, which include bovine, equine, porcine, hamster and human fibronectin, will be combined to give a current model for the structure of fibronectin.

A. Primary structure

1. Sodium dodecyl sulphate gel electrophoresis.

Sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis has been used to examine plasma, amniotic fluid and cellular fibronectin. While the results for amniotic fluid and cellular fibronectin are similar, the results for plasma fibronectin suggest that it might be structurally different.

Non-reduced amniotic fluid and cellular fibronectins migrate as a single diffuse band which corresponds to a Molecular Weight of 450,000 (Mosesson *et al.* 1975) to 480,000 (Pande *et al.* 1981). Some high molecular weight material from cellular fibronectin did not enter the gel and was found to correspond to multimers of the above molecule (Hynes and Destree, 1977; Keski-Oja *et al.* 1977 and Yamada *et al.* 1977). Examination of the multimers showed a high degree of inter-chain disulphide bonds which are thought to account for the insolubility of these multimers (Ruoslahti and Vaheri, 1974 and Yamada *et al.* 1977), however, the 450,000 M.W. molecule is also less soluble than the amniotic fluid derived molecule (Mosesson and Amrani, 1980).

Oldberg and Ruoslahti (1982) have recently shown that the inter-reaction of fibronectin with collagen and proteoglycans in the extra-cellular matrix are the cause of the insolubility of fibronectin from this source. Reduced cellular fibronectin migrates as a single broad

band which corresponds to a Molecular Weight of 210,000-250,000 (Hogg, 1974; Yamada and Weston, 1974 and Mosesson *et al.* 1975), while the molecular weight of amniotic fluid fibronectin is 235,000 (Balian *et al.* 1979a) to 240,000 (Pande *et al.* 1981). SDS gel electrophoresis, therefore, suggests that cellular and amniotic fluid fibronectin is a dimer of two identical polypeptide chains.

Non-reduced plasma fibronectin also migrates as a single band of about 450,000 M.W. (Mosesson *et al.* 1975 and Sekiguchi *et al.* 1981). Reduced plasma fibronectin, however, migrates as a closely spaced doublet (Mosesson *et al.* 1975 and Mosher, 1975). The exact molecular weights of the two bands is uncertain but estimates of the difference vary from 5,000 (Kurkinen *et al.* 1980) to 20,000 (Sekiguchi *et al.* 1981). Both molecular weights are in the same range as that given for reduced cellular fibronectin above. However, Yamada *et al.* (1980) have estimated that the molecular weight of plasma fibronectin is 5,000-10,000 lower than cellular fibronectin.

It, therefore, appears that plasma fibronectin is a dimer of non-identical polypeptide subunits which are possibly both different to the polypeptide subunit of cellular and amniotic fluid fibronectin (Table 1.4). The plasma fibronectin dimer is probably composed of non-identical subunits as SDS gel electrophoresis only resolves a single band (Yamada *et al.* 1980), but as the band is diffuse it may comprise two bands, each a dimer of identical subunits (Sekiguchi *et al.* 1981) or three bands formed by two homodimers and a heterodimer.

Isoelectric focussing of SDS treated plasma and amniotic fluid fibronectin revealed similar banding patterns with an apparent isoelectric point of 5.0 (Pande *et al.* 1981).

SDS gels have also been used in the mapping of the functional domains of fibronectin (Chapter 1.V.C).

TABLE 1.4.

THE APPARENT MOLECULAR WEIGHTS OF PLASMA, CELLULAR AND AMNIOTIC FLUID FIBRONECTINS

(for references see text)

	Source of Fibronectin		
	Plasma	Cellular	Amniotic Fluid
Dimer	450,000	480,000	480,000
Monomer	210,000 - 215,000 230,000 - 230,000	240,000	240,000

2. The amino acid composition of fibronectin

The amino acid composition of fibronectin from plasma, amniotic fluid and the cell surface, are very similar (Table 1.5 and Vuento *et al.* 1977 and Pande *et al.* 1981). Fibronectin has an unusually high threonine content (Balian *et al.* 1979b) and is relatively rich in proline and poor in cystine residues (Mosesson *et al.* 1975 and Mosher, 1975). The amino-terminus is blocked by pyroglutamate in all the forms studied except for chick cell-associated fibronectin (Yamada *et al.* 1977). Pearlstein *et al.* (1980) have calculated that a maximum of 22 disulphide bonds may be present in the dimer. However, there are probably less as, at least, three free sulphydryl groups are thought to be required for cell binding (Fukuda and Hakomori, 1979). Petersen *et al.* (1981) have reported the presence of 10 intrachain disulphide bridges in the first 259 residues from the amino terminus and 2 in the first 52 residues from the carboxyl terminus. An interchain disulphide bridge is located close to the carboxyl terminus (Chen *et al.* 1977). Vibe-Pedersen *et al.* (1982) were only able to locate 1 free thiol group (per subunit) close to a carbohydrate residue in a 27 amino acid sequence from the central 170,000 M.W. region of the molecule. Smith *et al.* (1982) have narrowed the location of this thiol group to a 80,000 M.W. region in the carboxyl half of the molecule.

The complete amino acid sequence of fibronectin has still to be elucidated, although 1050 of the proposed 1880 amino acids of a subunit of bovine plasma fibronectin have been sequenced (Skorstengaard *et al.* 1982). The sequencing studies have revealed 3 different domains of internal homology (Vibe-Pedersen *et al.* 1982) one of which has 5 copies in the amino terminal 259 residues (Petersen *et al.* 1981). Amino terminal sequencing has been used to study the structure of the functional domains of fibronectin (Pande and Shively, 1982) (Chapter 1.V. C).

TABLE 1.5.

THE AMINO ACID COMPOSITION OF FIBRONECTIN

Amino Acid	Residues/1,000 residues				
	Plasma			Amniotic Fluid	Cellular
	Human[a]	Hamster[b]	Porcine[c]	Human[d]	Human[e]
Aspartate	94	94	91	95	93
Threonine	103.7	104	104	101	103
Serine	74.7	73	80	78	76
Glutamate	117	120	114	122	118
Proline	83.7	85	87	92	74
Glycine	84.3	86	84	88	98
Alanine	42.3	44	44	43	47.5
Half-Cystine	25	29	16	25	20.5
Valine	76.3	72	84	73	71.5
Methionine	9.7	10	11	11	12.5
Isoleucine	43.7	47	46	42	41.5
Leucine	54	56	56	56	58
Tyrosine	42.3	41	40	36	38.5
Phenylalanine	24.7	24	22	25	23
Histidine	21.7	19	21	20	19.5
Lysine	36	36	38	32	35.5
Arginine	52.3	53	51	50	52.5
Tryptophan	21.5	-	21	11	36

[a] from Mosesson *et al.* (1975), Mosher (1975) and Balian *et al.* (1979b);
[b] Sekiguchi *et al.* (1981);
[c] Isemura *et al.* (1981);
[d] Balian *et al.* (1979b);
[e] from Yamada *et al.* (1977) and Balian *et al.* (1979b).

The amino-terminal 13 residues of fibronectin from plasma, amniotic fluid and the cell are identical (Pande *et al.* 1981). The tryptic peptides of fibronectin from various sources show extensive correspondence (Balian *et al.* 1979b), but each type does have unique bands on SDS electrophoresis (Pande *et al.* 1981). However, 2-dimensional peptide maps of cellular and plasma fibronectin, as well as the two subunits of plasma fibronectin appear identical (Birdwell *et al.* 1980).

3. The carbohydrate content of fibronectin.

The amount and type of sugar residues attached to fibronectin is dependent upon both its origin in the body and the species from which the sample is taken. There is generally 4-6 oligosaccharide units on each fibronectin monomer, which account for 4-7% of the weight of the fibronectin molecule. Because of the differences in the carbohydrate composition of plasma, amniotic fluid and cellular fibronectin (Table 1.6), it is easiest to consider them separately.

a) Plasma fibronectin - the oligosaccharide units are biantennary asparagine-linked carbohydrate chains with branching predominantly at 3,6-linked mannose and with an equal distribution of 4- and 6-linked intrachain galactose. Sialic acid is the predominant terminal sugar. N-acetylglucosamines are also present and traces of fucose and N-acetylgalactosamine have been reported. In man there are 4-6 sites of attachment and the carbohydrate accounts for 4.8% of the weight of the fibronectin monomer (Mosesson *et al.* 1975; Vuento *et al.* 1977; Yamada *et al.* 1977; Carter *et al.* 1978; Balian *et al.* 1979b; Fukuda and Hakomori, 1979; Takasaki *et al.* 1980 and Pande *et al.* 1981). The predominant carbohydrate structure in hamsters is almost identical (Fukuda *et al.* 1982).

b) Amniotic fluid fibronectin - the oligosaccharide units are more complex than those of the plasma form, with 3,6- and 2,4-linked

TABLE 1.6.

THE CARBOHYDRATE COMPOSITION OF FIBRONECTIN FROM MAN

Residue	% Dry Weight Plasma[a]	Amniotic Fluid[b]	Cell[c]
Fucose	Trace	0.4	-
Mannose	1.1	1.1	1.35
Galactose	0.95	1.7	1.00
Xylose	-	-	0.03
N-acetylglucosamine	1.65	2.5	1.50
N-acetylgalactosamine	0.1	0.7	-
Sialic acid	0.75	0.6	0.60
	4.55	7.0	4.48

[a] from Vuento *et al.* (1977) and Balian *et al.* (1979b);

[b] Balian *et al.* (1979b);

[c] from Vuento *et al.* (1977) and Yamada *et al.* (1977).

mannose and 3- and 4-linked, but no 6-linked, galactose. The terminal sugars are fucose, mannose, galactose, N-acetylglucosamine and sialic acid. N-acetylgalactosamine is also present. The carbohydrate content is 6.8% by weight (Balian *et al.* 1979b and Pande *et al.* 1981).

c) Cellular fibronectin - there is a core region of N-acetyl-glucosamine and mannose attached to asparagine residues which has terminal galactose, fucose and sialic acid residues. Xylose has been reported to be present. The carbohydrate content is similar to that of plasma (Mosesson *et al.* 1975 and Yamada *et al.* 1977). The cell-associated fibronectin has differnt carbohydrate groups in different species. Chick embryo fibronectin has twice as much sialic acid, glucose and xylose (Yamada *et al.* 1977) and hamster embryo fibronectin has 3 terminal galactose and 2 terminal sialic acid residues (Fukuda and Hakomori, 1979); the sialic acid is linked C_3 to galactose while human is linked C_4 or C_6 (Pearlstein *et al.* 1980).

Olden *et al.* (1978) have shown that fibronectin without carbohydrate moieties has a normal rate of synthesis and secretion and unchanged biological activities, but it is 2-3 times more sensitive to proteolysis. It therefore appears that the role of the carbohydrate moieties is to protect the fibronectin molecule from proteolytic cleavage. Bernard *et al.* (1982) have confirmed this observation and shown the collagen-binding region of fibronectin (Chapter I.V. C), which normally contains 3-4 carbohydrate structures, is completely digested by proteases if the structures are removed.

It is possible that the difference in carbohydrate composition of plasma and amniotic fluid fibronectin accounts for their different behaviour on SDS gel electrophoresis (Balian *et al.* 1979b and Pande *et al.* 1981), but there may be other causes (see Chapter I V C).

4. Phosphorylation of fibronectin

Covalently bound phosphorylation of fibronectin occurs intracellularly and is the same on fibroblast surface, plasma and fibroblast secreted fibronectin (Ledger and Tanzer, 1982). Phosphate groups are only present as phosphoserine(Ali and Hunter, 1981 and Ledger and Tanzer, 1982) which are restricted in a 20,000-50,000 M.W. region near one of the ends of the molecule (Ledger and Tanzer, 1982).

B. Immunological studies

The various forms of fibronectin show complete immunological identity (Mosesson *et al.* 1975 and Mosher, 1975). The antigenic activity of fibronectin closely reflects the structural intactness of the monomer (Vuento *et al.* 1980 and Koteliansky *et al.* 1982a). The reduction of disulphide bonds in the presence of 8M urea followed by alkylation decreased the antigenic activity by 90%. Vuento *et al.* (1980) reported a rapid loss of antigenic activity at 60^{o}C, but their results have been criticised because the assay used was sensitive to protein solubility (Wallace *et al.* 1981).

Several groups of workers have raised monoclonal antibodies to a variety of antigenic determinants on the fibronectin molecule (Atherton and Hynes, 1981; Atherton *et al.* 1981; Koteliansky *et al.* 1982a; Sekiguchi *et al.* 1982; Smith and Furcht, 1982 and Smith *et al.* 1982). The monoclonal antibodies have been used as an aid to map the functional domains of fibronectin (Chapter 1.V.C) and to locate a species-specific determinant (Chapter 1.VI.B), as well as to define two areas of difference between cellular and plasma fibronectin (Chapter 1.V.C).

C. The location of functional domains

If a protein consists of several functional domains, a simple method of generating specific fragments, which are structurally and functionally intact, is limited proteolysis. This is because an

extended polypeptide chain is accessible to proteases and is readily hydrolysed, whereas any cleavage sites located within a folded domain tend to be buried orinaccessible because of steric hindrance (Yamada *et al.* 1980). Studies which have utilised enzyme degradation have suggested that fibronectin contains domains connected by such an extended polypeptide chain (Balian *et al.* 1979a), and have located a sequence of four amino acids, Pro- Gln- Pro- His which is repeated in these chains (Pande and Shively, 1982). Therefore, a number of proteases have been used in conjunction with SDS electrophoresis (for molecular weight determinations) and affinity chromatography (for functional determinations) to map the different protease resistant functional domains of fibronectin. Recent studies have also used monoclonal antibodies as an additional aid. The general procedures for these studies have been discussed clearly elsewhere (Yamada *et al.* 1980; Sekiguchi *et al.* 1981; Hayashi and Yamada, 1981 and Vartio, 1982).

A composite arrangement of the plasma fibronectin molecule is shown in Figure 1.1. The data for the map is drawn from the following references: Chen *et al.* (1977); Jilek and Hörmann (1977); Iwanaga *et al.* (1978); Balian *et al.* (1979a) and (1979b); Gold *et al.* (1979); Furie *et al.* (1980); McDonald and Kelly (1980); Mosher and Procter (1980); Mosher *et al.* (1980); Sekiguchi and Hakomori (1980); Yamada *et al.* (1980); Akiyama *et al.* (1981); Hayashi and Yamada (1981); Isemura *et al.* (1981); McDonald *et al.* (1981); Petersen *et al.* (1981); Pierschbacher *et al.* (1981); Sekiguchi *et al.* (1981); Ehrisman *et al.* (1982); Hayashi and Yamada (1982); Hörmann *et al.* (1982); Isemura *et al.* (1982); Pande and Shively (1982); Sekiguchi *et al.* (1982); Smith and Furcht (1982); Smith *et al.* (1982) and Vartio (1982).

FIGURE 1.1: A MODEL FOR THE STRUCTURE OF PLASMA FIBRONECTIN

A. Proposed binding sites (not in linear order within domains) - A: actin; F: fibrin; S: *Staphylococcus aureus*; T: Transglutaminase cross link sites; H: heparin; C: collagen; P: polyamine; D: DNA; X: cell attachment site; FN: fibronectin self-association sites.

In addition, another polyamine site is present in domains 5-7 and a fibronectin self-association site is present in domains 1-4.

⇧ : known differences with cellular fibronectin, approximate locations.

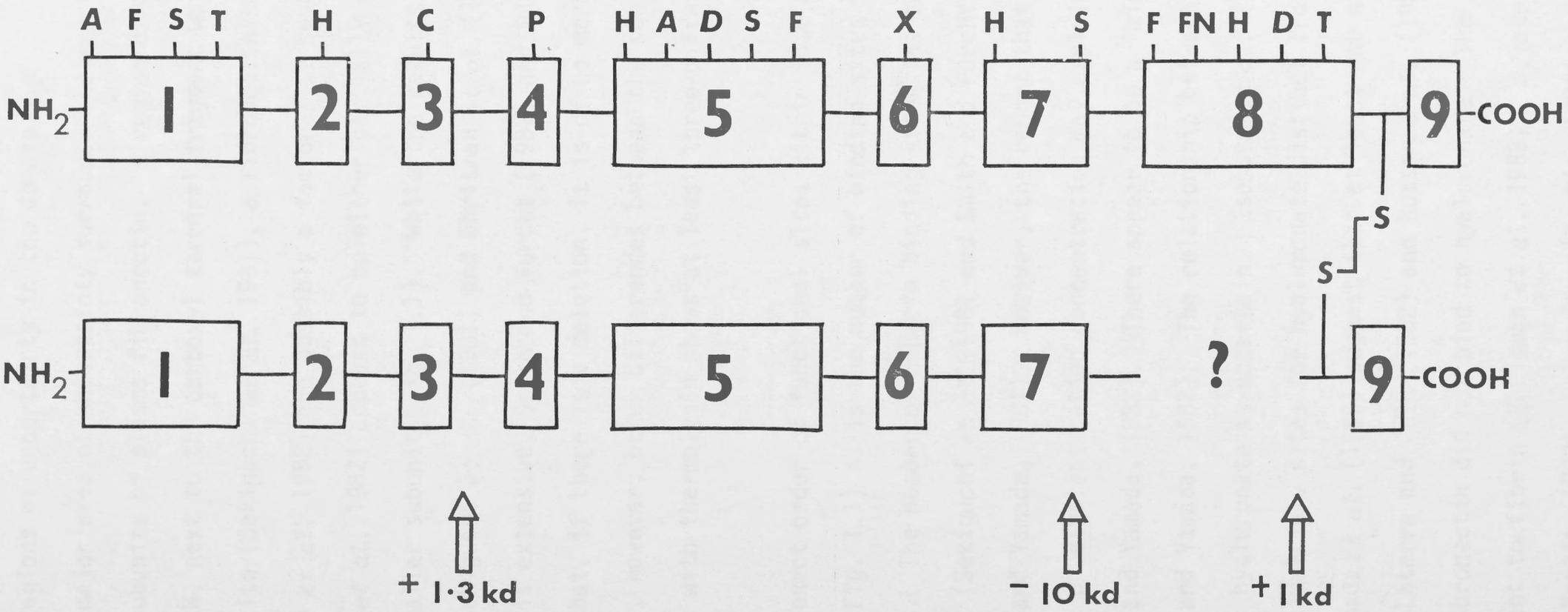
B. Sulphydryl Groups

Domains 1 and 2 have 10 intrachain disulphide bonds; domain 9 has 2 intrachain disulphide bonds; domain 6 contains a free sulphydryl group; S-S: interchain disulphide bond

Carbohydrate Residues

Domains 3 and 4 contain 4 groups; domain 6 contains a single group.

The domain sizes shown do not represent their sizes in the fibronectin molecule.


A F S T
H
C
P
H A D S F
X
H
S
F FN H D T
NH2
1
2
3
4
5
6
7
8
9
COOH
S
S
NH2
1
2
3
4
5
6
7
?
9
COOH
+ 1·3 kd
− 10 kd
+ 1 kd

1. Regions of uncertainty in the domain map

The major area of uncertainty surrounds the exact difference between the two subunits of plasma fibronectin. A region of between 20,000-25,000 M.W. next to the carboxyl terminal fragment which contains a fibrin binding site (Sekiguchi *et al.* 1981), a fibronectin self-association site (Ehrisman *et al.* 1982) and possibly a factor XIII susceptible site (Hörmann *et al.* 1982) appears to be either partially or totally absent in the smaller subunit (Fig. 1.1). While the results of Isemura (1981), Sekiguchi *et al.* (1981) and Ehrisman *et al.* (1982) suggest that the deletion is extensive, Smith and Furcht (1982) and Smith *et al.* (1982) suggest that, if there is a deletion, it is of no more than 5000 M.W. There are, however, other differences between the subunits; proteolytic digestion with thermolysin shows at least three differences (Akiyama 1981).

The exact order of functional sites within some domains is uncertain (Fig. 1.1) as is the number of binding sites for certain macromolecules. The number of heparin binding sites has been reported as between 2 (Sekiguchi *et al.* 1981 and Smith and Furcht, 1982) and 5 (Hayashi and Yamada, 1981). However, the report that heparin binding is dependent on divalent cation concentration may explain these differences (Hayashi and Yamada, 1982). There appear to be a minimum of three sites (Hayashi and Yamada, 1982). The relationship between these sites and those for hyaluronate is uncertain. Isemura *et al.* (1982) suggest that there are multiple sites for hyaluronate distinct from those for heparin, while Yamada *et al.* (1980) suggest that at least one site is shared. In contrast Laterra and Culp (1982) and Rosso *et al.* (1982) found that plasma fibronectin did not bind to hyaluronate. The number of sites for attachement to fibrin (Ehrisman *et al.* 1982). *S. aureus* (Kurkinen *et al.* 1980 and Vartio 1982), polyamines (Vartio, 1982) and DNA (McMaster and Zardi, 1982) are also uncertain. In addition Koteliansky *et al.* (1982b) have shown that fibronectin has an affinity

for four actin-binding proteins, vinculin, α-actinin, tropomyosin and myosin, but it is uncertain if binding sites for these proteins are present on the fibronectin molecule. It appears that the larger subunit of plasma fibronectin contains at least 22 functional sites and the smaller subunit at least 20.

2. Differences between cellular and plasma fibronectin

SDS gel electrophoresis after protease cleavage suggested that differences in the polypeptide sequence and the molecular weight of cellular and plasma fibronectin exist (Yamada and Kennedy, 1979 and Chapter 1.V.A). At present three definite differences have been found by digestion with thermolysin. However, others may be present as of twentynine fragments generated fifteen show a different migration on SDS gels (Hayashi and Yamada, 1981). There is no evidence for two different subunits in cellular fibronectin, but the relationship of the single subunit to the proposed two subunits of plasma fibronectin is not known (Chapter 1.VI.A.1). Two of the identified differences are an extra 1,300 M.W. segment near the collagen binding site and an extra 1,000 M.W. segment near the carboxyl terminal in plasma fibronectin (Hayashi and Yamada, 1982). These two segments probably correspond to differences located by monoclonal antibodies close to the collagen binding site (Atherton and Hynes, 1981) and close to the carboxyl terminal (Atherton *et al.* 1981). The third polypeptide difference involves an extra 10,000 M.W. segment in cellular fibronectin in a region containing a heparin binding site (Hayashi and Yamada, 1982 and Fig. 1.1).

D. Secondary and tertiary structure

Electron microscopy has shown the fibronectin molecule to be approximately 160 nm long (but ranges from 120 nm) and 2 nm in diameter (Erikson *et al.* 1981). There are no visible globular units and the

two subunits are joined at the carboxyl termini (Engel *et al.* 1981 and Erikson *et al.* 1981). It is uncertain whether the subunits are joined end to end (Erikson *et al.* 1981) or whether they enclose an angle of 70^{o} with each arm measuring about 60nm in length (Engel *et al.* 1981). The subunit arms show a limited flexibility with three distinct regions at which preferential flexion occurs (Engel *et al.* 1981). It is probable that these three regions are proteolytic susceptible areas between the functional domains.

The sedimentation coefficients reported for fibronectin range from 7.2-7.6 (Pearlstein *et al.* 1980) to 12-14 (Vaheri and Mosher, 1978). The former suggests an assymetric non-globular protein, while the latter suggests a spherical, globular protein. Yamada and Olden (1978) also suggested that the molecule is assymetric. None of these results agree entirely with those from electron microscopy.

The results from circular dichroism are also contradictory. In some reports no secondary structure is revealed (Yamada and Olden, 1978 and Koteliansky *et al.* 1981a) while in others considerable β-structure is predicted (Mosesson *et al.* 1975). Fibronectin does appear to contain some β-structure as infrared spectroscopy indicates that an anti-parallel β-structure accounts for 35% of the molecule (Koteliansky *et al.* 1981a). Neither of these techniques reveal any evidence for fibronectin being a globular protein.

Thermal stability, differential scanning colorimetry and intrinsic fluorescence studies suggest that fibronectin contains at least three domains connected by flexible segments which allow the domains independent movement in solution (Alexander *et al.* 1979; Koteliansky *et al.* 1981b and Wallace *et al.* 1981). Mosesson and Amrani (1980) have suggested that the conformational features can be accounted for by interactions between aromatic side chain groups within the peptide

backbone and the presence of a β-pleated sheet and β-turns. However, further studies are required before the secondary and tertiary structures of fibronectin are fully elucidated.

E. Summary

A structural model for plasma fibronectin based on the results given above is shown in Figure 1.1. The essential features of the structure of fibronectin that have been revealed are:

a) All forms of fibronectin consist of two polypeptide subunits. In the plasma form of the molecule they are apparently non-identical, while in the cellular form they appear to be identical;

b) Fibronectin molecules from all sources are immunologically identical and have very similar amino acid compositions;

c) The carbohydrate residues attached to fibronectin differ on fibronectin from different sources. These residues protect the molecule from proteolysis;

d) Fibronectin consists of at least three functional domains connected by flexible polypeptide chains;

e) Three differences between cellular and plasma fibronectin have been localised by the use of proteolytic cleavage and monoclonal antibodies;

f) At least two differences exist between the subunits of plasma fibronectin; one is within the cell spreading domain, the other is an extra fibrin-binding domain in the larger subunit;

g) All the differences found are located in the internal part of the molecule, and are therefore not the result of simple proteolytic cleavage.

These results indicate that the adhesive and binding interactions between cells, extracellular macromolecules and fibronectin can be explained by a combination of the appropriate active sites. A prediction

of this model is that cell substratum interaction only requires the monomer while cell-cell adhesion requires at least the dimer (Yamada *et al.* 1980).

VI. THE EVOLUTION AND GENETICS OF FIBRONECTIN

A. Genetics

1. How many structural loci?

There have been few genetically oriented studies of fibronectin Consequently, it is not known whether or not the different forms are under the control of a single structural locus. Some of the results obtained from studies of the function (Section III) and the structure (Section V) of fibronectin are pertinent to this problem.

a) The functional properties of plasma and cellular fibronectin are different. Cellular fibronectin is a more affective haemaglutinin and is able to restore normal morphology and social behaviour to transformed fibroblasts (Yamada and Kennedy, 1979).

b) SDS polyacrylamide gel electrophoresis suggests that cellular and amniotic fluid fibronectins are heavier than plasma fibronectin and that while cellular and amniotic fluid fibronectins consist of identical subunits, the subunits of plasma fibronectin have different molecular weights (Mosesson *et al.* 1975; Mosher, 1975 and Balian *et al.* 1979b).

c) Proteolytic cleavage indicates that there are a number of internal differences in amino acid sequence in fibronectins. The collagen-binding domain is longer in plasma fibronectin and the larger subunit of plasma fibronectin has an extra fibrin-binding domain and a longer cell spreading domain than the smaller subunit (Yamada *et al.* 1980; Isemura *et al.* 1981 and Sekiguchi *et al.* 1981).

There are a number of possible explanations for the above results, some of which involve the presence of more than one structural locus for fibronectin (Yamada *et al.* 1980; Ehrisman *et al.* 1982 and Laterra

and Culp, 1982). At present it is impossible to be certain how many loci exist, but a copy of a fibronectin gene has been isolated and cloned (Fagan *et al.* 1981). Therefore, with available recombinant DNA technology, an answer should be available in the near future.

2. The chromosomal location of the fibronectin structural loci

Owerbach *et al.* (1978) suggested that a gene required for the expression of fibronectin was located on human chromosome number 8 as in the human/mouse cell hybrids which they studied fibronectin production was concordant with the presence of chromosome 8 and an enzyme marker for chromosome 8, glutathione reductase (GSR). However, the fibronectin assay used was not species specific and when one of their positive cell lines was re-examined with specific anti-human and anti-mouse antisera the fibronectin was found to be murine (Klinger and Ruoslahti, 1980). Studies utilising a non-species specific antisera suggested that a structural locus for fibronectin was on chromosome 11 (Eun and Klinger, 1979 and 1980). Smith *et al.* (1979) also suggested that a structural locus was present on chromosome 11 but, in addition, a regulatory locus was present on chromosome 3.

Klinger and Ruoslahti (1980) apparently confirmed the presence of a structural locus on chromosome 11 by the use of human specific antisera to fibronectin. Fibronectin fibril formation was syntenic with chromosome 11 and an enzyme marker for chromosome 11, lactate dehydrogenase A. However, they reported the presence of one cell line with a chromosome 11 which did not produce fibronectin fibrils. A possible criticism of their assay is that it measures the formation of fibronectin fibrils rather than the production of fibronectin. It is, therefore, possible that the locus on chromosome 11 controls the formation of fibrils (Chapter 1.II. A).

Rennard *et al.* (1981b) and Church (1981) have used a species specific enzyme immunoassay and have shown a 100% concordance between the production of fibronectin and chromosome 8 and GSR, thereby indicating that a structural locus for fibronectin is present on chromosome 8.

A possible explanation of the contradictory results of the above studies is that more than one structural locus for fibronectin is present in the human genome. One locus may be present on chromosome 8 and another on chromosome 11. In the cell hybrids examined it is possible that the locus being expressed is different. Until further studies are carried out the location of the structural gene(s) remains uncertain, but is likely to involve chromosome 8.

B. Conservation of the fibronectin molecule during evolution

The role of fibronectin in cell-cell adhesion, cell-substratum interactions and the mononuclear phagocytic system suggests that the fibronectin molecule could show a considerable degree of conservation during evolution. Studies on mammals and birds with an antisera raised against human fibronectin in rabbits, indicate that this may be the case. All species studied to date have shown immunogenic cross reactivity, for example, chicken (Blumberg and Robbins, 1975; Linder *et al.* 1975; Yamada *et al.* 1975 and Koteliansky *et al.* 1982a), mouse (Yamada *et al.* 1976a), rhesus monkeys (Mosher, 1976b and Koteliansky *et al.* 1982a), bovines (Iwanaga *et al.* 1978 and Birdwell *et al.* 1980), rat (Molnar *et al.* 1979b), hamster (Fukuda and Hakomori, 1979) and porcines (Isemura *et al.* 1981). The structure and functions of fibronectin from these species is also similar.

Fibronectin has also been detected in the elasmobranch, *Torpedo californica* (Engvall *et al.* 1978), the sea urchin, *Sphaerechinus granularis* (Spiegal *et al.* 1980), the horseshoe crab, *Limulus* (Akiyama *et al.*

1981), the marine sponges, *Microconia porifera* (Akiyama *et al.* 1981) and *Tethya* (Labat-Robert *et al.* 1981a) and the freshwater sponge, *Ephydatia mulleri* (Labat-Robert *et al.* 1981a), but not the earthworm (Akiyama *et al.* 1981) with anti-human fibronectin antisera. As the sponges (Porifera) represent the earliest forms of multicellular life, these results suggest that the fibronectin molecule has been highly conserved during phylogenesis. As some of the antigenic sites of fibronectin appear to be conformationally determined (Vuento *et al.* 1980 and Wallace *et al.* 1981), the above results would suggest that some of the functional domains present in mammalian fibronectin existed in some of the earliest multicellular organisms to evolve.

It is possible to raise monoclonal antibodies with species-specificities, one such antibody to a site in the central region of the plasma fibronectin molecule will cross react to human and rhesus macaque plasma fibronectin, but not to rat, calf, pig and chick plasma fibronectin (Koteliansky *et al.* 1982a).

Chapter 2

I. INTRODUCTION

In view of the biological processes in which fibronectin is involved and its apparent role as a substrate for factor XIII in blood coagulation and wound healing (see Chapter 1.III) in addition to the absence of any definite information on the genetic control of the various forms of fibronectin (see Chapter 1.VI.A), it was decided to develop a procedure by which structurally abnormal, as well as normal, variants of fibronectin could be rapidly identified.

Fibronectin does not have a biological activity that could readily be used to develop a chromatogenic or fluorescent stain to detect its presence. However, the relatively high plasma concentration of fibronectin, 325±76 µg/ml (Stathakis *et al.* 1981), permits the localisation of the molecule by immunological techniques. Because crossed-immunoelectrophoresis does not permit the screening of large numbers of samples and is difficult to interpret from a genetic view, it was decided to locate plasma fibronectin by immunofixation after electrophoresis (Alper and Johnson, 1969). A procedure for the electrophoresis of fibronectin on thin layer agarose gels which would allow good resolution of the fibronectin molecule was therefore developed.

The existence of genetic variants of plasma fibronectin, which are detectable by electrophoresis, would give some clues to the genetic control of the plasma form. In addition the examination of cellular fibronectin from individuals with variants of the plasma form should enable a better understanding of the genetic relationship of the two forms to be reached.

Studies of the structure of plasma and cellular fibronectin have suggested that the plasma form of the protein is a dimer of non-identical subunits, while the cellular (and amniotic fluid) form consists of two identical subunits (see Chapter 1.V). The relationship of the cellular fibronectin subunit to the two subunits of plasma fibronectin is uncertain (Yamada *et al.* 1980; Ehrisman *et al.* 1982 and Laterra and Culp, 1982).

As fibronectin is known to be a dimer (Mosesson *et al.* 1975; Mosher, 1975 and Balian *et al.* 1979b), the electrophoretic pattern of genetic variants in a heterozygote can be predicted. If a protein is a dimer of identical subunits, as appears to be the case for cellular fibronectin, the heterozygous presence of a variant with a different electrophoretic mobility would result in a three banded pattern being observed after electrophoresis. The random assembly of the two types of subunit into dimers would result in the production of homodimers identical to those found in homozygotes for each form and a heterodimer with an electrophoretic mobility midway between the two homodimers. Whereas, if a protein is a dimer of non-identical, non-allelic subunits, the presence of a variant of one of the subunits would result in a two-banded pattern. The constant subunit would form a dimer with each of the variant and the usual form of the other subunit. If the non-identical subunits are, in fact, allelic then a three-banded pattern would be expected after electrophoresis. The three bands would represent dimers between the product of each allele with a modified product of the same allele (the fast and slow bands) and the product of each allele with a modified product of the other allele (the middle band). However, if the mutation in the variant allele altered the modification process, the exact banding pattern would be difficult to predict. The modification could arise because of differential gene processing or post-translational processes.

Five possible genetic explanations of the observed structures of plasma and cellular fibronectin, as well as the expected electrophoretograms of plasma and cellular fibronectin when a variant is present in the heterozygous state, are discussed below and are illustrated in Figure 2.1:

Model 1:

There is a single structural gene for both cellular and plasma fibronectin. The smaller plasma subunit is formed as a result of post-translational modifications of 50% of the subunits in plasma. This model would predict a three-banded pattern after electrophoresis, for both plasma and cellular fibronectin (Figure 2.1.a).

Post-translational modifications are, however, an unlikely explanation for the two plasma subunits. Plasma and cellular fibronectins are known to have slightly different functional properties (Yamada and Kennedy, 1979), but the removal of carbohydrate groups and the alteration of sulphydryl and amino groups do not affect the functions of fibronectin (Olden *et al.* 1978 and Pearlstein *et al.* 1980). In addition, the structural differences, both between cellular and plasma fibronectin and between the subunits of plasma fibronectin, are located internally rather than at the terminal ends of the molecule (Yamada *et al.* 1980; Atherton and Hynes, 1981; Atherton *et al.* 1981; Hayashi and Yamada, 1981 and 1982 and Sekiguchi *et al.* 1981). Therefore, a proteolytic modification is unlikely. Finally, it is difficult to propose a post-translational process which affects exactly 50% of the subunits.

Model 2:

There is a single structural gene for both plasma and cellular fibronectin. The smaller plasma subunit is the result of differential processing of the transcript of this gene. This model would also predict a three-banded pattern after electrophoresis, for both cellular

FIGURE 2.1: THE EXPECTED ELECTROPHORETOGRAMS OF PLASMA AND CELLULAR FIBRONECTIN, IF GENETIC VARIANTS ARE PRESENT IN THE HETEROZYGOUS STATE, FROM FIVE DIFFERENT GENETIC MODELS (see text).

a) Models 1 and 2

b) Model 3

c) Model 4

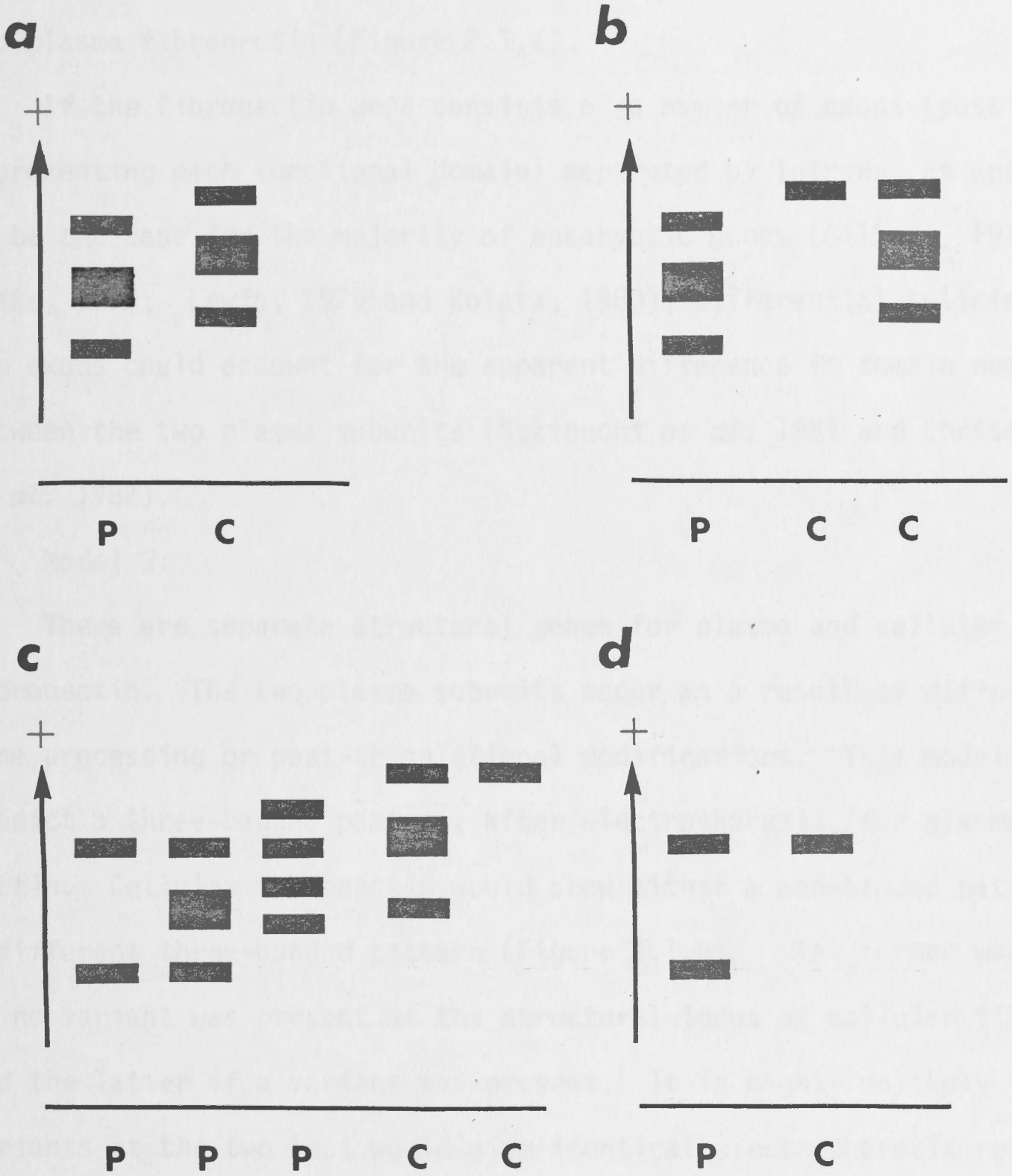
d) Model 5

e) Model 5

f) Model 5

P = plasma fibronectin; C = cellular fibronectin

The five models and the electrophoretograms are explained in the text.


a
+
P
C
b
+
P
C
C
c
+
P
P
P
C
C
d
+
P
C
e
+
P
C
f
+
P
P
C

and plasma fibronectin (Figure 2.1.a).

If the fibronectin gene consists of a number of exons (possibly representing each functional domain) separated by introns, as appears to be the case for the majority of eukaryotic genes (Gilbert, 1978; Blake, 1979; Lewin, 1979 and Kolata, 1980), differential splicing of the exons could account for the apparent difference in domain number between the two plasma subunits (Sekiguchi *et al.* 1981 and Ehrisman *et al.* 1982).

Model 3:

There are separate structural genes for plasma and cellular fibronectin. The two plasma subunits occur as a result of differential gene processing or post-translational modifications. This model would predict a three-banded pattern, after electrophoresis, for plasma fibronectin. Cellular fibronectin would show either a one-banded pattern or a different three-banded pattern (Figure 2.1.b). The former would occur if no variant was present at the structural locus of cellular fibronectin and the latter if a variant was present. It is highly unlikely that the variants at the two loci would give identical electrophoretic results.

The arguments against post-translational modifications are similar to those for Model 1.

Model 4:

There are distinct structural genes for cellular fibronectin and the two subunits of plasma fibronectin. The predictions of this model for the electrophoretograms if genetic variation occurs are more complex. If variation occurs at only one of the plasma fibronectin loci, a two-banded pattern would be expected, as only one of the subunits would be present in the variant form. But, if variation occurs at both plasma fibronectin loci a three- or four-banded pattern would be expected depending on the charge differences of the variants. The predicted

banding pattern for cellular fibronectin would be independent of that of plasma fibronectin and would be the same as for Model 3 (Figure 2.1.c).

The occurrence of gene families, which have apparently arisen by gene duplication, appears to be fairly common in the eukaryotic genome (Efstradiadis *et al.* 1980).

Model 5:

There is a common structural gene for the subunit of cellular and the large subunit of plasma fibronectin and a distinct structural gene for the small plasma fibronectin subunit. This model predicts three possible electrophoretic patterns. Firstly, if the variant occurred at the locus for the small plasma fibronectin subunit, then plasma fibronectin would show a two-banded pattern and cellular fibronectin a one-banded pattern (Figure 2.1.d). Secondly, if the variant occurred at the other structural locus, plasma fibronectin would again show a two-banded pattern, but cellular fibronectin would show a three-banded pattern (Figure 2.1.e). Finally, the presence of variants at both loci would produce a three- or four-banded pattern in plasma fibronectin and a three-banded pattern in cellular fibronectin (Figure 2.1.f). It is unlikely that the three-banded patterns would show identical mobility.

This model could be correct if the two extra segments of 1300 M.W. near the collagen binding site, and 1000 M.W., near the carboxyl terminal, and the deficient 10000 M.W. segment of plasma fibronectin, compared to cellular fibronectin (Hayashi and Yamada, 1982), are all in the smaller plasma subunit.

The models described above are not exclusive and it is possible that a more complex model will explain the genetic control of fibronectin.

The relationship of fibronectin from amniotic fluid, cerebrospinal fluid and milk to the plasma and cellular forms, could be examined in a similar way.

The electrophoretic technique developed in this study was used to screen plasma samples from a number of human populations, from different racial groups and different areas of the world, for genetic variants of plasma fibronectin in an attempt to test the five models given above. In addition, since post-translational modifications can alter the mobility of proteins, the effect of desialysation and treatment with β-mercaptoethanol on the electrophoretic mobility of plasma fibronectin was examined. Because of the extreme conservation of human plasma fibronectin found by these studies, plasma samples from a variety of domestic animals were also screened for genetic variants.

II. MATERIALS AND METHODS

A. Purification of fibronectin

Citrated plasma from normal blood donors was obtained from out-dated whole blood collected by the Canberra Red Cross Blood Transfusion Service, by centrifugation at 4000g for 10 minutes. Fibronectin was purified on a gelatin-Sepharose affinity column by a modification of the method described by Engvall and Ruoslahti (1977).

1. Preparation of the gelatin-Sepharose

The cynaogen bromide (CNBr) activated Sepharose 4B required for the affinity chromatography column was prepared as described by Axén *et al.* (1967). Gelatin was coupled to the activated Sepharose 4B by the procedure outlined below:

a. 100g of CNBr activated Sepharose was washed on a glass filter with 1mM HCl for 15 minutes.

b. 1g of gelatin was boiled in 100ml of 0.1M $NaHCO_3$ containing 0.5M NaCl.

c. The Sepharose was added to the gelatin mixture and shaken for 2 hours at room temperature.

d. The Sepharose was washed with 0.1M $NaHCO_3$ containing 0.5M NaCl.

e. The Sepharose was added to 500 ml of 1M ethanolamine, pH 8, and shaken for 2 hours at room temperature.

f. The gelatin-Sepharose was washed with 0.1M acetate buffer, pH 4, containing 1M NaCl and 0.1M borate buffer, pH 8, containing 1M NaCl, alternately for three cycles.

2. Column chromatography

A volume of approximately 200ml of the gelatin-Sepharose was poured into a column and washed with buffer 1, containing 50mM Tris, 20mM sodium citrate and 5mM ε-amino caproic acid at pH 7.5. Plasma (800ml) was applied to the column at a flow rate of 30ml/hr. The column was then washed

with buffer 1 until the $O.D_{280}$ of the eluate was zero. After washing the column with 600ml of buffer 1 containing 1M NaCl, fibronectin was eluted by the application of buffer 1 containing 5M urea. Fractions were tested at $O.D_{280}$ for protein content.

The eluted fibronectin was dialysed against three changes of 100 volumes of buffer 1 for 24 hours to remove urea. The homogeneity of the final fibronectin preparation was checked by agarose gel electrophoresis.

B. Production of anti-fibronectin IgG

Antiserum was produced in rabbits to purified fibronectin by sub-cutaneous injections, in multiple sites on the rabbits back, of an emulsion of aqueous protein and Complete Freunds adjuvant. Approximately 20µg of protein was injected at intervals of one week for four weeks. Antiserum was harvested after the fourth injection. Antiserum was stored in 2ml aliquots at $^{-}20°C$ until use. The specificity of the antisera was checked on Ouchterlony diffusion plates.

C. Agarose electrophoresis

As a result of experimentation with purified fibronectin, a barbital buffer, pH 8.6, was found to give the best resolution. The electrode buffer contained barbituric acid 20mM, sodium barbitone, 70mM and calcium lactate 2mM. The gel buffer was a 2 in 3 dilution of the electrode buffer. The barbituric acid was dissolved in boiling water prior to being added to the rest of the buffer.

Thin layer gels were prepared on 15 x 17cm glass plates. The plates were warmed to 60°C and 30ml of 1% (w/v) agarose (Sigma Type 1) in the gel buffer was spread over each plate and allowed to solidify at room temperature for 20 minutes. Sample slots (14/gel) were cut by pressing a plastic slot former into the gel 3cm from the cathodal end of the plate. Samples of 4µl of thawed plasma were then applied to the gel.

Electrophoresis was carried out horizontally with the gel plate placed on a cooling block between the electrode tanks. The gel was connected to the electrode tanks by a double layer of Whatman 3MM chromatography paper. Electrophoresis was carried out at 13V/cm until a haemoglobin marker had migrated 2.5cm towards the anodal end of the gel.

D. Immunofixation

After electrophoresis fibronectin was specifically localised by overlaying the gel with a 1 in 4 dilution (in physiological saline) of the antiserum. The antiserum was spread over the gel with a sealed and bent pasteur pipette tip, taking care not to touch the gel surface. The gel was incubated at room temperature, in a moist environment, for 1-2 hours. Care must be taken, at this stage, to keep the gel level to prevent the antisera from flowing to one side. After immunofixation, the gel was rinsed briefly under running tap water, covered with a wet filter paper and compressed for 10-30 minutes under approximately 5mm of absorbent paper covered by a perspex plate and a 2kg weight. After compression, the gel was soaked overnight at 4°C in at least 1L of 150mM NaCl to remove non-immunoprecipitated protein.

The successful application of the immunofixation technique depends on achieving the optimum balance between the amount of antigen in the gel and the titre of the antibody applied to the gel surface. The correct dilution of the antisera is therefore dependent on the initial titre of the antibody and must be determined empirically by testing different dilutions of each batch of antiserum.

After soaking overnight in 150mM NaCl, the gels were washed under running tap water for 10 minutes and again compressed as described above. The gels were then dried before being placed in a 0.2% (w/v) Coomassie blue R solution (45% (v/v) methanol: 10% (v/v) acetic acid: 45% (v/v) water) for 5 minutes. The gels were then washed in a destain solution

(2.5% (v/v) acetic acid: 2.5% (v/v) Teepol: 95% (v/v) water) and rinsed under running tap water. Zones of protein fixed by the anti-fibronectin antisera react with the Coomassie blue to form blue bands.

E. Origin of samples

1. Human samples

Plasma or serum samples from 2,981 individuals from 17 different populations were screened for genetic variants. Plasma or serum samples from these individuals had previously been collected and stored in liquid nitrogen, or at $^{-}$20°C for periods ranging from a few months to 10 years in the Department of Human Biology, John Curtin School of Medical Research. The plasma samples were collected on a variety of anticoagulants.

2. Animal samples

Plasma samples from a variety of animals, including goats, dogs, cats, sheep, highland ponies, horses, donkeys, mice, cattle, camels and pigs were collected on citrate, as an anticoagulant and stored at $^{-}$20°C for 1-6 months before use.

F. Treatment of samples

Several human samples and one of each of the animal samples were treated with 7mU of neuraminidase/µl of sample and dialysed overnight as described by Awdeh (1976). In addition, some samples were incubated at room temperature with ¼ volume of 0.5% β-mercaptoethanol (v/v) for 30 minutes before electrophoresis.

III. RESULTS

A. Purification of fibronectin

The application of 5M urea to the affinity column produced a single peak of protein (Figure 2.2). Each 9ml fraction was tested for protein content by agarose gel electrophoresis in the buffer system described above and gel staining with Coomassie blue after having first fixed the proteins with picric acid. All the fractions containing detectable protein levels showed a single band (Figure 2.3), indicating that if any contaminants were present in the purified fibronectin they were very minor.

The antisera raised against purified fibronectin showed two lines of cross-reactivity against plasma on Ouchterlony gels. When the antisera was used for immunofixation after agarose electrophoresis a faint band was present at a position which corresponds to the electrophoretic mobility of albumin in the buffer system used. It was, therefore, concluded that albumin was a minor contaminant in the purified fibronectin.

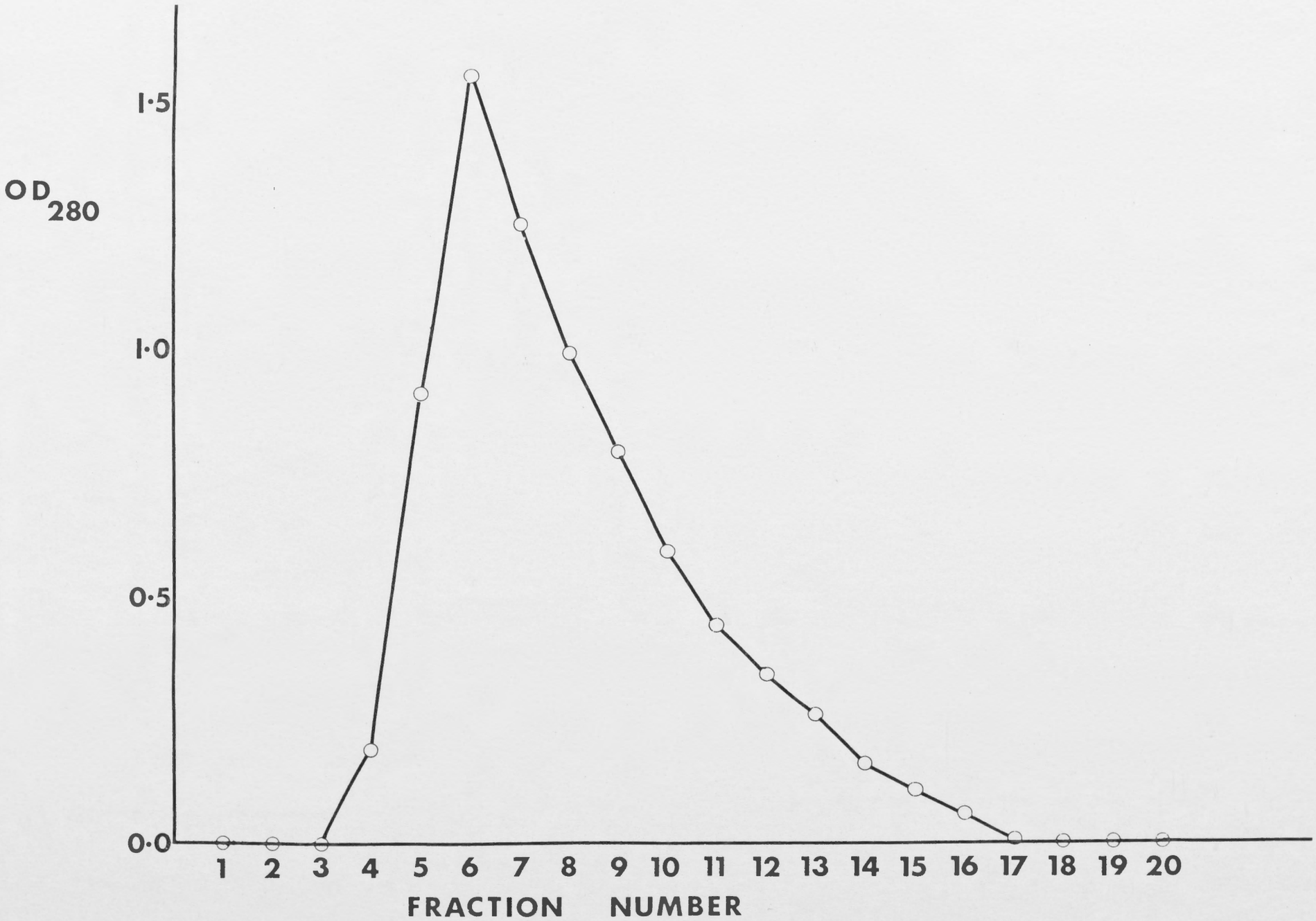
B. Human samples

The single band of fibronectin obtained after electrophoresis of citrated plasma is shown in Figure 2.4. The use of heparin as an anticoagulant was found to result in significant losses of fibronectin to concentrations which were below the level of detection. Serum samples contained sufficient fibronectin levels for detection. Table 2.1 shows the results of a survey of 2,981 individuals from a wide range of racial groups.

A single sample from Ternate, a remote Indonesian island, presented an abnormal phenotype. The relative electrophoretic mobility of this variant form is shown in Figure 2.5a) and a schematic representation

FIGURE 2.2: THE ELUTION PROFILE OF PLASMA FIBRONECTIN FROM THE AFFINITY COLUMN WITH 5M UREA

Urea was applied at fraction 1 and each fraction contained 9ml of eluate.


OD280
1·5
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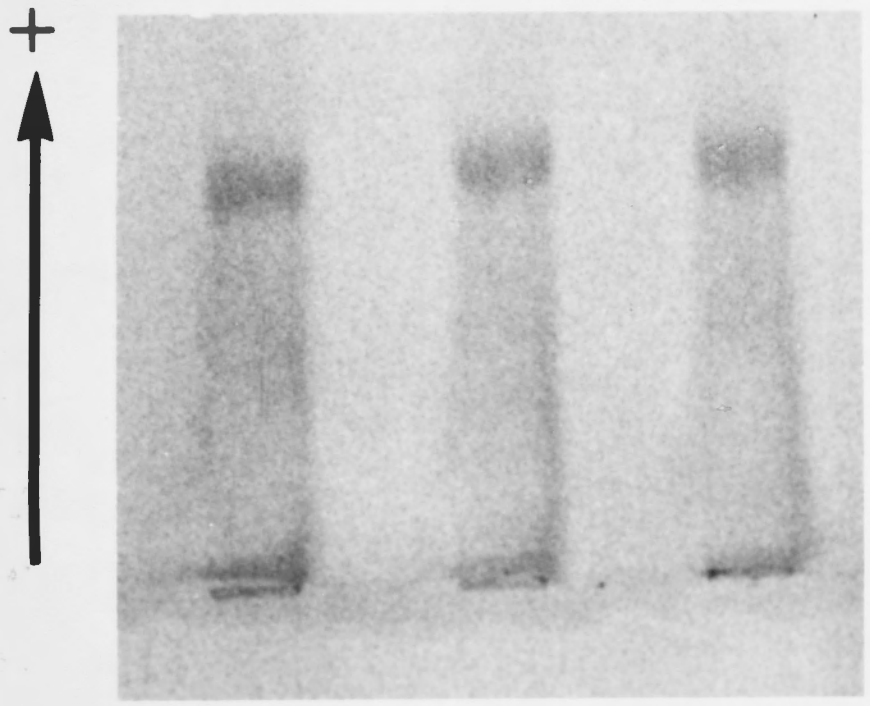
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FRACTION NUMBER

FIGURE 2.3: AGAROSE ELECTROPHORESIS OF PURIFIED PLASMA FIBRONECTIN

F = Purified fibronectin

FIGURE 2.4: AGAROSE GEL ELECTROPHORESIS OF PLASMA FIBRONECTION: THE COMMON PHENOTYPE

C = Common phenotype of plasma fibronectin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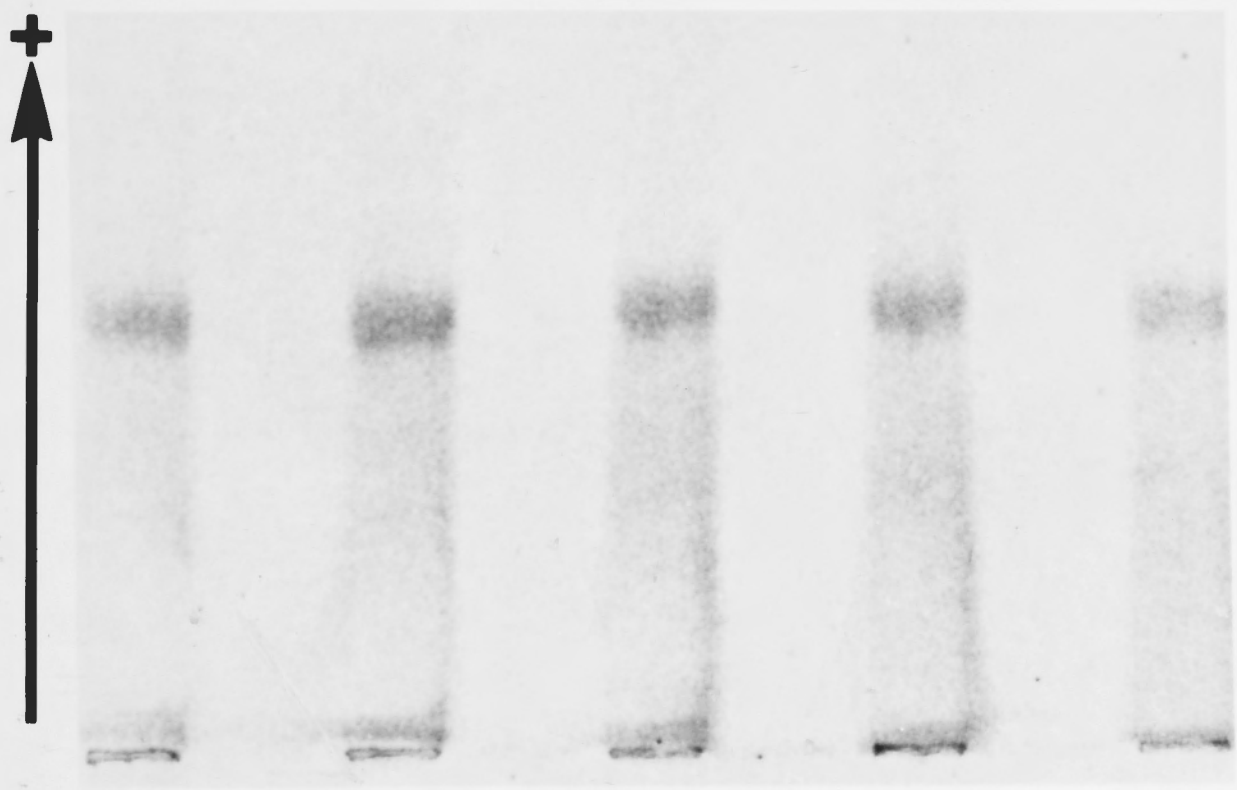
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TABLE 2.1.

ELECTROPHORETIC SCREENING OF HUMAN PLASMA AND SERUM FIBRONECTIN

Population	Number Tested	Number Ascertained	Variants
AUSTRALIA			
Caucasian	235	234	-
Aboriginal	198	52	
PAPUA NEW GUINEA			
Tep Tep (Morobe District)	326	313	-
Simbai (Schrader Mt.)	128	88	-
Buka Is.	76	76	-
INDONESIA			
Ternates (Moluccas)	213	210	1
Galelarese (Halmahera)	100	100	-
Bimanese (Sumbawa)	100	100	-
THAILAND	93	93	-
JAPAN	149	78	-
CHINA	118	83	-
INDIA	70	42	
FIJI			
Melanesian	277	277	-
CAROLINE IS.	224	224	
AMAZON INDIANS	135	125	-
PIMA INDIAN	201	201	-
SOUTH AFRICA			
Negroid	223	203	-
CASPIAN SEA	115	114	-

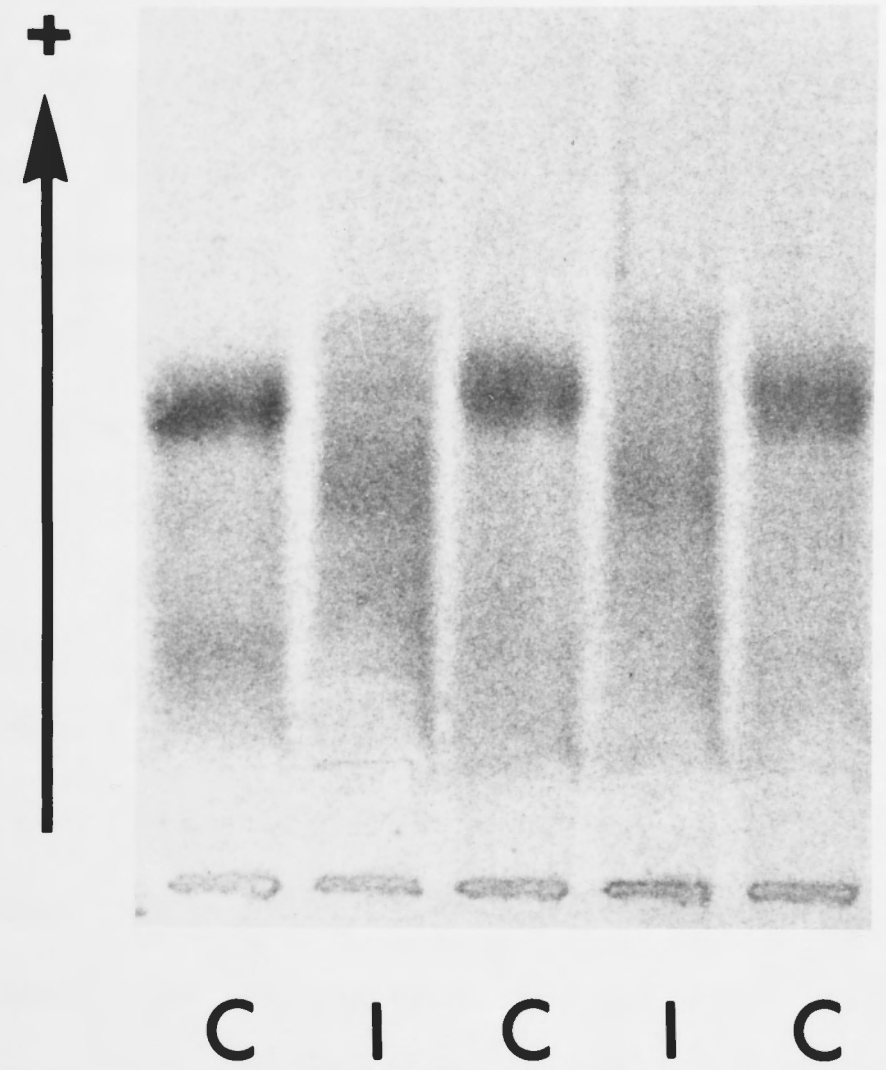
FIGURE 2.5: THE ELECTROPHORETIC PHENOTYPE OF THE INDONESIAN VARIANT OF PLASMA FIBRONECTIN

a) Electrophoresis of the Indonesian variant

b) A schematic representation of the Indonesian variant

C = Common fibronectin phenotype; I = Indonesian variant

a



b

of the variant is illustrated in Figure 2.5.b). Treatment of this sample or the normal form with β-mercaptoethanol did not alter the electrophoretic pattern (Figure 2.6). The effect of neuraminidase treatment on the normal phenotype and the variant form are illustrated in Figure 2.7. The latter is only shown schematically as the photographic reproduction of the gel was very poor.

C. Animal samples

The common phenotype exhibited by dogs, cats, sheep, goats, horses, highland ponies, donkeys, mice, cattle, camels and pigs and their mobility relative to human fibronectin, are shown in Figure 2.8 and Table 2.2. The results of a survey of 14 dogs, 47 cats, 62 sheep, 38 goats, 151 horses, 6 highland ponies, 33 donkeys, 3 mice, 55 cattle, 2 camels and 62 pigs are given in Table 2.3. The phenotype expressed by horses, donkeys, highland ponies, goats and sheep varied in some individuals as illustrated for horses in Figure 2.9. While treatment with β-mercaptoethanol had no effect on any of the phenotypes, neuraminidase treatment did alter the mobility of the most anodal band in all species studied and in all phenotypes expressed (Figure 2.10).

FIGURE 2.6: THE EFFECT OF β-MERCAPTOETHANOL TREATMENT ON THE ELECTROPHORETC MOBILITY OF PLASMA FIBRONECTIN

The Indonesian variant showed a loss of resolution similar to that seen here in the common phenotype.

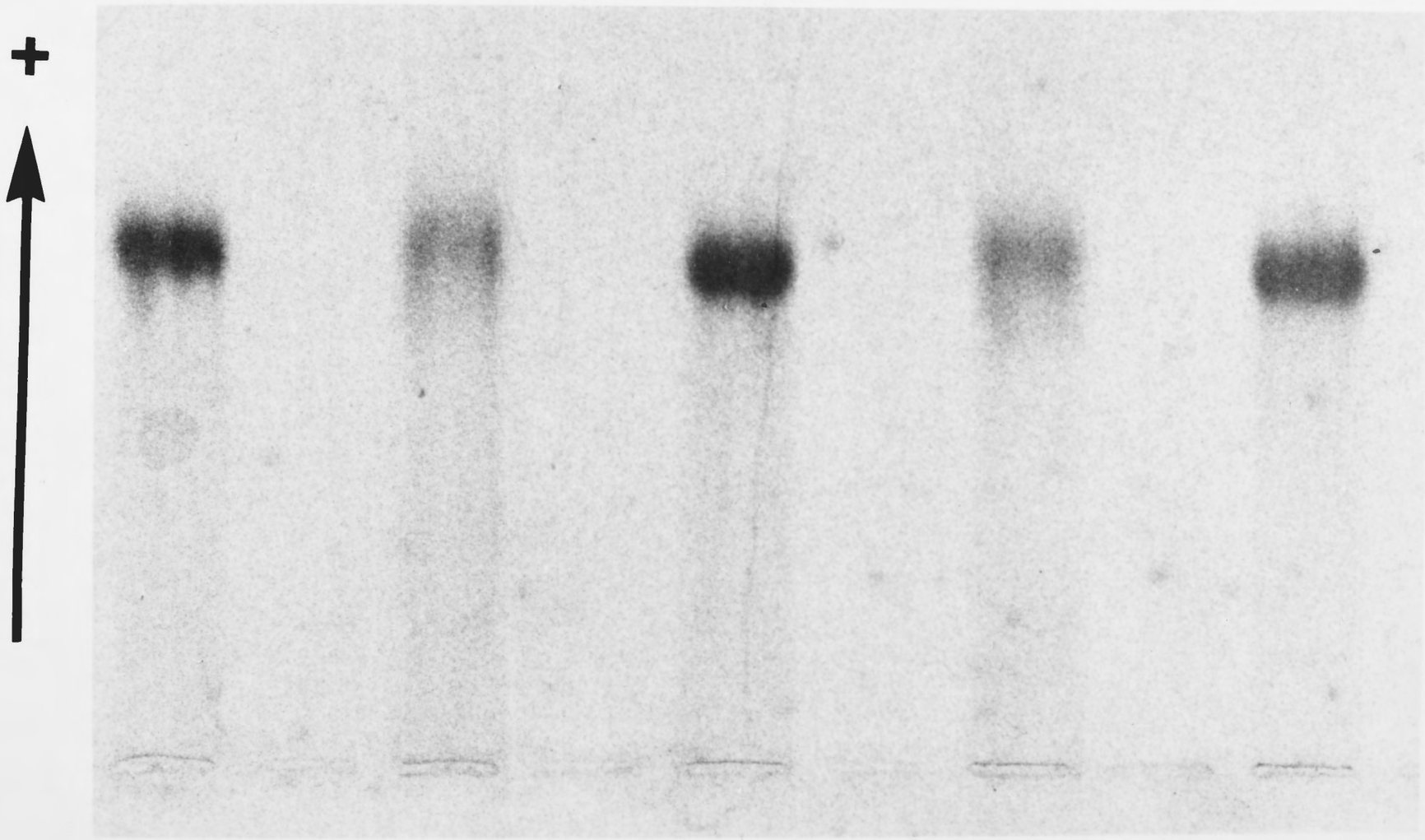

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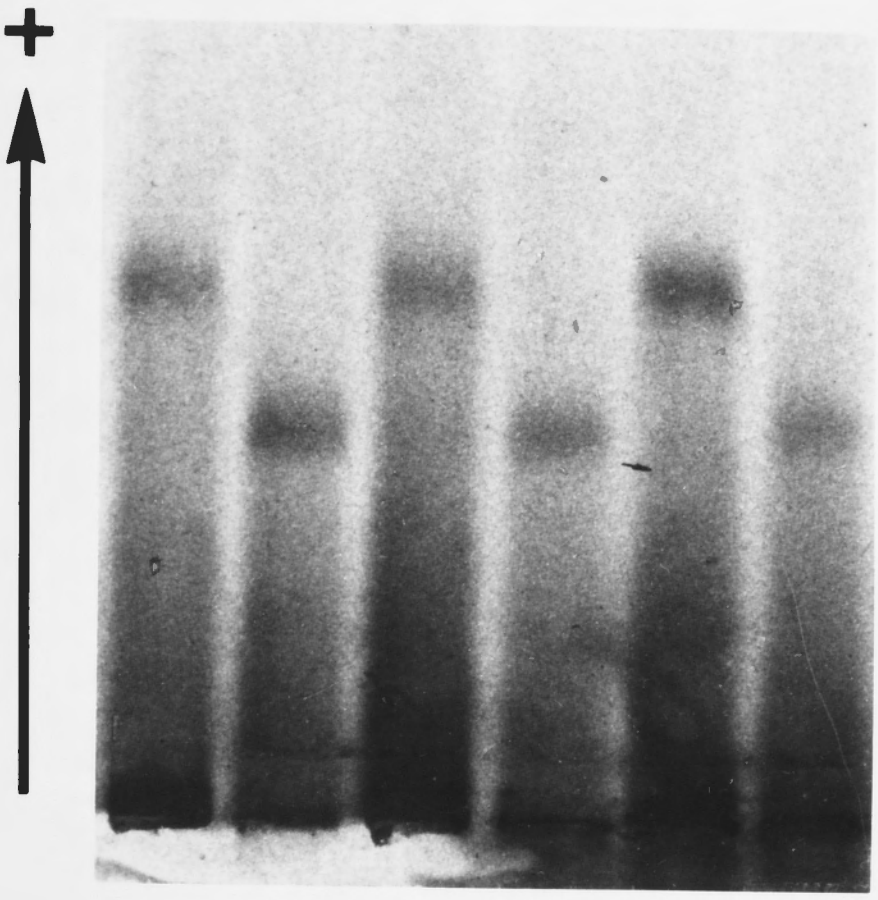
FIGURE 2.7: THE EFFECT OF NEURAMINIDASE TREATMENT ON THE ELECTROPHORETIC MOBILITY OF PLASMA FIBRONECTIN

a) Neuraminidase treatment of the common form of fibronectin

b) A schematic representation of the effects of neuraminidase treatment of the common form and the Indonesian variant of plasma fibronectin.

C = common phenotype; I = Indonesian variant;
N = Neuraminidase treated

a



b

TABLE 2.2.

THE MOBILITY OF PLASMA FIBRONECTIN FROM 11 ANIMALS

RELATIVE TO THAT OF HUMAN PLASMA FIBRONECTIN

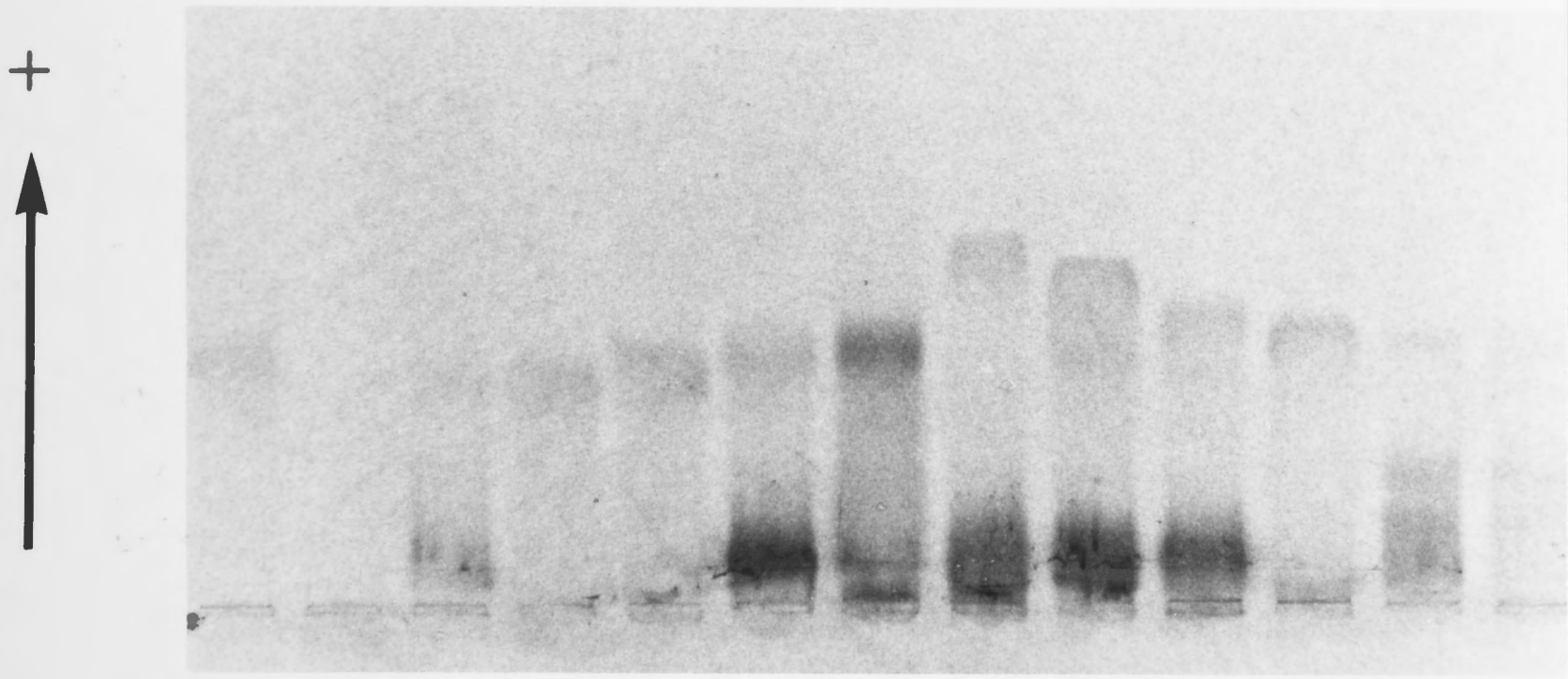
Animal	Relative Mobility
Horse	1.00
Donkey	1.00
Highland Pony	1.00
Goat	1.23
Dog	0.92
Cat	0.85
Sheep	1.35
Mouse	0.96
Cattle	1.08
Camel	0.89
Pig	0.98

FIGURE 2.8: THE ELECTROPHORETIC PHENOTYPES OF PLASMA FIBRONECTIN FROM ANIMALS AND MAN

M = mouse; C = camel; Ca = cat; D = dog; P = pig; H = human; S = sheep; G = goat; Ct = cattle; Ho = horse; Do = donkey and HP = Highland pony

FIGURE 2.9: THE PHENOTYPIC VARIANTS OF PLASMA FIBRONECTIN SHOWN BY THE HORSE (see text for explanation)

1 = 1 band; 2 = 2 bands; 3 = 3 bands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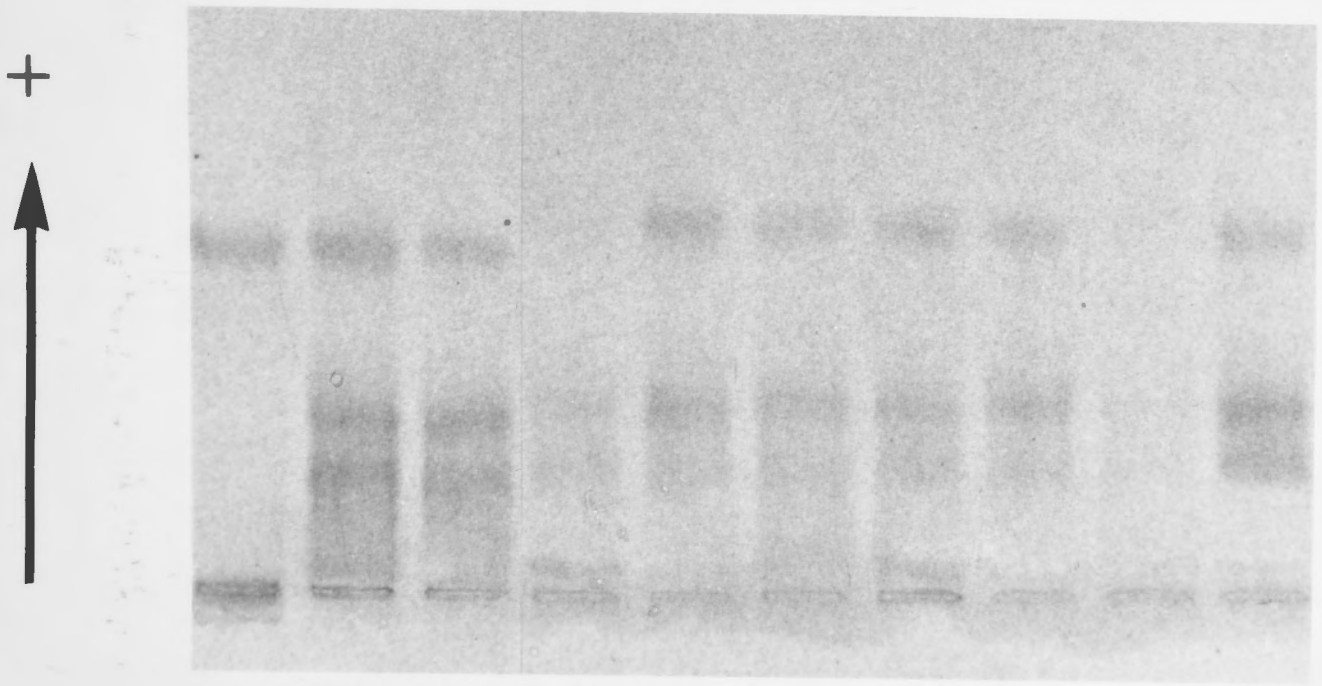
HP


+
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3
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2
3

TABLE 2.3.

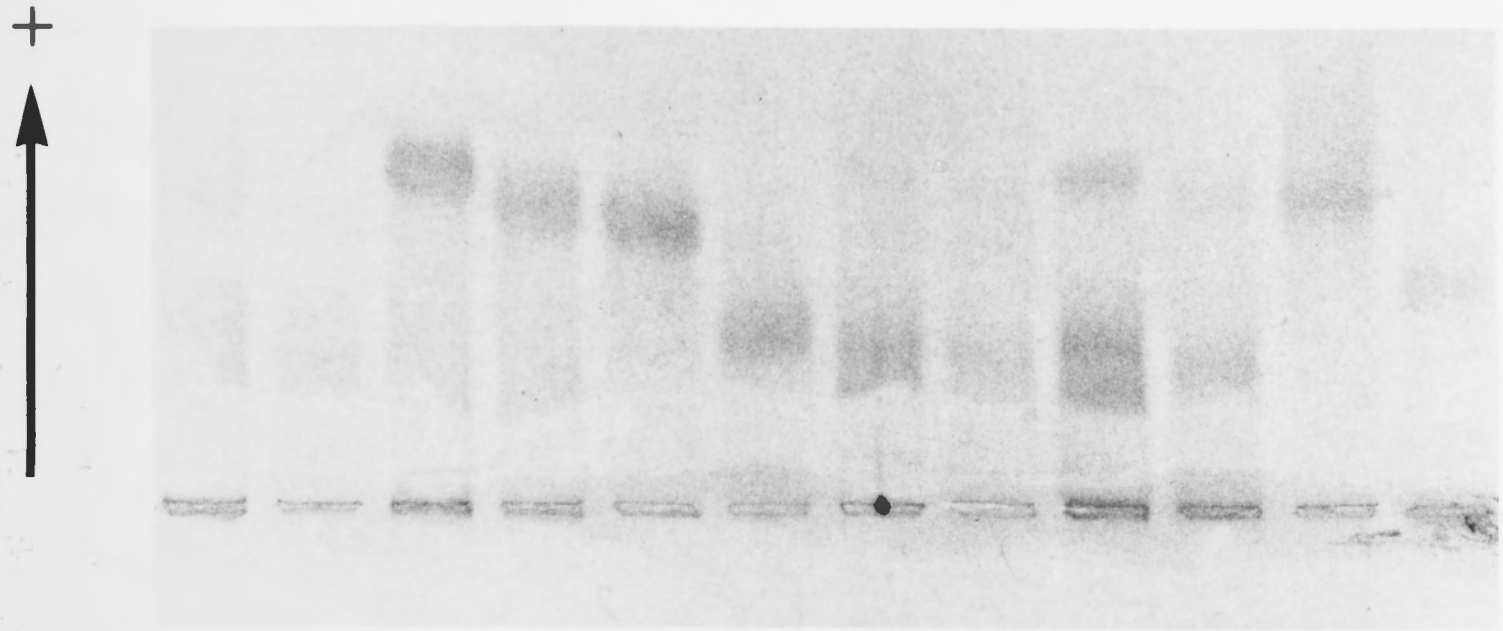
ELECTROPHORETIC SCREENING OF ANIMAL PLASMA FIBRONECTIN

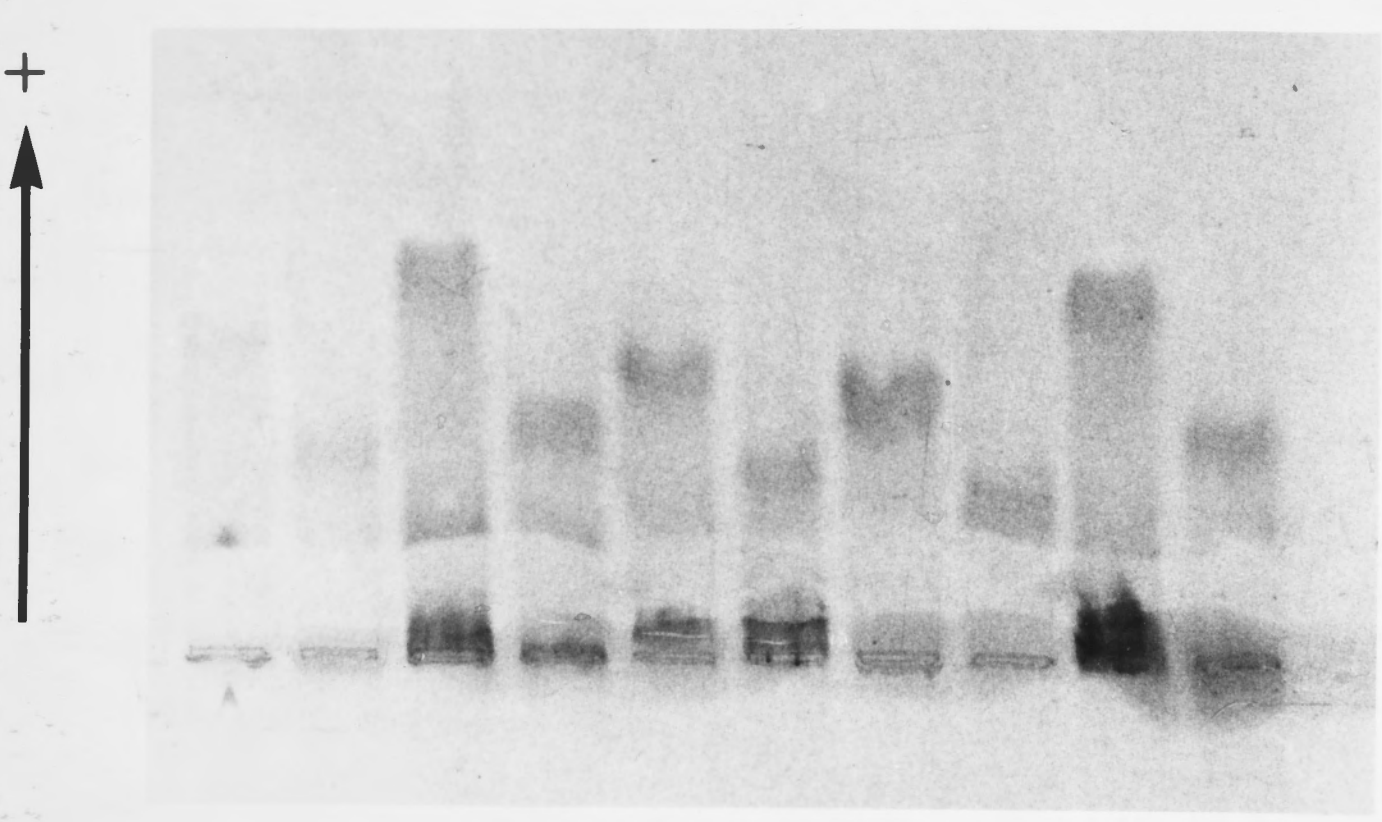
	Number Tested	Number Ascertained	Phenotype One Band	Two Bands	Three Bands
Horse	151	151	39	9	103
Donkey	33	33	-	11	22
Highland Pony	6	6	-	-	6
Goat	38	38	30	-	8
Dog	14	14	14	-	-
Cat	47	36	36	-	-
Sheep	62	62	48	-	14
Mouse	3	3	3	-	-
Cattle	55	54	54	-	-
Camel	2	2	2	-	-
Pig	62	62	62	-	-

FIGURE 2.10: THE EFFECT OF NEURAMINIDASE TREATMENT ON THE ELECTROPHORETIC MOBILITY OF FIBRONECTIN FROM ANIMAL PLASMA SAMPLES

Upper gel: C = camel; Ho = horse; Ca = cat; Do = donkey; HP = Highland pony; M = mouse; N = neuraminidase treated

Lower gel: Ct = cattle; S = sheep; P = pig; D = dog; G = goat; N = neuraminidase treated


+
C N Ho N Ca N Do N HP N M N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Ct N S N P N D N G N

IV. DISCUSSION

A. Human plasma fibronectin

The single band of fibronectin obtained after electrophoresis (Figure 2.4) was the same from serum and plasma and was not altered by the type of anticoagulant used. Although in some populations, where samples were collected in heparin, ascertainment of the phenotype of every sample was not obtained. This was due to an apparent drop in the concentration of soluble plasma fibronectin. Fibronectin has been shown to become insoluble in the presence of heparin (Mosesson and Amrani, 1980).

During the formation of a blood clot between 20% and 50% of the plasma fibronectin is incorporated into the clot (Chen *et al*. 1977). The fact that the fibronectin bands from plasma and serum samples are identical indicates that the fibronectin remaining in serum fibronectin which has not been incorporated into the clot and is not the result of some proteolytic event which might release part of the fibronectin molecule from the clot. Additionally, the identity of plasma and serum fibronectin suggest that fibronectin is not a substrate for the proteases released during blood coagulation.

1. The Indonesian variant

Of the 2,623 samples which gave positive results, only one sample from Ternate (The Moluccas Islands) in Indonesia presented an abnormal phenotype. The phenotype is difficult to photograph (Figure 2.5.a) and is therefore shown schematically (Figure 2.5.b). The plasma sample was normal for 4 other protein systems and red cells from that individual collected at the same time were normal for 20 enzyme and protein systems.

Treatment of the sample with β-mercaptoethanol, prior to electrophoresis, did not affect the four-banded phenotype. However, neuramin-

idase treatment, to remove sialic acid groups, while not affecting the number or relative distribution of the bands in the variant phenotype did alter the overall mobility of the four bands. The change in mobility for each band was identical to the mobility change shown by the normal fibronectin band after neuraminidase treatment (Figure 2.7). This suggests that each of the four bands has the same sialic acid content as the normal fibronectin molecule. Therefore, if the three extra bands result from proteolytic cleavage of fibronectin the segment not containing sialic acid residues either does not cross-react with the antisera or has an electrophoretic mobility such that it is not detected in the buffer system used.

Unfortunately, it has not been possible to test a fresh sample from that individual or to study other family members. Until such investigations can be completed it is not possible to make a definite conclusion about the origin of the abnormal phenotype.

2. Genetic control of plasma fibronectin

As only one sample with a variant phenotype was found in the screening programme carried out, it has not been possible to test the validity of the five models discussed above. The abnormal four-banded phenotype does fit the predictions of models 4 and 5. However, the four bands are not equally spaced as these models predict. Additionally, these models would only produce four bands if variants are present at two loci. The lack of detectable variation in any other samples means that it is extremely unlikely that this individual would have two detectable variants. Therefore, it would appear that either the variant phenotype is not genetic in origin, or none of the models are correct.

However, until the origin of the Indonesian variant is proven one way or the other, no conclusions can be drawn concerning the genetic control of fibronectin. The absence of electrophoretically detectable variation of plasma fibronectin from both man and animals indicates

that a different experimental approach is needed to find an answer to the problem of the genetic control of fibronectin. Fagan *et al.* (1981) have reported the cloning of a fibronectin gene. It therefore seems probable that studies such as this, which utilise the available recombinant DNA technology, will provide the solution to the problem.

3. Genetic conservation of the fibronectin molecule

Since antibodies raised against human fibronectin cross-react with fibronectin from many other species (Chapter 1.VI.B) including Porifera (Akiyama *et al.* 1981 and Labat-Robert *et al.* 1981a), which represent the earliest forms of multicellular life, it is apparent that the fibronectin molecule has been highly conserved during phylogenesis.

The finding of only one possible genetic, electrophoretically detectable variant in the 2,623 samples that gave positive results suggests that this evolutionary conservation is present within species as well as between species. It is probable that the major reason for the extreme evolutionary conservation shown by the fibronectin molecule is the large number of molecular interactions that the molecule is involved in (Chapter 1.III). The large numbers of functional sites on the fibronectin molecule suggests that an alteration in amino acid sequence is more likely to affect its function. McConkey (1982) has demonstrated that some proteins known to be involved in the complexities of intracellular organisation show very little genetic variation after examination by high-resolution two-dimensional polyacrylamide gel electrophoresis.

B. Animal studies

The antibodies raised against human plasma fibronectin crossreacted with plasma fibronectin from dogs, cats, sheep, goats, horses, highland ponies, donkeys, mice, cattle, camels and pigs. Therefore a number of plasma samples from each of these animals were examined to see if a genetic answer could be obtained from animals. However, most of the animals exhibited a single electrophoretic phenotype (Figure 2.8)

again demonstrating the lack of detectable genetic variation of plasma fibronectin.

1. Variant samples

Three species of animals exhibited variant phenotypes, while in goats and sheep two phenotypes were present, horses showed three phenotypes (Figure 2.9). The one-banded phenotype is similar to that expressed in other species. The two- and three-banded phenotypes appear to be derived from the one-banded form. In the three-banded forms an extra two relatively more cathodal bands are present. The relative intensity of these two bands to the more anodal band, varied between samples. As the intensity of the two bands increased the intensity of the anodal band diminished. In those samples where the cathodal bands were most intense the anodal band was absent resulting in the two-banded phenotype.

It therefore appears that the variant phenotypes are not genetic in origin. It is possible that they are the result of degradation of the fibronectin molecule or that they represent fibronectin bound to other plasma components. The recent reports of slower moving components after crossed-immunoelectrophoresis of human plasma fibronectin from some individuals (Höfelar *et al.* 1982; Klingemann *et al.* 1982 and Suzuki and Abe, 1982) which are thought to result from fibronectin bound to another plasma component, give support to the latter suggestion.

2. Relative mobility of the animal samples

The electrophoretic mobility of plasma fibronectin from the animals tested and man is shown in Figure 2.8. As can be seen from Figure 2.10 although treatment with neuraminidase alters the mobility, differences still exist between the animals and man. Therefore, the mobility differences are due to changes in amino acid content and not to a variation of

the carbohydrate content of the fibronectin molecule between species.

C. The chromosomal location of the fibronectin structural loci

As discussed in Chapter 1.VI.A.2 there is conflicting evidence as to the chromosomal location of the fibronectin structural gene(s) (Owerbach *et al.* 1978; Eun and Klinger, 1979 and 1980; Smith *et al.* 1979; Klinger and Ruoslahti, 1980; Church, 1981 and Rennard *et al.* 1981b). Several recent studies have suggested that the structural gene(s) are on chromosome 11 and that the locus on chromosome 8 controls the expression of fibronectin on the cell surface (Gerald and Miller, 1982 and Robson and Khan, 1982). In addition, Zardi *et al.* (1982b) have used species-specific monoclonal antibodies to suggest that a fibronectin gene is located on chromosome 2.

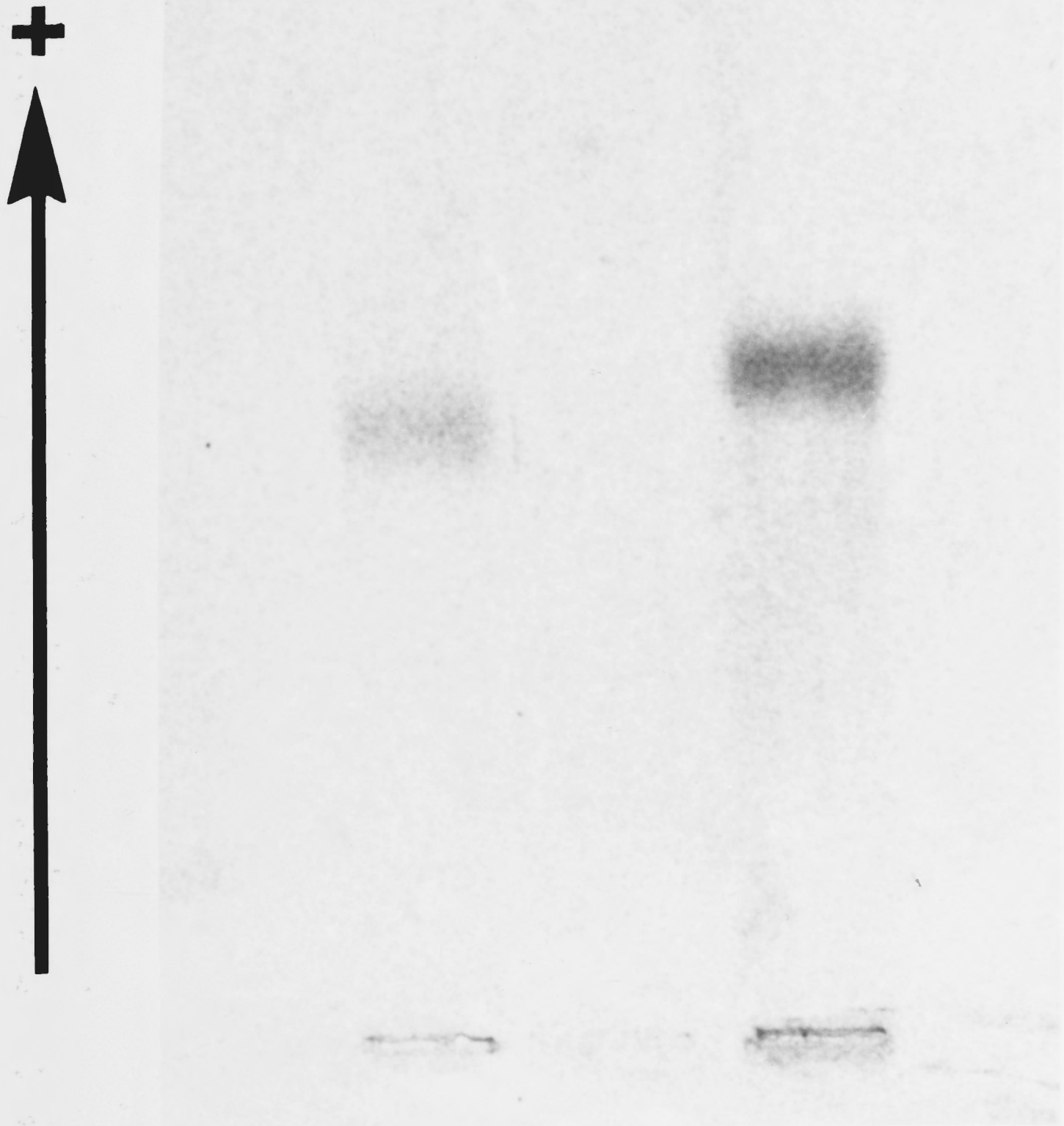
It is possible that the electrophoretic technique described here could be used to map the locus of the structural gene for cytoplasmic fibronectin by the use of mouse/human somatic cell hybrids without the need for species specific antisera as there is a clear mobility difference between human and mouse plasma fibronectin (Figure 2.11). The problems involved with the cell surface expression of fibronectin would also be eliminated by the use of this technique.

D. Abnormal fibronectins

The electrophoretic procedure described here could be used as a diagnostic tool in those individuals with suspected fibronectin abnormalities (Donati *et al.* 1977; Remuzzi *et al.* 1977; Furcht *et al.* 1979 and Arneson *et al.* 1980) as well as patients with wound healing and bleeding disorders of unknown cause, as an abnormal fibronectin molecule may well exhibit a variant phenotype. As fibronectin appears to play an integral role in embryogenesis (Chapter 1.III.D) the occurrence of a fibronectin molecule with an abnormal function in the homozygous state may lead to spontaneous abortion. Therefore, the

FIGURE 2.11: THE DIFFERENCE IN ELECTROPHORETIC MOBILITY BETWEEN PLASMA FIBRONECTIN FROM MOUSE AND HUMAN SAMPLES

M = mouse; H = human


+
M
H

study of families with a history of spontaneous abortion, where no other cause has been diagnosed, could reveal the presence of abnormal fibronectin molecules.

V. CONCLUSIONS

An electrophoretic technique which permits the rapid screening of plasma samples for abnormal fibronectins, by the process of immunofixation, has been described. This technique was used to study samples from 2,981 individuals and a number of animals in a search for genetic variants of plasma fibronectin. Only one possible genetic variant was found in an individual from Indonesia, thereby suggesting that the fibronectin molecule is a highly conserved molecule within species.

Chapter 3

I. HISTORICAL ASPECTS

A. Nomenclature

Blood coagulation factor XIII is the zymogen of an enzyme generated in the final stages of the blood coagulation cascade pathway. A variety of names have been suggested for both the zymogen and the active enzyme (Table 3.1) most of which are related to the function or mode of action of the enzyme. The International Committee on Haemostasis and Thrombosis officially named the protein factor XIII in 1963 (Hunter *et al.* 1964). However, Lorand and Konishi (1964) suggested that factor XIII was found in plasma as an inactive zymogen which was activated in the presence of thrombin. Thus, the term factor XIII was used for the zymogen only and the term factor XIIIa was used for the active enzyme. Although some of the other suggested names are still used in the literature, the above two names will be used throughout this discussion.

B. History

Robbins (1944) used dilute acids and alkalis to differentiate between two forms of fibrin and concluded that a factor in serum, which required the presence of calcium ions, was responsible for the formation of the insoluble fibrin. Laki and Lorand (1948) came to similar conclusions with urea as the solvent. However, it was not until Duckert *et al.* (1960) described a haemorrhagic diathesis that was attributable to a congenital deficiency of this plasma factor, then termed fibrin stabilizing factor, that the importance of plasma factor XIII in blood coagulation was proven.

Lorand and Jacobsen (1962) suggested that factor XIIIa cross-

TABLE 3.1.

ALTERNATIVE NOMENCLATURE FOR FACTOR XIII AND FACTOR XIIIa

Name	Abbreviation	Form	Reference
Fibrin-stabilising factor	FSF	Zymogen	Lorand (1950)
Lacki-Lorand factor	L-L factor	Zymogen	Loewy and Edsall (1954)
Fibrinase	-	Active	Loewy *et al.* (1961)
Plasma transamidase	-	Active	Chandrasekar *et al.* (1964)
Polymerase	-	Active	Lorand and Konishi (1964)
Plasma transglutaminase	-	Active	Loewy (1968)
Fibrinoligase	-	Active	Lorand (1972)
Protransglutaminase	-	Zymogen	Folk and Finlayson (1977)

linked an amino group (donor) of one fibrin molecule with a carboxyl group (acceptor) of another fibrin molecule. Work in the late 1960's and early 1970's (Folk and Chung, 1973 and Folk and Finlayson, 1977) showed that factor XIIIa was a transglutaminase and elucidated the mode of action of the enzyme (Chapter 3.IV.A). Lorand and Konishi (1964) proposed that the circulating factor XIII in plasma was inactive and that thrombin and calcium ions were required for activation. This was confirmed by later work and the mechanism of activation is now well documented (Alami *et al.* 1968 and Lorand *et al.* 1980).

In the late 1960's and early 1970's factor XIII was found in platelets (Kiesselbach and Wagner, 1966), the placenta (Bohn and Schwick, 1971) and the uterus (Chung, 1972) as well as in plasma. This wider distribution of factor XIII, together with the observation of wound healing abnormalities in some factor XIII deficient patients (Duckert *et al.* 1960 and Beck *et al.* 1961) and the discovery that factor XIII cross-links several proteins other than fibrin (Chapter 3.IV), has led to factor XIII being considered as an enzyme important in the wound healing process rather than a specific blood coagulation factor.

II. THE STRUCTURE OF FACTOR XIII

Factor XIII has been shown to be present in plasma, platelets, placenta, the uterus, the prostate gland, liver and the spleen (Chung, 1972 and Ikematsu, 1981). Other transglutaminases are present in some of these tissues as well as other body tissues. These transglutaminases are distinct from factor XIII as judged by their physical, chemical, immunological and enzymatic properties (Chung, 1972).

A. The origin of Factor XIII

The tissue forms of factor XIII appear to be synthesised in the tissues where they are found (Ikematsu, 1981). However, the origin of the platelet and plasma forms was for some years a subject of debate. In the early 1970's a number of studies (Kiesselbach and Wagner, 1972; Loewy, 1972 and McDonagh and Wagner, 1972) showed that platelet factor XIII is synthesised in the megakaryocytes of the bone marrow. These studies also suggested that the platelet was not a major source of the plasma form as had previously been thought (Hampton *et al.* 1972).

Although a high correlation between platelet count and plasma factor XIII activity has been reported in animal models (Rider *et al.* 1978) other studies have demonstrated that liver hepatocytes synthesise both subunits of the plasma zymogen (see below) and that they are apparently the sole source of the B subunit of plasma factor XIII (Lee and Chung, 1976; Ikematsu, 1981 and Rasche, 1982), as had been suggested previously (Bohn, 1972).

It therefore appears that the B subunits of plasma factor XIII are produced in the liver, while the A subunits may be synthesised by a number of sources (Ikematsu, 1981).

B. The structure of plasma Factor XIII

In 1961, Loewy *et al.* reported that plasma factor XIII was a trimer of 300,000-350,000 M.W. with each subunit being 110,000 M.W. Further studies in the 1960's and early 1970's gave conflicting results, for example, Lorand *et al.* (1968) reported a total molecular weight of 160,000 and Bannerjee *et al.* (1972) reported subunit weights of 70,000 M.W. and 35,000 M.W. At this time Schwartz *et al.* (1971a and 1973) utilised SDS polyacrylamide gel electrophoresis and sedimentation equilibrium in the ultracentrifuge to produce a model for the structure of plasma factor XIII which is generally accepted as being correct.

Plasma factor XIII consists of two different subunits, designated A and B (Schwartz *et al.* 1971a) of 75,000 and 88,000 M.W. respectively. These subunits form a tetramer of A_2B_2 of 320,000$\pm$20,000 M.W. which is joined by noncovalent bonds. Schwartz *et al.* (1971a and 1973) also clearly demonstrated that the enzymatic activity of plasma factor XIII was found only in the A subunits. Although this model has gained general acceptance Lorand *et al.* (1980) proposed that the most common circulating form in plasma is a heterodimer, AB, of 160,000 M.W. The results of other workers (Folk and Finlayson, 1977) and genetic analysis of the two subunits (Board, 1979 and 1980) do not agree with this proposal and confirm that plasma factor XIII consists of a dimer of two A subunits joined to two B subunits.

Bohn *et al.* (1973) reported that there was an excess of B subunits over A subunits in plasma, thus suggesting that some B subunits are not combined with A subunits. The finding that B subunits are synthesised at a greater rate than A subunits (Lee and Chung, 1976) gave a possible explanation to this observation. In contrast, studies by McDonagh (1982) have suggested that no excess of B subunits are present in plasma.

C. The structure of other Factor XIII's

Before the existence of two different subunits in plasma factor XIII was shown, the relationship of factor XIII from other sources to the plasma form, was uncertain (Bohn, 1970; Bohn and Schwick, 1971; Folk and Chung, 1973 and Folk and Finlayson, 1977). However, with the reports of A and B subunits in plasma factor XIII it soon became apparent that factor XIII from platelets, placenta, uterus, prostate gland and spleen consisted of a dimer of two A subunits of 146,000± 10,000 M.W. (Schwartz *et al.* 1971a and 1973; Chung, 1972 and Ikematsu, 1981). The identity of the two subunits with the A subunit of plasma was confirmed by a variety of studies (Folk and Chung, 1973 and Folk and Finlayson, 1977).

D. The primary structure of the A and B subunits

The amino acid composition of the A subunit from plasma, platelet and placenta is identical, while the amino acid composition of the B subunit is different from that of the A subunits (Table 3.2 and Schwartz *et al.* 1971a and 1973 and Bohn, 1972). The complete amino acid sequence of both subunits is unknown, although the amino-terminal 42 amino acids of the A subunit have been sequenced (Takagi and Doolittle, 1974). Chung *et al.* (1974) have shown that while the A subunit contains six sulphydryl groups and no intrachain disulphide bonds the B subunit contains no sulphydryl groups and sixteen or seventeen disulphide bonds. A free sulphydryl group next to a glutamine residue is essential for the enzymatic activity of the A subunit (Alami *et al.* 1968; Holbrook *et al.* 1973 and Chung *et al.* 1974). This active site is located in a 56,000 M.W. thrombin fragment of the A subunit (Folk and Finlayson, 1977).

Schwartz *et al.* (1971a) reported that only the B subunit contained carbohydrate moieties. However, Bohn (1972) demonstrated that, although

TABLE 3.2.

THE AMINO ACID COMPOSITION OF FACTOR XIII

Amino Acid	Platelet[a,b,c] Factor XIII	Placenta[b] Factor XIII	Plasma Factor XIII[a,c] A Subunit	B Subunit
	Residues/100 Residues			
Lysine	5.6	5.2	5.8	7.4
Histidine	1.9	1.9	2.0	2.6
Arginine	6.1	6.2	5.8	4.4
Aspartic acid	12.7	12.3	12.9	9.2
Threonine	6.6	6.3	6.3	7.2
Serine	7.0	6.3	7.5	7.6
Glutamic acid	11.6	11.1	12.3	13.8
Proline	5.2	4.9	5.4	7.8
Glycine	8.1	7.1	9.4	8.5
Alanine	6.1	5.3	6.3	3.6
Valine	8.5	9.8	7.8	3.9
Half-cysteine	0.9	N.D.	0.8	5.0
Methionine	2.6	2.6	1.9	1.3
Isoleucine	4.3	5.1	3.7	3.7
Leucine	7.0	6.8	7.2	8.4
Tyrosine	3.9	4.4	3.7	6.5
Phenylalanine	4.3	4.7	4.2	3.5
Tryptophan	N.D.	N.D.	N.D.	N.D.

N.D. - Not determined

[a] Schwartz *et al.* 1971a

[b] Bohn, 1972

[c] Schwartz *et al.* 1973

the B subunit contains more carbohydrate groups, some are present on the A subunit (Table 3.3). The carbohydrate groups are not essential for enzymatic activity (Bohn, 1972).

Microheterogeneity of both A and B subunits has been reported (Bohn, 1970; Schwartz *et al.* 1973; Barbui *et al.* 1974; Curtis 1974; Credo *et al.* 1977 and Lorand *et al.* 1980). The role of genetic variation in the causation of this heterogeneity is discussed in Chapter 4.

E. The interaction of A and B subunits

The exact control mechanism of the levels of subunits in plasma has been of interest since the observation that the level of B subunit is reduced in patients who are deficient in or have reduced levels of the enzymatically active A subunit (Barbui *et al.* 1978). An explanation of the concomitant reduction of B subunit levels has resulted from the study of the effect on B subunit levels of the administration of A subunits to patients deficient in A subunits, and the levels of B subunits after an operation (Ikematsu, 1981; Rodeghiero *et al.* 1981a and McDonagh, 1982). It appears that the level of A subunits exerts a positive feedback on the level of B subunits, so that as the concentration of A subunits rises the liver is stimulated to either release stored B subunits or increase the rate of synthesis of B subunits.

F. The role of B subunits

The enzymatic activity of factor XIII is present in the A subunit, therefore the presence of the B subunits in the plasma molecule has led to speculation about the role that the B subunit has *in vivo*. It has been suggested that it promotes the secretion of factor XIII from the liver (Schwartz *et al.* 1971a), but no supportative evidence exists. Several workers have demonstrated that the B subunit must disassociate from

TABLE 3.3.

THE CARBOHYDRATE CONTENT OF FACTOR XIII (Bohn, 1972)

Carbohydrate Group	Plasma[a] Factor XIII %	Platelet[b] Factor XIII %	Placenta[b] Factor XIII %
Hexose	1.9	1.2	0.98
Fucose	0.2	0.0	0.0
Acetylhexosamine	1.6	0.16	0.28
Sialic acid	1.2	0.15	0.21
Total	4.9	1.5	1.47

[a] A and B subunits

[b] A subunits only

the A subunit for the active site to be exposed (Chung *et al.* 1974; Cooke, 1974; Lorand *et al.* 1974 and Seelig and Folk, 1980). Therefore, the B subunits could control the rate of activation of the zymogen by thrombin (Folk and Finlayson, 1977) or protect the active site. The B subunit stabilises the A subunits in plasma by extending their halflife (Lee and Chung, 1976), possibly by decreasing the rate of proteolytic degradation (Seelig and Folk, 1980), although it does not decrease plasmin digestion of the A subunit (Rider and McDonagh, 1981). It is also possible that the B subunits act as transport proteins (Losowsky and Miloszewski, 1977).

III. ACTIVATION OF THE FACTOR XIII ZYMOGEN

The scheme for the activation mechanism of the factor XIII zymogen is outlined in Figure 3.1. The mechanism is different for the plasma zymogen compared to the other zymogens because of the presence of B subunits (Fig. 3.1a), but in all cases it is basically a two step process. The first step is a proteolytic cleavage event and the second is a conformational alteration.

A. The thrombin catalysed proteolytic cleavage of the A subunit

Studies in the 1960's indicated that factor XIII could be activated by thrombin, as well as several other proteases, in the presence of calcium ions (Ca^{2+}) (Folk and Chung, 1973). However, Tyler (1970) demonstrated that the thrombin catalysed proteolysis of factor XIII did not require Ca^{2+}, although Ca^{2+} were required for factor XIIIa catalytic activity. Schwartz *et al.* (1971a) utilised SDS polyacrylamide gel electrophoresis to clearly show that thrombin removed a 4000 M.W. peptide from the A subunit. Takagi and Doolittle (1974) sequenced this 36 amino acid peptide and showed that it was the amino-terminal 36 amino acids of the A chain. Factor XIII is cleaved by thrombin at an arginyl-glycine bond after the sequence Val-Pro-Arg (Takagi and Doolittle, 1974). Several studies have confirmed the report of Schwartz *et al.* (1971a) that no change occurs in the B subunit (Folk and Finlayson, 1977).

Several other proteases have been shown to catalyse the cleavage of A subunits to A' subunits; trypsin and crude papain and reptilase (Schwartz *et al.* 1973), venom from *Agkistrodon rhodostoma*, *Bitis gabonica*, *Bothrops jararaca* and *Bothrops atrox* (Schwartz *et al.* 1973 and Folk and Finlayson, 1977). Plasmin (Folk and Finlayson, 1977) and Factor Xa (McDonagh and McDonagh, 1975) have also been reported

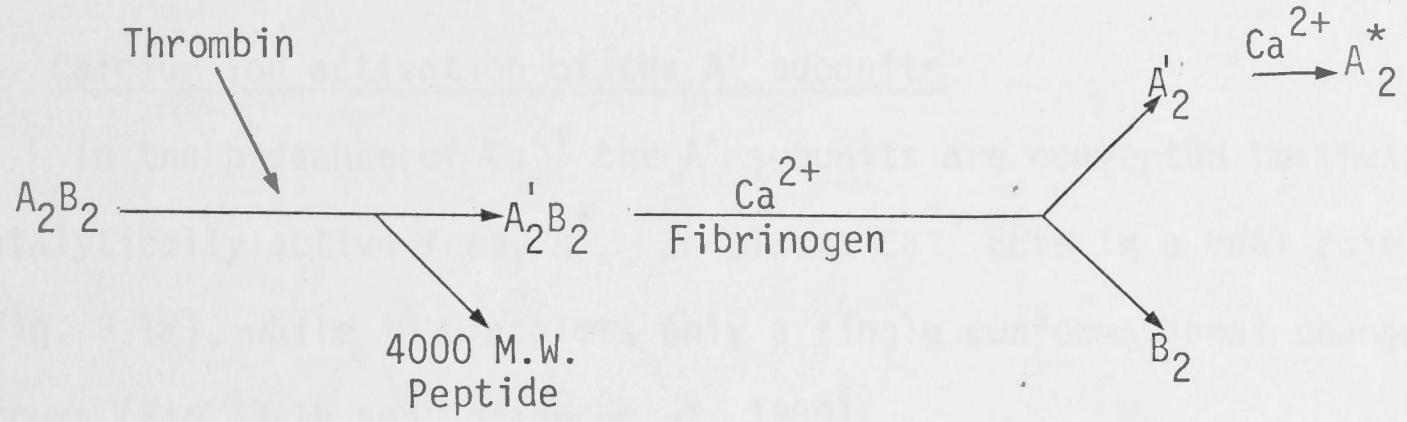
FIGURE 3.1.

THE ACTIVATION OF THE FACTOR XIII ZYMOGEN

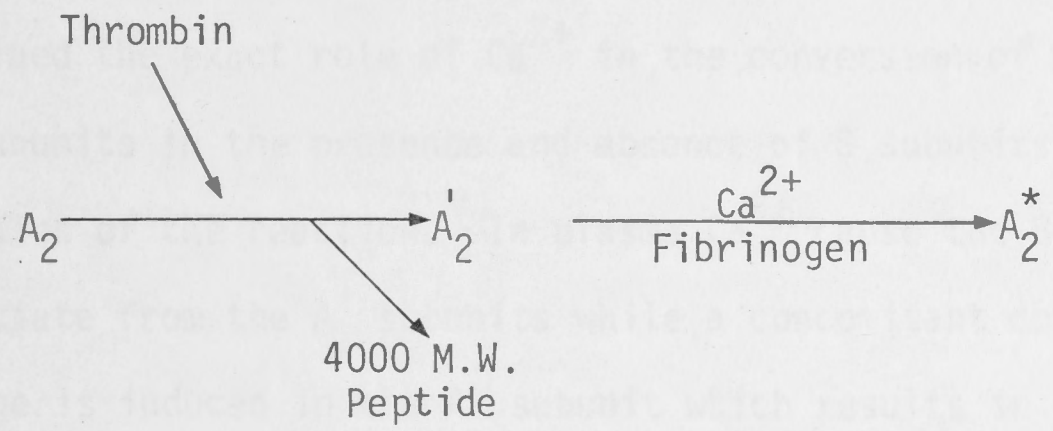
a) The plasma zymogen; b) the platelet zymogen

A' - thrombin cleaved A subunit; A^* - enzymatically active A subunit

a)



b)



to activate factor XIII, but these results have not been confirmed by others (Schwartz *et al.* 1973; Folk and Finlayson, 1977 and Rider and McDonagh, 1981).

The A' subunits show no enzymatic activity in the absence of Ca^{2+} (Lorand *et al.* 1980) and remain associated with the B subunits in plasma (Schwartz *et al.* 1973), but the proteolytic cleavage is an essential first step in the activation process (Chung *et al.* 1974)

B. Calcium ion activation of the A' subunits

In the presence of Ca^{2+} the A' subunits are converted to their catalytically active form, A^*. In plasma Ca^{2+} acts in a dual role (Fig. 3.1a), while in platelets only a single conformational change occurs (Fig. 3.1b and Lorand *et al.* 1980).

A series of studies in the early 1970's (Chung and Folk, 1972; Chung *et al.* 1974; Cooke, 1974; Cooke and Holbrook, 1974; Curtis *et al.* 1974; Lorand *et al.* 1974 and Folk and Finlayson, 1977) examined the exact role of Ca^{2+} in the conversion of A' subunits to A^* subunits in the presence and absence of B subunits, as well as the kinetics of the reaction. In plasma Ca^{2+} cause the B subunits to dissociate from the A' subunits while a concomitant conformational change is induced in the A' subunit which results in the exposure of the active site (Lorand *et al.* 1980). Platelet factor XIII also requires the presence of Ca^{2+} for the conformational change which results in the exposure of the active site and the formation of factor XIIIa. However, the appearance of enzymatic activity is more rapid in platelets, thus suggesting that, in plasma, the disassociation of the tetramer, A'_2B_2, is the rate determining step (Lorand *et al.* 1980).

The published Ca^{2+} concentrations required for activation *in vitro* were generally higher than the plasma Ca^{2+} levels (Chung and Folk, 1972; Lorand *et al.* 1974; Folk and Finlayson, 1977 and Lorand *et al.* 1980)

which suggested that another factor could be involved in the activation process *in vivo*. Credo *et al.* (1978) demonstrated that the presence of fibrinogen reduced the Ca^{2+} requirement to physiological levels. Further studies showed that a specific domain on the α-chain of fibrinogen was responsible for the reduced Ca^{2+} requirement (Lorand *et al.* 1980). The probable *in vivo* importance of this interaction is supported by results that suggest that factor XIII circulates in plasma bound to fibrinogen by the A subunits (Greenberg and Shuman, 1981 and 1982). However, previous studies have failed to detect any complex between fibrinogen and factor XIII (Folk and Chung, 1973).

C. Summary

Factor XIII is activated to factor XIIIa in a precise sequential manner in the presence of thrombin, Ca^{2+} and fibrinogen (Fig. 3.1 and Lorand *et al.* 1980). Nelson and Lerner (1978) have reported the presence of 1-1.5% factor XIIIa in plasma. The importance of this observation is uncertain. The report that in platelets factor XIIIa is the usual form (Tsukada, 1982) is in contradiction to previous results (Nelson and Lerner, 1978) and needs to be confirmed.

IV. BIOLOGICAL REACTIONS OF FACTOR XIII

A. The chemical reaction

Factor XIIIa, as do all transglutaminases, catalyses a Ca^{2+}-dependent acyl-transfer reaction in which γ-carboxamide groups of peptide-bound glutamine residues are the acyl donors. Primary amino groups of lysyl residues of a variety of compounds function as acyl acceptors. The result is the formation of γ-glutamyl-ε-lysyl bonds between two proteins (Figure 3.2; Folk and Chung, 1973 and Folk and Finlayson, 1977). The studies which demonstrated that the formation of the γ-glutamyl-ε-lysyl bond is catalysed by transglutaminases and which analysed the properties of the bond, have been reviewed elsewhere (Folk and Chung, 1973; Finlayson, 1974; Folk and Finlayson, 1977 and Chapter 3.IV.C).

B. The mechanism of reaction

A scheme for the mechanism of the formation of γ-glutamyl-ε-lysyl bonds is illustrated in Figure 3.3. In this scheme initial velocity and product inhibition results define glutamine as, A (the first substrate to add to enzyme) and ammonia as, P (the first product released). The acyl-enzyme intermediate, F, may react with water to form glutamic acid, R, or with the primary amino group of lysine, B, to form the γ-glutamyl-ε-lysyl crosslink, Q (Folk, 1969; Chung *et al*. 1970; Chung and Folk, 1972; Folk and Chung, 1973 and Folk and Finlayson, 1977). Comprehensive kinetic studies of factor XIIIa, which used the acetylated B chain of oxidised insulin and methylamine as substrates, conformed to the above mechanism (Chung and Folk, 1972).

FIGURE 3.2.

THE FORMATION OF THE γ-GLUTAMYL-ε-LYSYL BOND BY FACTOR XIIIa

(adapted from Losowsky and Miloszewski, 1977)

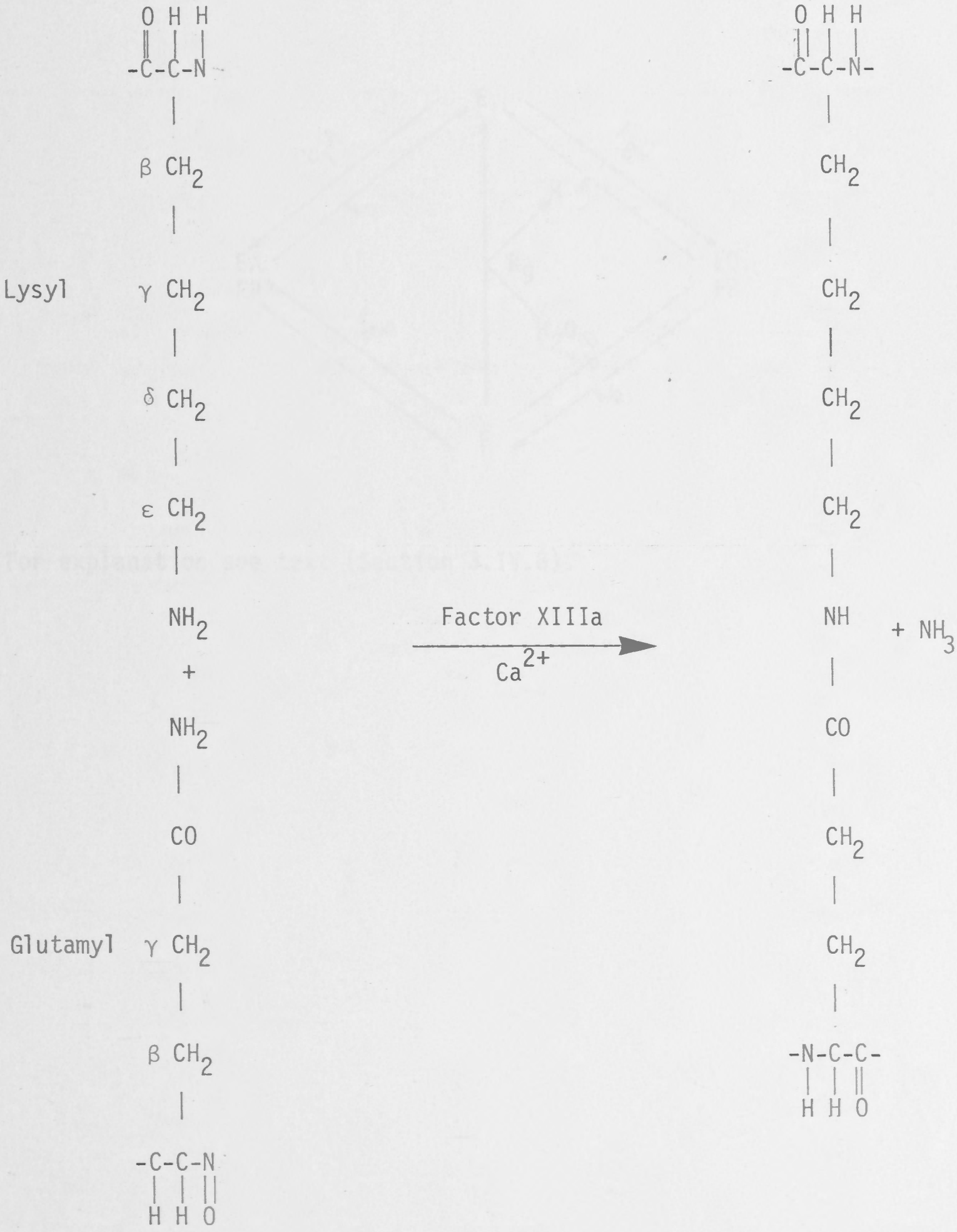
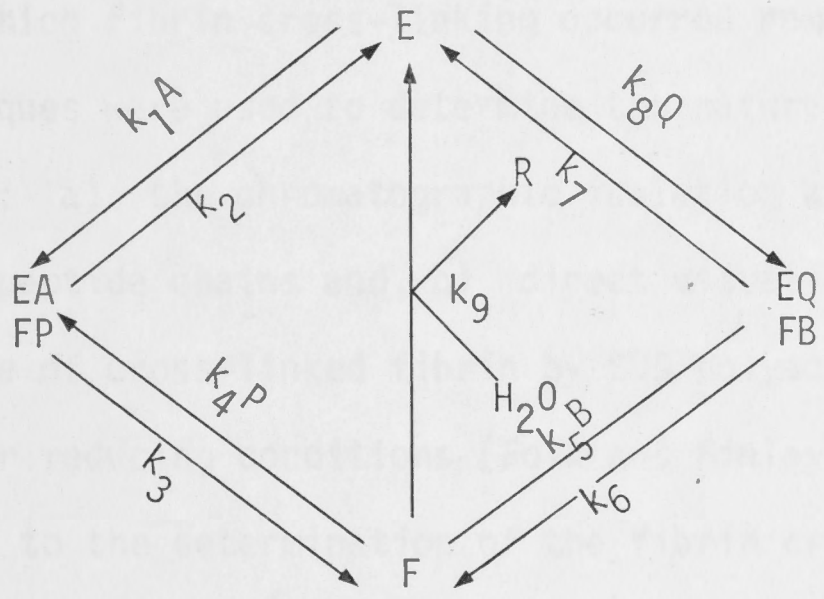


FIGURE 3.3.

THE MECHANISM OF THE FACTOR XIIIa CATALYSED REACTION

(from Chung and Folk, 1972)



For explanation see text (Section 3.IV.B).

C. Fibrin cross-linking

In the course of blood coagulation in mammals fibrin, formed from fibrinogen by the catalytic action of thrombin (reviewed by Doolittle, 1981), is converted to an insoluble polymeric lattice. By the late 1960's the role of factor XIIIa in catalysing this polymerisation had been established (Duckert *et al.* 1960 and Alami *et al.* 1968). However, the process by which fibrin cross-linking occurred remained uncertain. Two basic techniques were used to determine the nature of the cross-linking reaction: a) the chromatographic isolation and subsequent analysis of polypeptide chains and, b) direct visualisation of the subunit structure of cross-linked fibrin by SDS polyacrylamide electrophoresis under reducing conditions (Folk and Finlayson, 1977). The studies that led to the determination of the fibrin cross-linking process have been extensively reviewed elsewhere (Folk and Chung, 1973; Finlayson, 1974 and Folk and Finlayson, 1977) and will only be summarised here.

Mammalian fibrin is composed of two each of α-, β- and γ-chains (Doolittle, 1981). It was generally presumed that factor XIIIa produced insoluble fibrin by cross-linking through the formation of γ-glutamyl-ε-lysyl bonds between these chains (Lorand *et al.* 1968; Matcić and Loewy, 1968 and Pisano *et al.* 1968). The first definitive evidence of cross-links between fibrin monomers came from the results of Chen and Doolittle (1969 and 1970) and Takagi and Iwanaga (1970), but which chains were cross-linked was uncertain. McKee *et al.* (1970) utilised the SDS polyacrylamide gel system to show that γ-chain dimers were rapidly formed, followed by a slower cross-linkage of the α-chains. β-chains were not involved in fibrin cross-linking. Final proof of the occurrence of cross-links between γ-chains to form dimers and α-chains to form insoluble polymers was provided by Chen

and Doolittle (1971) and McDonagh *et al.* (1971b).

Pisano *et al.* (1971 and 1972) demonstrated that the cross-links catalysed by factor XIIIa were γ-glutamyl-ε-lysyl bonds. In clots formed in normal plasma there are six cross-links per mole of fibrin, four are between α-chains and two are between γ-chains, whereas in factor XIII deficient plasma little or no cross-links are found in clots. With the identification of the γ-glutamyl-ε-lysyl bonds the position of the cross-links in the γ-chains and the antiparallel arrangement of the γ-chains in the dimer was soon established (Chen and Doolittle, 1971; McDonagh *et al.* 1972 and Sharp *et al.* 1972). The intermolecular nature of the γ-chain cross-links was confirmed by Moroi *et al.* (1975).

Factor XIIIa, *in vitro*, catalyses the formation of γ-glutamyl-ε-lysyl bonds between two γ-chains and between the α-chains of fibrin and thus cross-links fibrin molecules to form an insoluble lattice. Factor XIIIa from a variety of sources forms identical patterns of cross-linkage (Schwartz *et al.* 1971a; Chung, 1972; Folk and Chung, 1973 and Schwartz *et al.* 1973). The *in vivo* situation appears to reflect the *in vitro* results although the level of α-chain cross-linking may be less (Pisano *et al.* 1972 and Folk and Finlayson, 1977). This could be because not all the sites are available due to the cross-linking of other proteins to fibrin (Chapter 3.IV.D) or because of the reported factor XIIIa inhibitory activity of plasma (de Cataldo and Baudo, 1972).

Factor XIIIa also cross-links fibrinogen, but at a slower rate, apparently due to a shift from zero order to first order kinetics (Chung, 1972 and Kanaide and Shainoff, 1975). The *in vivo* significance of this reaction is uncertain (Folk and Finlayson, 1977) as it is unlikely that FXIIIa would be generated without the concomitant

conversion of fibrinogen to fibrin.

D. Interaction with other proteins

Factor XIIIa has been shown to catalyse the formation of γ-glutamyl-ε-lysyl bonds between fibronectin molecules (Mosher, 1975 and Keski-Oja *et al.* 1976), plasma fibronectin and α-chains of fibrin (Mosher, 1975; Mosher, 1976a and Tamaki and Aoki, 1981), collagen and γ-chains of fibrin (Nyman and Duckert, 1975), fibronectin and collagen (Mosher *et al.* 1979 and 1980), α-chains of fibrin and α_2-plasmin inhibitor (Sakata and Aoki, 1980 and Tamaki and Aoki, 1981), between myosin molecules (Cohen *et al.* 1979), between actin molecules (Cohen *et al.* 1980, but see Mui and Ganguly 1977), between dansylcadaverine and α_2-macroglobulin (Mosher, 1976a and Wierzbicki and Mosher, 1982), between fibronectin and *Staphylococcus aureus* (Mosher and Procter, 1980) and between monodansylcadaverine and a 110,000 M.W. protein (Mosher, 1976a) which could be a subunit of factor VIII related antigen (Folk and Finlayson, 1977 and Hada *et al.* 1982).

The biological importance of these interactions is discussed in Chapter 3.V, as well as in Chapter 1 (for the fibronectin interactions).

V. BIOLOGICAL FUNCTIONS OF FACTOR XIII

A. Haemostasis

The ability of the circulation system to maintain blood fluid within the vessels and to prevent excessive blood loss upon injury, is defined as haemostasis (Murano, 1980). The importance of factor XIII in the blood coagulation system was shown in 1960 by the effects of the absence of factor XIII (Duckert *et al.* 1960 and Chapter 3.VI). Basically, the blood coagulation process is the formation of an insoluble fibrin clot at the site of injury. As described above (Chapter 3.IV.C), factor XIII is responsible for the final step in this process, the covalent cross-linking of fibrin molecules by the formation of inter-chain γ-glutamyl-ε-lysyl bonds. Although the presence of factor XIII in the circulation system is essential for normal haemostasis (Duckert *et al.* 1960 and Duckert, 1972), the formation of a cross-linked fibrin clot does not appear to account for the effects of its absence on the haemostatic process (Folk and Finlayson, 1977).

Studies in the late 1970's and 1980's (Chapter 3.IV.D) have shown that factor XIII probably cross-links several other haemostatically important proteins into the clot and is also probably involved in clot retraction by platelets (see below).

1. Clot stability

a) mechanical: Duckert (1972) proposed that the cross-linking of fibrin could promote haemostasis by producing a mechanically stronger and more rigid clot. Cross-linked fibrin has a greater rigidity than non-cross-linked fibrin, although the differences are small (Mockros *et al.* 1974 and Roberts *et al.* 1974). It has been suggested that this is due to the prevention of slippage of fibrin monomers under stress (Gerth *et al.* 1974). Cross-linked fibrin also has an increased

elastic modulus; this increase is dependent on α-chain cross-linking (Shen *et al.* 1974 and 1975). These results are supported by the thrombo-elastogram of factor XIII deficient patients being subnormal and decreasing at a greater rate than normal (Duckert, 1960 and Folk and Finlayson, 1977). It is, therefore, possible to imagine a weaker, non-cross-linked clot, buffeted by circulating forces, being distorted, dismembered or dislodged (Hartert, 1974 and Folk and Finlayson, 1977).

Additional support for this hypothesis is provided by the fact that bleeding in factor XIII deficient patients normally occurs 24-36 hours after injury (Duckert, 1972) and that α-chain cross-linking which provides rigidity occurs at a later stage (Chapter 3.IV.C). However, only 1-5% of normal plasma factor XIII levels provide haemostatic protection (Folk and Finlayson, 1977) whereas considerably higher levels are required for α-chain cross-linking over a shorter period *in vitro* (Pisano *et al.* 1972).

The observation that fibronectin is covalently crosslinked to fibrin by factor XIII at a similar rate to that at which fibrin α-chain cross-linking occurs (Mosher, 1975; Tamaki and Aoki, 1981 and Chapter 1.III.E) offers a further mechanism by which the clot can be anchored and stabilised. The fibronectin could then bind to and be cross-linked to the surrounding collagen matrix (the molecular interactions of fibronectin are discussed in Chapter 1.III). Factor XIIIa has been reported to mediate fibronectin-collagen cross-linking (Mosher *et al.* 1979 and 1980). Also the clot may be stabilised by the possible formation of direct fibrin-collagen cross-links (Nyman and Duckert, 1975).

b) solubility: The presence of factor XIII in plasma was originally determined by its ability to render fibrin clots insoluble in a variety of reagents (Alami *et al.* 1968 and Lorand, 1972). The insolubility of

cross-linked fibrin clots in 2% acetic acid is dependent on γ-chain cross-links (Schwartz *et al.* 1971b and Moroi *et al.* 1975), while in 1% monochloroacetic acid α-chain cross-links are also required (Scully 1976). The biological significance of these solubility tests is uncertain apart from the indication that cross-linked clots are more stable.

c) proteolysis: *In vivo* fibrinolysis of the clot is catalysed by plasmin. The two major natural inhibitors of plasmin are α_2-macroglobulin and α_2-plasmin inhibitor (Hedner, 1973 and Aoki, 1979), but only α_2-plasmin inhibitor (α_2PI) prevents clot proteolysis (Aoki, 1979). Schwartz *et al.* (1973) and Gaffney and Brasher (1973) suggested that extensive fibrin α-chain cross-linking substantially increased clot lysis time. These reports, combined with the results of several other studies, led to the general assumption that α-chain cross-linking by factor XIIIa made fibrin less susceptible to fibrinolytic enzymes (Lorand and Jacobsen, 1962; Gormsen *et al.* 1967; Henderson and Nussbaum, 1969; McDonagh *et al.* 1971a; Gormsen and Federsen, 1972 and Gaffney and Whitaker, 1979). However, studies which utilised purified proteins rather than plasma, failed to show any difference in lysis rates between cross-linked and non-cross-linked fibrin clots (Haverkate, 1975 and Rampling, 1978).

These anomalies are apparently explained by the observation that factor XIII catalyses the rapid covalent incorporation of α_2-PI into the fibrin clot (Sakata and Aoki, 1980 and Tamaki and Aoki, 1981) and the fact that α_2-PI levels are lower in serum than plasma (Sakata and Aoki, 1980). Therefore, the reduced susceptibility to proteolysis of fibrin clots formed in the presence of factor XIIIa *in vivo* and *in vitro* is probably due to the covalent attachment of α_2-PI to the clot rather than the formation of fibrin cross-links (Ichinose and Aoki, 1982 and Sakata

and Aoki, 1982).

2. Clot retraction

Kiesselbach and Wagner (1972) showed that platelet factor XIII, which comprises 30-50% of factor XIII in blood, is not needed for the formation of the fibrin clot. The physiological function of platelet factor XIII was, therefore, unknown. The interaction of fibrin, myosin and actin from platelets is an important process in clot retraction (Cohen and de Vries, 1973 and Cohen *et al.* 1975). As the association of these proteins occurs in the presence of platelet factor XIII (Lopaciuk *et al.* 1976), it is possible that the formation of cross-linkages between them may stabilise the interactions. Cohen *et al.* (1979 and 1980) have shown that *in vitro* factor XIIIa does catalyse the cross-linking of actin and of myosin (light and heavy chains). In addition calmodulin reduces the Ca^{2+} requirement to levels obtainable in the platelet cytosol (Cohen and Kahn, 1981).

It is, therefore, hypothesised that the factor XIIIa catalysed cross-linking of fibrin, actin and myosin plays an important role in stabilising the irreversibly contracted gel mass seen in platelets following the release reaction and during clot retraction. Adenosine triphosphate (ATP) may have a regulatory role in this process (Cohen *et al.* 1980). The observation that platelets in factor XIII deficient plasma fail to develop tension and long pseudopods supports this hypothesis (Gerrard *et al.* 1981).

3. Others

The above discussion involves the role of factor XIII in the later stages of haemostasis. A possible early role could be in the process of local clotting at the site of injury if the ability of factor XIIIa to irreversibly cross-link fibrin (Ly *et al.* 1974 and Kanaide and Shainoff, 1975) reduces the concentration of fibrin monomer necessary

for gel formation (Shen *et al.* 1975).

The physiological importance of the factor XIIIa, cofactor enhanced, incorporation of primary amines into the protease inhibitor, α_2-macroglobulin, is uncertain but it is possible that factor XIIIa could irreversibly bind proteases produced during blood coagulation to α_2-macroglobulin and thus aid in their inactivation (Mosher 1976a and Wierzbicki and Mosher, 1982).

B. Wound healing

Approximately 20-25% of factor XIII deficient patients show abnormal wound healing processes (Duckert, 1972), which suggest that factor XIII is involved in these processes. However, why 75-80% of deficient patients have apparently normal wound healing is difficult to explain. Studies which have utilised animal models, as well as some *in vitro* studies support the idea that factor XIII is necessary for normal wound healing (Beck *et al.* 1961; Marktl and Rudas, 1974; Folk and Finlayson, 1977; Bruhn, 1981 and Chung *et al.* 1982). The observation that plasma factor XIII levels are reduced to 40% of normal at a time after major surgery when fibroblast activity would be expected also suggests that factor XIII is used in the wound healing process (Letheby *et al.* 1974).

The likelyrole of factor XIII in wound healing became apparent from the ability of factor XIIIa to cross-link proteins other than fibrin. The importance of the factor XIIIa catalysed cross-linking of fibronectin has been discussed elsewhere (Chapter 1.III.E and see Bowness, 1982). In addition the possibility that wound strength is increased by the factor XIIIa mediated polymerisation of collagen may be important (Nyman and Duckert, 1975 and Soria *et al.* 1975). Through its ability to cross-link a number of proteins that are vital to the wound healing process, factor XIII appears to play an important role in normal wound

healing.

C. Pregnancy

The level of factor XIII in plasma falls steadily throughout pregnancy to a level 50-60% of normal, possibly to counterbalance an increased rate of fibrin formation and thus protect against thrombo-embolic complications (Coopland *et al.* 1969 and Ebert *et al.* 1981, but see Padar 1972). However, it is possible that the reduced levels represent an increased consumption of factor XIII during pregnancy since factor XIII is known to bind to fibrin (Folk and Chung, 1975).

As pregnant women deficient in factor XIII abort spontaneously unless they receive adequate replacement therapy (Lorand *et al.* 1980), it is apparent that factor XIII activity is required for a normal pregnancy. Factor XIII is present in the plasma, placenta and uterus and is absent from all three sites in deficient patients, therefore it is uncertain in which site the deficiency is important. The fact that plasma therapy enables pregnancy to reach term does not mean that factor XIII in the placenta and uterus is not important in normal pregnancies (Folk and Finlayson, 1977).

One role of factor XIII in pregnancy could be to cross-link fibronectin from the amniotic fluid (Chen *et al.* 1978) or from the plasma with a large amount of fibrin from the maternal blood in the placental basement membrane so that a safe and effective anatomical barrier exists between the mother and foetus (Folk and Finlayson, 1977). A second role for factor XIII is suggested by the ability of factor XIIIa to cross-link actin and myosin in muscle (Cohen *et al.* 1979 and 1980). It is possible that, as hypothesised by Alving and Laki (1967), the uterine factor XIIIa serves as a catalyst for the connection of muscle proteins into a contractile network necessary for delivery.

Recent investigations into the mechanisms by which the embryo is protected against rejection by the mother's immune system during its first days in the uterus have suggested a role for factor XIII (Mukherjee *et al.* 1981). It appears that factor XIII cross-links uteroglobulin, a protein synthesised by the uterus, to transplantation antigens on the embryonic surface, thereby hiding them from the mother's immune system. As mentioned previously the fact that women deficient in factor XIII are protected from spontaneous abortions by replacement therapy suggests that the presence of factor XIII in the blood system is sufficient for the process described above to occur.

D. The mononuclear phagocytic system

The interaction of factor XIII with *Staphylococcus aureus* and fibronectin and its possible role in the mononuclear phagocytic system has been discussed in Chapter 1.III.F.

VI. FACTOR XIII IN DISEASE

A. Factor XIII deficiency

Lorand *et al.* (1980) in an extensive review have classified factor XIII deficiency into three groups: I. Hereditary absence of factor XIII; II. Acquired inhibitors of factor XIII and III. Lowered levels of factor XIII in association with another disease state. In group I factor XIII is deficient in all body tissues and fluid, whereas, in II and III only the plasma levels are reduced. Each group will be considered separately here.

1. Congenital factor XIII deficiency

In 1960, Duckert *et al.* reported the first case of a bleeding disorder due to a congenital deficiency of factor XIII. Since that initial report approximately 100 further cases have been diagnosed (Lorand *et al.* 1980). The clinical manifestations have been comprehensively reviewed (Britten, 1967; Alami *et al.* 1968; Duckert, 1972; Losowsky and Miloszewski, 1977; Kitchens and Newcomb, 1979; Lorand *et al.* 1980 and Miloszewski and Losowsky, 1982). The characteristic clinical observation is haemorrhage from the umbilical cord within days or weeks of birth (Losowsky and Miloszewski, 1977). Other common clinical findings are listed in Table 3.4.

Although the bleeding diathesis is mild in some reported cases, in the majority it is serious. The life of the patient is marred and put at risk by the recurrent and severe bleeding which occurs 24-36 hours after mild trauma. In early life intracranial haemorrhage is often a cause of death (Lorand *et al.* 1980). However, a long term prophylactic treatment which allows patients to live a normal life, is possible.

Since an initial report of successful treatment (Ottaviani and Mandelli, 1966) factor XIII from a variety of sources has been used

TABLE 3.4. THE COMMON CLINICAL MANIFESTATIONS OF FACTOR XIII DEFICIENCY

Umbilical Haemorrhage

Epistaxis

Intracranial Bleeding

Recurrent Abortion

Haemarthrosis

Intramuscular Bleeding

Abnormal Wound Healing

Superficial Bruising

Subcutaneous Bleeding

in treatment, including fresh frozen plasma and placental concentrates (Britten, 1967; Bohn, 1972 and Losowsky and Miloszewski, 1977). The danger of the development of antibodies has not been reported to be a problem (Losowsky and Miloszewski, 1977) with the possible exception of one patient (Lorand *et al.* 1969a). Because of the relatively long half-life of factor XIII *in vivo*, 10-14 days (Miloszewski and Losowsky, 1970; Ikkala, 1972 and Lorand *et al.* 1980) and the fact that only 1-5% of normal plasma levels achieve haemostasis (Britten, 1967 and Duckert, 1972), replacement therapy is only required once in every 5-6 weeks (Losowsky and Miloszewski, 1977). The readily available prophylactic treatment negates the necessity for antenatal diagnosis and selective abortion in this disorder, as has been suggested (Fried *et al.* 1981).

Congenital factor XIII deficiency is now generally accepted as being an autosomal recessive disease (see Chapter 3.VII. A), and is apparently present in most races (Lorand *et al.* 1980). In the majority of cases plasma factor XIIIa activity is either absent or below 1% of normal (McDonagh, 1982), although levels of up to 1.7% of normal have been reported (Rodeghiero and Barbui, 1980). As detected by immuno-precipitation, which will not detect levels below 5% of normal, A sub-units are absent and B subunits are at concentrations of about 50% of normal (Bohn *et al.* 1973; Israels *et al.* 1973; Barbui *et al.* 1974; Rodeghiero and Barbui, 1980 and Ikematsu, 1981). However, the presence of inactive A subunits has been suggested in some cases (Ukita *et al.* 1976 and Forman *et al.* 1977) as well as the total absence of A and B subunits in one case (Girolami *et al.* 1978). All this data suggests that there is probably genetic heterogeneity of factor XIII deficiency (see Chapter 3.VII.B).

In most heterozygotes factor XIIIa activity and the plasma levels of A and B subunits are between those of deficient homozygotes and

normal controls (Board *et al.* 1980; Ikematsu, 1981 and McDonagh, 1982).

Various procedures have been used for the diagnosis of factor XIII deficiency: Thromboelastography (Duckert *et al.* 1960); clot histology (Alami *et al.* 1968); clot solubility (Loewy *et al.* 1961; Lorand and Konishi, 1964; Barry and Delage, 1965 and Kitchens and Newcomb, 1979); SDS polyacrylamide gel visualisation of fibrin cross-linking (Lorand *et al.* 1969b and Schwartz *et al.* 1971b); amine incorporation rate assays for enzyme activity (Lorand *et al.* 1969a; Dvilansky *et al.* 1970; Lorand and Gotah, 1970; Lorand *et al.* 1971; Lorand *et al.* 1972 and Curtis and Lorand, 1976); fluorescent polarization analysis (Yamada and Meguro, 1977); electrophoresis on N-dimethylcasein agarose (Lorand *et al.* 1979) and the localisation of A subunits on agarose gels, by a fluorescent technique, after high voltage electrophoresis (Board, 1979; Stenberg and Stenflo, 1979; Board *et al.* 1980 and Stenberg *et al.* 1980).

While the enzyme assays offer the most accurate measure of enzyme activity (Lorand *et al.* 1980) they are rather complex for most diagnostic laboratories and it has, therefore, been argued that gel electrophoresis should be routinely used (Stenberg *et al.* 1980). In the case of heterozygote detection, problems arise with the enzyme rate assays because of the wide range of activity in normal subjects (Lorand *et al.* 1980) and the possibility that an unstable A subunit with raised activity would not be detected in combination with a normal A subunit (Castle *et al.* 1981). The preferred method of heterozygote detection would therefore be the electrophoretic system of Board (1979), which enables individuals to be genotyped (see Chapter 3.VII and Chapter 4).

2. Acquired inhibitors

In 1972, Lewis suggested that inhibitors of factor XIII could act in four ways; a) inactivation of the zymogen; b) blockage of activation; c) depression of enzyme activity and d) blockage of fibrin cross-linkage

sites. Evidence exists for the presence of all four types of inhibitors in the few reported cases (Lewis, 1972 and Lorand *et al.* 1980). In most cases the inhibition is associated with drug therapy, in particular with the use of isoniazid in antituberculous therapy (Lewis, 1972). As isoniazid is a potent inhibitor of factor XIIIa this may not appear surprising, but inhibition persists after drug therapy has ceased (Lewis, 1972 and Lorand *et al.* 1980). It has been shown that inhibition is due to the presence of immunoglobin G (IgG) which cross reacts with factor XIII (Lorand *et al.* 1980 and Ikematsu, 1981). The reason why IgG is produced against the factor XIII molecule is not known. Recently Galanikis *et al.* (1982) have demonstrated the presence of inhibitors specific for factor XIIIa in the gas-phase, water-soluble components of cigarette smoke. The possible importance of factor XIIIa inhibition in the pathophysiology of smoking is discussed by these workers.

3. Factor XIII deficiency in other disease states

Reduced levels of factor XIII have been reported in a wide range of conditions; malignant disorders (Lorand *et al.*1980; Zuck *et al.* 1981; Gramse *et al.* 1982 and Matsuda, 1982); anaemias; haemorrhagic disease and renal disease (Lorand *et al.* 1980); uraemia (Nussbaum and Morse, 1964); iron intoxication (Henriksson *et al.* 1979); erosive gastritis (Nilsson *et al.* 1975); cerebral infarction (Matsuda, 1982); arteriosclerosis (Alkjaersig *et al.* 1977); antibiotic-associated pseudomembranous colitis (Kuratsuji *et al.* 1982); disseminated intravascular coagulation (DIC) (Merskey *et al.* 1967; Ikematsu, 1981; Rodeghiero *et al.* 1981b and Matsuda, 1982); Henoch-Schönlein's purpara (Henriksson *et al.* 1975); septicaemia (Holst *et al.* 1981; Egbring *et al.* 1982; Jochum *et al.* 1982 and Matsuda, 1982); liver disease (Lorand *et al.* 1980 and Matsuda, 1982) and after surgery (Letheby *et al.* 1974 and Ikematsu, 1981). The mechanism of reduction in several of the above conditions is now reasonably clear.

Liver disease: As discussed previously, plasma factor XIII is synthesised largely in the liver (Chapter 3.II.A) and evidence from a variety of sources suggests that reduced hepatic synthesis is the cause of decreased levels of plasma factor XIII in some liver disorders (Yavugil *et al.* 1967; Mandel and Gerhold 1969; Walls and Losowsky, 1969; Cucuianu *et al.* 1973 and 1976; Biland *et al.* 1979; Lorand *et al.* 1980 and Matsuda, 1982).

Leukaemia and Septicaemia: Approximately 30% of acute myeloid leukaemia patients (Gramse *et al.* 1982) and 33% of septicaemia patients (Egbring *et al.* 1982) have markedly reduced plasma factor XIII levels. In both cases the cause appears to be direct proteolysis of the molecule, probably by elastase (Holst *et al.* 1981; Gramse *et al.* 1982 and Jochum *et al.* 1982).

Surgery and DIC: The probable reason for the low factor XIII levels in these two conditions is the utilisation and consumption of the molecule in wound healing and the coagulation process respectively (Ikematsu, 1981).

B. Other diseases

Factor XIII and tissue transglutaminases have been implicated in the development of neoplasms (Yancey and Laki, 1972; de Petro *et al.* 1980 and Bruhn, 1981). While factor XIIIa has been shown to prevent focal appearance and cause the reversion to normal cell morphology of Rous-sarcoma virus-induced transformed cells *in vitro* (de Petro *et al.* 1980), it is probable that, *in vivo* tissue transglutaminases are more important as transglutaminase activity is reduced in transformed cells (Birckbichler *et al.* 1977). While Yancey and Laki (1972) suggested that metastasis is inhibited by transglutaminases, Bruhn (1981) claims that tumour cells are stimulated by factor XIII. Therefore, any role for factor XIII in neoplastic development remains uncertain.

It has been suggested that the liberation of thrombin and the subsequent activation of plasma factor XIII in arteriosclerotic lesions could induce the proliferation of cell wall cells and cause intima fibrosis (Bruhn, 1981) rather than the platelet-induced smooth muscle proliferation hypothesis of Ross (1980). However, previous work has suggested that tissue transglutaminases are probably more important than factor XIII in arteriosclerosis (Laki *et al.* 1972).

VII. THE GENETICS OF FACTOR XIII

A. The mode of inheritance of congenital factor XIII deficiency

The initial reports of factor XIII deficiency indicated that the disorder had an autosomal recessive mode of inheritance (Duckert 1960; Barry and Delage, 1965; Lorand, 1965 and Fisher *et al.* 1966), but because of reportedly lower factor XIIIa activity in males (as measured by clot solubility) and the apparent absence of male to male transmission in two families, Hampton *et al.* (1966) suggested an X-linked recessive mode of inheritance. However, further studies demonstrated that the defect in these families was due to the presence of an abnormal fibrinogen, fibrinogen Oklahoma (Hampton, 1968 and 1970 and Hampton and Morton, 1970). Although, in a review of the literature, Britten (1967) showed that the disorder fitted an autosomal recessive model by four recognised criteria, an X-linked model was again proposed because of an excess of affected males (Ratnoff and Steinberg, 1968 and Steinberg and Ratnoff, 1969). By 1971, the male to female ratio in reported cases was closer to unity and further studies of the initial family reported by Duckert had supported the autosomal recessive model (Lorand *et al.* 1970; McDonagh *et al.* 1971c and Duckert 1972). However, those families with only males affected, had lower levels of consanguinity than families with both sexes affected thus suggesting two forms of the disorder with X-linked and autosomal modes of inheritance respectively (Ratnoff and Steinberg, 1972).

In the early 1970's more specific and accurate assays became available for the measurement of factor XIII activity (Chapter 3.VI.A.1). Studies which have utilised these assays have failed to reveal any cases that need to invoke the X-linked model, thus suggesting that congenital factor XIII deficiency is an autosomal recessive disorder (McDonagh *et al.* 1974; Barbui *et al.* 1978 and Kitchens and Newcomb,

1979). However, because of the difficulty in identifying heterozygotes caused by the wide range of activity of factor XIII in normal subjects (Walls and Losowsky, 1969 and Lorand *et al*. 1980) some authors felt that X-linked inheritance could not be ruled out in some cases (Losowsky and Miloszewski, 1977 and Lorand *et al*. 1980).

Board (1979 and 1980) utilised an agarose gel electrophoresis system (see Chapter 4) to demonstrate that genes for both the A and B subunits of factor XIII are located on autosomes. The same technique has shown that a null allele, *FXIIIA*QO* and an allele with an unstable product, *FXIIIA*3* at the A locus are inherited in an autosomal manner (Board *et al*. 1980 and Castle *et al*. 1981). It therefore appears that factor XIII deficiency is an autosomal recessive disorder and is not an X-linked condition (see also Chapter 4).

B. Genetic heterogeneity of factor XIII deficiency

The possibility of genetic heterogeneity of factor XIII deficiency was originally put forward because of the dispute over the mode of inheritance of the disorder (Hampton *et al*. 1972 and Ratnoff and Steinberg, 1972). Later work, which reported unusual immunological results in some deficient cases again suggested the presence of more than one genetic type of the disorder (Ukita *et al*. 1976 and Forman *et al*. 1977). Recently, two different alleles capable of causing factor XIII deficiency have been reported (Board *et al*. 1980; Castle *et al*. 1981 and see Chapter 4).

C. Genetic polymorphism of the A subunit locus

The development of a suitable thin layer agarose electrophoresis system and a specific fluorescent staining technique led to the discovery of a genetically determined polymorphism of the A subunit in Caucasian Australians (Board, 1979). Studies of additional populations have shown the polymorphism to be widely spread, with the frequency of

the different alleles varying between groups (Table 3.5). In addition to the three alleles which code for proteins with normal activity, a null allele and an allele which codes for an unstable protein are present at the A locus (Board *et al* 1980; Castle *et al*. 1981 and Chapter 4).

D. Genetic polymorphism of the B subunit locus

The B subunit of plasma factor XIII has no known enzymatic activity but can be localised after electrophoresis in agarose by immunofixation with specific antiserum (Board, 1980). The screening of several populations has revealed a large degree of genetic heterogeneity (Table 3.6).

Recently the use of isoelectric focussing of plasma samples has been advocated to separate the products of the different alleles (Kera *et al*. 1981). This technique concentrates the protein into very fine zones which enhances their staining intensity and makes the phenotypic band patterns easier to observe. Unfortunately the use of isoelectric-focussing, as described by Kera *et al*. 1981, does not clearly resolve the products of the *FXIIIB*1* and *FXIIIB*2* alleles (Board and Castle, 1982). Despite this fact it is likely that the *FXIIIB*2* allele is rare in the Japanese population studied by Kera *et al*. (1981) since no type 2 B subunits were detected by electrophoresis. However, the new rare variant reported in Japanese (Nakamura and Abe, 1982) is possibly the *FXIIIB*2* allele. The studies of Kreckl and Kühnl (1982) and Kühnl *et al*. (1982) have helped confirm the existence of the *FXIIIB*2* allele.

The phenotypes obtained by electrophoresis and isoelectricfocussing of the different combinations of the *FXIIIB*1*, *FXIIIB*2*, *FXIIIB*3* and *FXIIIB*4* alleles are shown in Figure 3.4. The *FXIIIB*4* allele has only been observed in a single Australian blood donor (Board and Castle, 1982).

TABLE 3.5

FREQUENCIES OF FACTOR XIIIA LOCUS ALLELES WITH FUNCTIONALLY NORMAL PRODUCTS IN VARIOUS POPULATIONS

Population	N	*FXIIIA*1*	*FXIIIA*2*	*FXIIIA*4*	Reference
Australian[†]	383	.795	.205	-	Board (1979)
Europeans[#]	239	.797	.203	-	Kreckl *et al.* (1982)
Japanese[§]	561	.887	.113	-	Nishigaki *et al.* (1981)
Japanese[§]	250	.900	.100	-	Kera and Nishimukai (1982)
Fijians					
Melanesian[§]	137	.783	.209	.008	Board and Coggan (1981)
Indian[§]	137	.768	.232	-	-ditto-
Loyalty Is.					
Melanesian[§]	133	.925	.075	-	-ditto-
Cook Is.					
Polynesian[§]	100	.980	.020	-	-ditto-

[†] Plasma and platelets

[#] Platelets

[§] Plasma

TABLE 3.6.

FREQUENCIES OF FACTOR XIIIB LOCUS ALLELES IN VARIOUS POPULATIONS

Population	N	*FXIIIB*1*	*FXIIIB*2*	*FXIIIB*3*	Reference
Australian	245	.747	.084	.169	Board (1980)
European	236	.725	.095	.180	Kreckl and Kühnl (1982) and Kühnl *et al.* (1982)
Japanese	182	.336	-	.664	Kera *et al.* (1981)
Japanese	310	.252	.013†	.735	Nakamura and Abe (1982)
Japanese	119	.303	-	.697	Nishigaki *et al.* (1982)
Fijian					
Melanesian	127	.61	.02	.37	Board and Castle (1982)
Indian	128	.66	.08	.26	-ditto-

† not reported as *FXIIIB*2*

FIGURE 3.4.

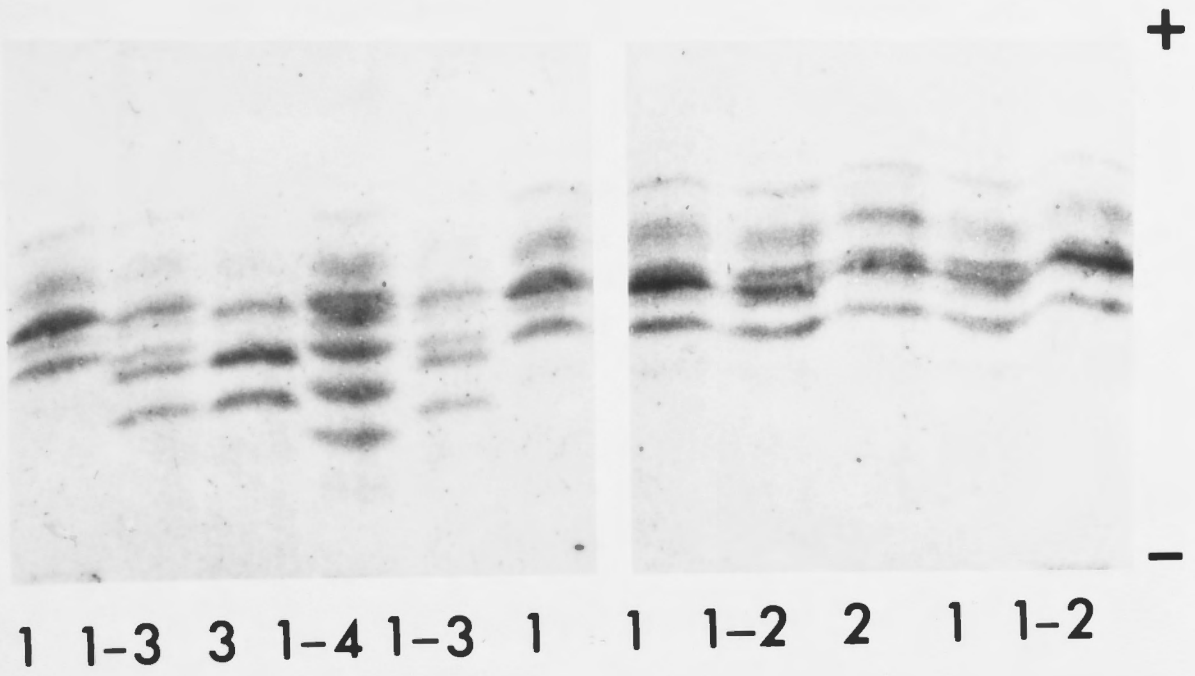
THE ELECTROPHORETIC PHENOTYPES SHOWN BY THE ALLELES AT THE FXIII-B LOCUS (from Board and Castle, 1982)

a) Isoelectric focussing of the four alleles at the FXIII-B locus

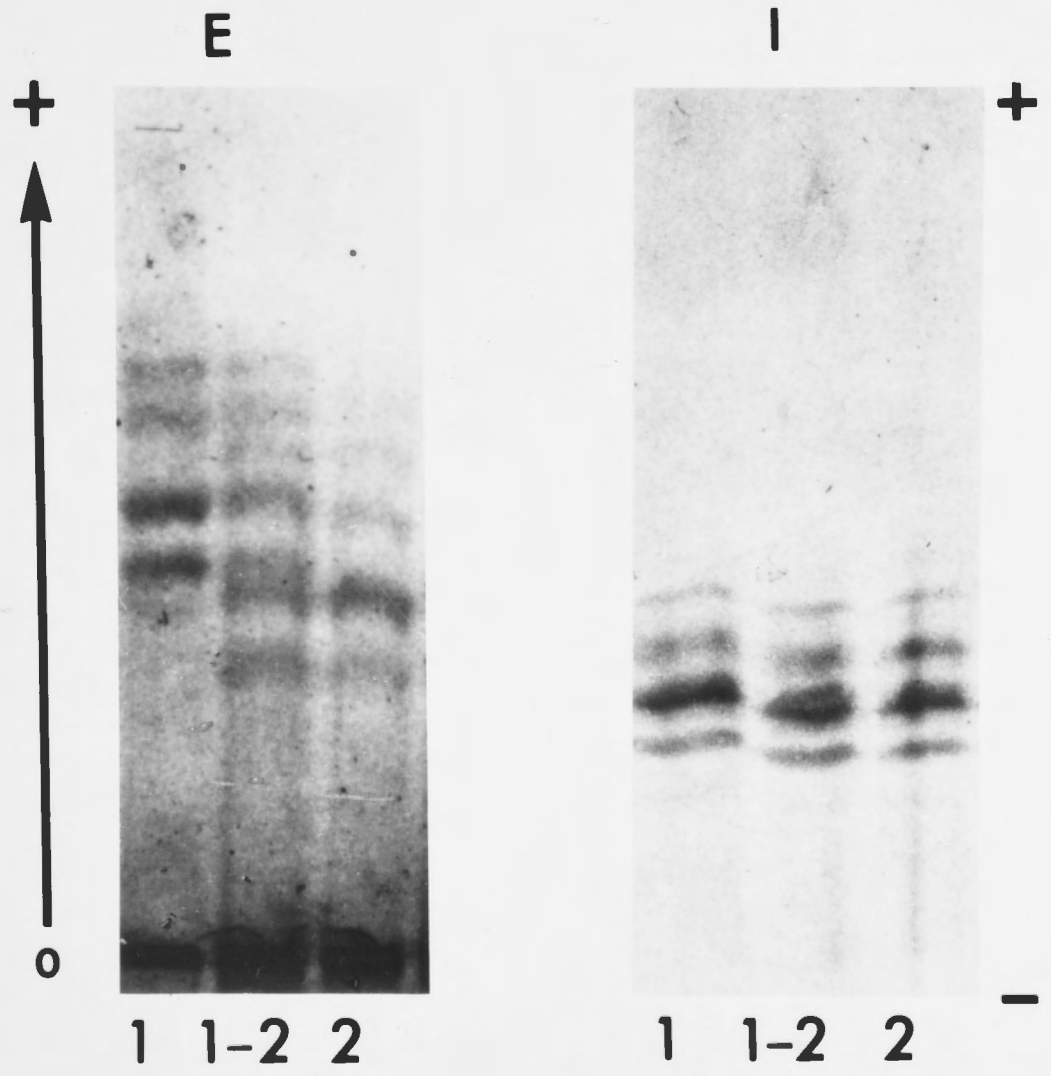
b) A comparison of the results of electrophoresis and isoelectric focussing of the Type 2 B subunits

E = electrophoresis; I = isoelectric focussing

a



b



E. The chromosomal locations of the A and B subunit loci

Neither loci has been assigned to a specific chromosome at the present time. However, there is no evidence of close linkage of the A subunit locus to the following markers; ACP1, 3cen, GC, MNSs, Ak1, ABO, 9cen, ESD, 13p, 14p, 15p, HP, PGP, ADA, 21p, C6, TF, GPT, K, IGK and JK and the factor XIIIA locus is excluded from the vicinity of 1p, the PEPC and Fy locus on chromosome 1 and from between the HLA and GLO loci on chromosome 6 the NP loci and 14p ter, the PI and GM loci on chromosome 14 and the centromere and GOT2 on chromosome 16 (King and Cook, 1981). In addition, deletion mapping techniques have been used to exclude the A subunit locus from the following chromosome segments 1q24, 4p15.1→p16.1, 4q23→q24, 4q27→q32, 6q22, 7q22, 13q13, 13q23→q31, 18q21→q23 and 21q21→qter and the B subunit locus from chromosome segments 4p15.1→16.1, 4q27→q31, 9p22→pter and 9pter→q11 (Mulley *et al.* 1980 and 1982; Cook *et al.* 1981 and King and Cook, 1981).

Chapter 4

I. INTRODUCTION

A. Genetic polymorphism of the A subunit locus

Bohn (1970) reported the occurrence of a charge heterogeneity of platelet factor XIII, thus suggesting that more than one type of A subunit was present in platelets. In 1973, Schwartz *et al.* noted the occurrence of two species of factor XIII A subunits from plasma in polyacrylamide gels containing urea at pH 3.2. It therefore appeared that there was a microheterogeneity of A subunits in man. As discussed briefly in Chapter 3. VII. C, this microheterogeneity can be explained by a genetic polymorphism at the A subunit locus.

Board (1979) developed a thin layer agarose gel electrophoresis system (pH 8.6), which gave good resolution of factor XIII A subunits, along with a specific fluorescent staining technique based on the incorporation of monodansyl cadaverine into casein by factor XIIIa (Lorand and Campbell, 1971). These two techniques were combined to demonstrate a genetic polymorphism at the A subunit locus in Australian blood donors. Two common alleles, designated *FXIIIA*1* and *FXIIIA*2*, were reported with frequencies of 0.79 and 0.21 respectively. Although this initial report indicated that the polymorphism was also present in New Guinea, Samoan, Chinese and Australian Aboriginal populations, no data was given.

Subsequently, several studies have extended the occurrence of this genetically determined polymorphism to German Europeans (Kreckl *et al.* 1982), Japanese populations (Nishigaki *et al.* 1981 and Kera and Nishimukai, 1982) and four Pacific Island populations, Loyalty Islanders (Melanesian), Cook Islanders (Polynesian) and Fijians (Melanesian and Indian) (Board and Coggan, 1981). These studies have shown that the

frequencies of the *FXIIIA*1* and *FXIIIA*2* alleles are different in the various population groups (Table 3.5). In addition, Board and Coggan (1981) have reported the occurrence of another allele, *FXIIIA*4* in Melanesians from Fiji at a frequency just below that generally recognised as being at a polymorphic level (Stern, 1973).

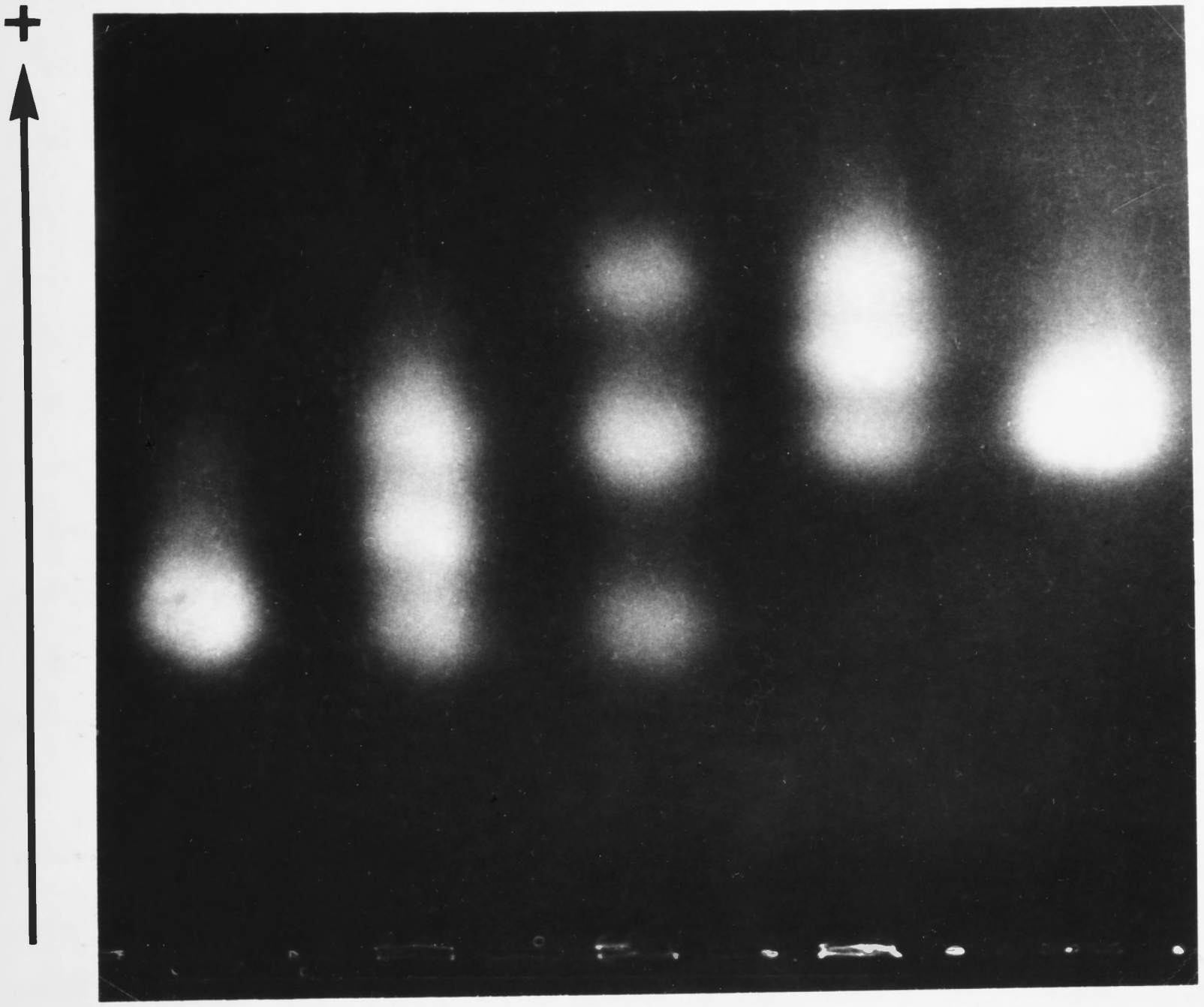
In the normal population, this polymorphic variation is not associated with any clinical abnormality. The products of the *FXIIIA*1* and *FXIIIA*2* alleles show similar enzyme activity, as measured by the rate of incorporation of ^{14}C putrescine into casein by unpurified plasma samples (Board *et al.* 1980).

The electrophoretic appearance of the various phenotypes observed for factor XIII A subunits are illustrated in Figure 4.1. Homozygotes present a single banded pattern, but heterozygotes present a more complex three banded phenotype. Since heterozygotes produce A subunits with different electrophoretic mobilities, their random assembly into dimers results in the production of homodimers identical to those found in homozygotes and heterodimers which have an electrophoretic mobility midway between the extremes of the fast and slow homodimers.

Since individual phenotypes for the FXIIIA locus can be readily determined from plasma samples by an inexpensive technique, the FXIIIA locus is potentially of considerable value as a genetic marker for population studies. Therefore it was decided to obtain gene frequency data for the FXIIIA locus from a number of populations. Particular emphasis, in these studies, was placed on population groups from Indonesia, Papua New Guinea and the Western Pacific. However, data was also obtained for South American Indians and for a population from the Caspian Littoral in the Middle East.

B. The genetics of factor XIII deficiency

The mode of inheritance of congenital factor XIII deficiency has


+
2
1—2
2—4
1—4
1

been discussed in Chapter 3.VII.A. As the published cases show several features characteristic of autosomal recessive inheritance: i) high incidence of consanguinity in the affected families; ii) absence of haemorrhagic symptoms in preceding generations although in several instances siblings have been affected with the disorder; iii) occurrence of the disorder in both sexes and iv) presence of subclinical laboratory defects in both parents of affected children (Britten, 1967) and both the A and B subunit loci of factor XIII are located on autosomal chromosomes (Board, 1979 and 1980), it is probable that congenital factor XIII deficiency is an autosomal recessive disorder.

Board *et al.* (1980) used the electrophoretic technique described above,in combination with a quantitative assay for factor XIIIa to show that factor XIII deficiency in a family originally described by Hamer and Rae (1971) was due to the presence of a null allele, *FXIIIA*QO* at the FXIIIA locus. However, studies that utilised immunological techniques have suggested the presence of more than one genetic type of this disorder (Ukita *et al.* 1976 and Forman *et al.* 1977) therefore suggesting that other alleles with abnormal or deficient products may exist in some populations.

In the course of the population screening programme mentioned above an apparently unstable A subunit protein was found in a normal New Zealand blood donor. Subsequent electrophoretic and quantitative studies in the family of the blood donor with this unstable protein revealed the same variation in his daughter, while his wife and son were both normal. As in the homozygous state, or in a heterozygous state with the previously reported null allele (Board *et al.* 1980), this variant protein would be expected to result in congenital factor XIII deficiency, it was considered to be of importance to investigate families which have reported cases of the disorder in an attempt to

determine the relative frequencies of the null and type 3 alleles in congenital factor XIII deficiency cases. Unfortunately, no cases of the disorder have been reported in Australia and it has only been possible to obtain samples from two additional families with confirmed cases and one family with a suspected case of congenital factor XIII deficiency for investigation.

II. MATERIALS AND METHODS

A. Agarose gel electrophoresis

The buffer system used was essentially the discontinuous system described by Ashton and Braden (1961). The electrode buffer contained 29 mM lithium hydroxide and 191 mM boric acid. The gel buffer was prepared by combining 9 vol solution containing 7.62 mM citric acid and 52 mM Tris with 1 vol electrode buffer. The two components of the buffer system were always prepared within 24 hours of use.

Thin layer agarose gels were prepared and set up for electrophoresis as described in Chapter 2. Electrophoresis was carried out at 18 V/cm until a haemoglobin marker migrated at least 6.5 cm to the anodal end of the gel. After electrophoresis of the samples for 5 minutes, the sample slots were overlaid with 1% agarose (w/v) dissolved in the gel buffer.

B. Localisation of factor XIIIa activity

After electrophoresis factor XIII A subunits were localised on the gel by their thrombin activated transamidase activity, incorporating monodansylcadaverine into casein (Board, 1979). Lorand and Campbell (1971) demonstrated that monodansylcadaverine was an excellent fluorescent substrate for factor XIIIa in the presence of casein. In the procedure used here monodansylcadaverine is covalently linked to casein in areas of factor XIIIa activity. The subsequent treatment with trichloroacetic acid (TCA) precipitates the casein in the gel, thus fixing the fluorescent complex at the site of factor XIIIa activity. Elution of the unreacted fluorescent substrate under running water removes the background fluorescence and leaves clearly defined fluorescent zones.

To identify the zones of factor XIIIa activity, the gels were overlaid with a filter paper strip (Whatman No. 1) soaked in a reaction mixture containing, 1M Tris. HCl (pH 7.4), 1 ml; 0.2M $CaCl_2$, 1 ml; 10 mg/ml casein (Sigma purified powder) dissolved in 1M Tris. HCl (pH 7.4), 3 ml; 0.025M monodansylcadaverine, 1 ml; β-mercaptoethanol, 20 µl and 2U thrombin (Ortho Diagnostics). The monodansylcadaverine was initially dissolved in a small amount of 0.5M HCl and then made up to volume with 1M Tris. HCl (pH 7.4). The gel was then incubated at 37°C, in a moist environment for 16 hours for plasma samples. Platelet extracts were only incubated for 4 hours.

After incubation, the filter paper containing the staining reagents was removed and the gel fixed by the application of a filter paper overlay containing 5% (w/v) TCA for 5 minutes. After fixation, the gel is again overlaid for a further 5 minutes with another filter paper soaked in 0.3 M Na_2HPO_4. The unincorporated monodansylcadaverine is removed by washing the gel under running tap water for 1-4 hours. The areas of factor XIIIa activity were viewed under short wave (254 nM) UV light. For most samples the zones of factor XIIIa activity can be viewed before the fixation and elution procedure due to a change in the emission wavelength of monodansylcadaverine bound to casein. However, the latter is necessary for good photographic reproduction of the gels.

C. Immunofixation

After electrophoresis, factor XIII A subunits were specifically localised by overlaying the gel with a 30% solution (v/v) of rabbit anti-human factor XIII subunit A antiserum (Behringwerke). The diluted antiserum was applied to the gel and the gel was processed and stained as described in Chapter 2.

D. Factor XIII assay

Thrombin activated plasma factor XIII activity was determined by the incorporation of ^{14}C putrescine (New England Nuclear) into α-casein (Sigma). The method was a modification of the procedure described by Dvilansky *et al.* (1970). The final putrescine concentration was elevated to 1 mM in this system. The reaction mixture contained, 0.3 M Tris. HCl (pH 7.5), 25 µl; 0.1 M $CaCl_2$, 25 µl; 1 mM EDTA; 25 µl; 0.5 M β-mercaptoethanol, 25 µl; 50 U/ml thrombin, 25 µl; 10 mM ^{14}C putrescine, 25 µl; 10mg/ml α-casein, 75 µl and plasma sample, 25 µl. Plasma samples were heated at 56°C for 3 minutes and centrifuged at 2000g for 5 minutes to remove fibrinogen prior to use in the assay.

The reaction was carried out at 37°C and the rate was determined at intervals by transferring 25 µl aliquots of the reaction mixture onto 2 cm Whatman 3MM chromatography paper discs which were immediately quenched in 5% TCA and processed as previously described by Mans and Novelli (1961). Because of the lag phase during enzyme activation, the rate of ^{14}C putrescine incorporation into casein did not reach its maximum until after 30 minutes. Aliquots were removed at 30 minute intervals up to 180 minutes and the reaction rates were calculated from least square regression of the incorporated radioactivity between 30 and 180 minutes.

E. Sample collection and preparation

The plasma samples for population screening had been collected previously, on a variety of anticoagulants, and had been stored for up to 10 years on liquid nitrogen or at -20°C. Platelets for screening were obtained from expired packs of concentrated platelets prepared by the Canberra Red Cross Blood Transfusion Service and the Auckland Blood Transfusion Centre. Platelets were separated from plasma by differ-

ential centrifugation and washed twice in ice cold 150 mM NaCl. Before electrophoresis platelets were treated with equal volumes of 1% Triton X100 to release the A subunits.

Blood from the families with suspected or confirmed factor XIII deficiency was collected onto citrate, as an anticoagulant, in other laboratories. Plasma was immediately prepared and shipped, by air, to the laboratory in Canberra where it was stored in liquid nitrogen for later evaluation.

Blood from the family with the apparently unstable A subunits was collected onto citrate, as an anticoagulant, in Auckland, New Zealand. Platelet-rich plasma was immediately prepared and shipped, by air, to the laboratory in Canberra. Platelets were prepared as described above. Determinations of activated plasma factor XIII activity were carried out within 48 hours of blood collection. Control samples were collected from laboratory workers and were treated in a similar fashion.

I. Populations studied

Indonesia: Eight populations from the Indonesian archipelago were studied. The populations were from Java, Lombok, Sumbawa, Timor, Celebes, Ternate and Northern Halmahera. The samples, therefore, represented the more Mongoloid people of the West and the people with Melanesian characteristics in the Eastern Region of Indonesia.

Papua New Guinea: Five populations from Papua New Guinea were studied. Three were from the mainland, the Western and Eastern Highlands and the Gulf District, the fourth was from Buka Island of the N.E. coast and the fifth was from Goodenough Island of the S.E. tip of Papua New Guinea.

Australia and New Zealand: A collection of Australian Aboriginal plasma samples was examined in addition to platelets from Australian and New Zealand blood donors.

Western Pacific: Collections from New Caledonia (Melanesians) to the North East of Australia, Guam (Micronesians) to the North of Australia and the Samoan and Tokelau Islands (Polynesians) to the East of Australia were examined.

Others: Plasma samples from Pima Indians (South America) and a population from the Caspian Littoral of the Middle East were also examined.

III. RESULTS

A. Population studies

Zones of factor XIIIa activity appeared as fluorescent bands 4-7 cm from the origin. As expected from previous studies, heterozygotes showed a symmetrical triplet pattern with three equidistant bands whose ratio was approximately 1:2:1. Homozygotes had a single band, although in some of the older collections of plasma samples a fainter anodic minor band was also present. It was considered that this anodic band represents a modification to the A subunits of factor XIII during prolonged storage.

1. Observed phenotypes

In addition to the three common phenotypes, 1-1, 1-2 and 2-2, two other three-banded and one two-banded phenotypes were seen. The two three-banded phenotypes were shown to correspond to the 1-4 and 2-4 phenotypes previously described in Fijian Melanesians (Figure 4.2).

The two-banded phenotype found in a New Zealand blood donor is described below (Chapter 4.IV.B).

2. Distribution of the observed phenotypes

Examination of plasma samples from the populations studied revealed that the two alleles for the A subunit, *FXIIIA*1* and *FXIIIA*2*, which are common in populations studied previously are also present in each of the populations studied here. The distribution of the observed phenotypes is given in Table 4.1.

Two of the Indonesian populations studied, the Yogyakarta and Macassarese and one of the Papua New Guinean collections from the Gulf District, contained individuals with phenotypes resulting from the presence of the *FXIIIA*4* allele. The new phenotype, 1-3, was not included in the data analysis as the *FXIIIA*3* allele codes for

Figure 4.2: A COMPARISON OF THE 1-4 AND 2-4 PHENOTYPES OBSERVED IN THIS STUDY WITH THOSE PUBLISHED BY BOARD AND COGGAN (1981):

(a) The 1-4 phenotype from Fiji (Board and Coggan, 1981)

(b) The 1-4 phenotype from Indonesia

(c) The 2-4 phenotype from Indonesia

(d) The 2-4 phenotype from Fiji (Board and Coggan, 1981)

(e) The 2-4 phenotype from Papua New Guinea

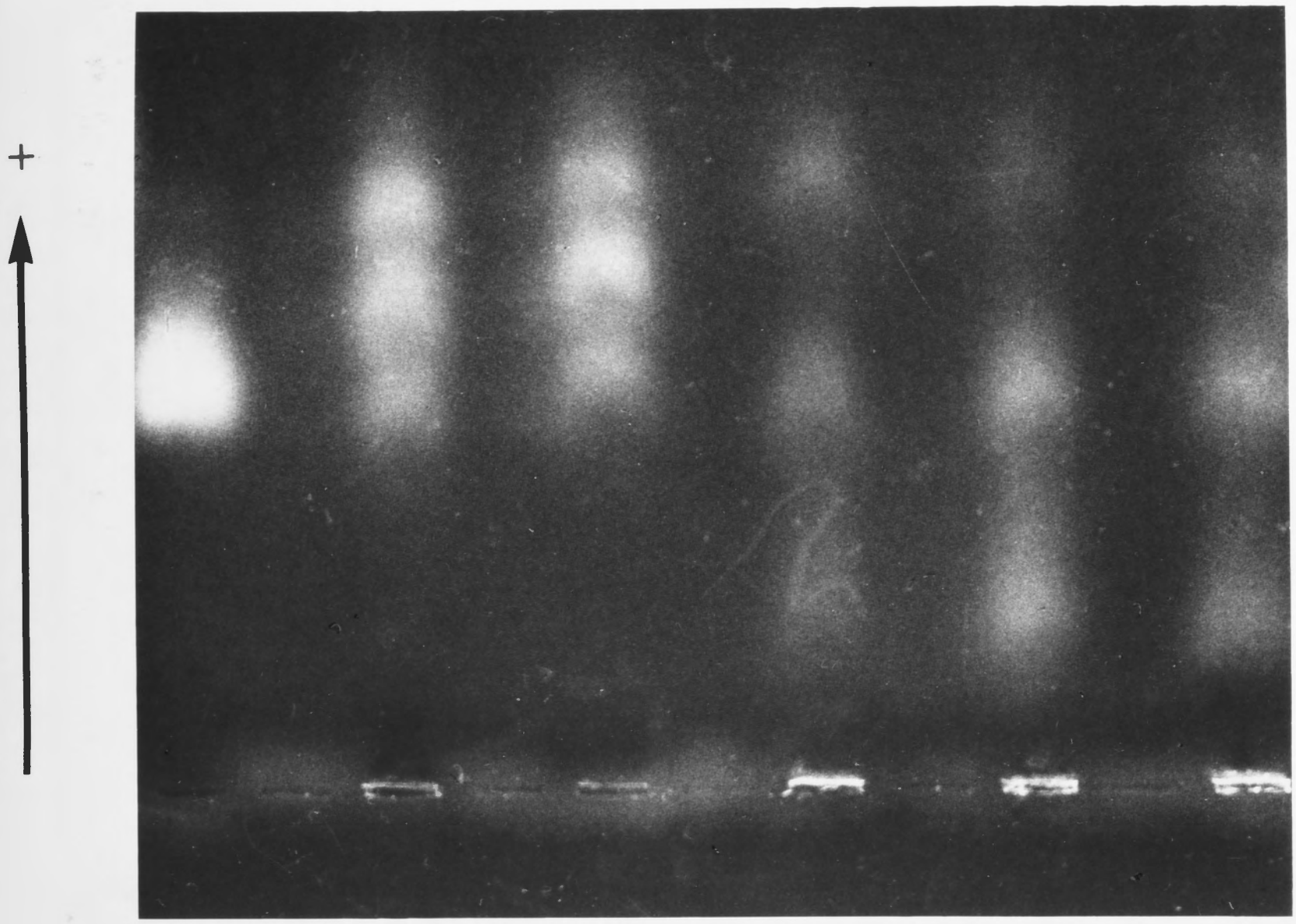

+
1-1
1-4
a
1-4
b
2-4
c
2-4
d
2-4
e

TABLE 4.1.

DISTRIBUTION OF FACTOR XIII A SUBUNIT PHENOTYPES

POPULATION		PHENOTYPE 1	2-1	2	1-4	2-4	TOTAL	χ^2	d.f.	p
INDONESIA										
Yogyakarta (Java)	Obs. No.	143	13	-	-	1	157			
	Exp. No.	142.36	13.33	0.31	0.95	0.05		21.750	3	0.01*
Lombok (Lombok)	Obs. No.	138	20	3	-	-	161			
	Exp. No.	136.05	23.90	1.05	-	-		4.288	3	0.05†
Bimanese (Sumbawa)	Obs. No.	147	12	2	-	-	161			
	Exp. No.	145.40	15.21	0.40	-	-		7.153	3	0.05†
Timorese (Timor)	Obs. No.	133	28	-	-	-	161			
	Exp. No.	134.22	25.57	1.22	-	-		1.460	3	0.05†
Macassarese (Celebes)	Obs. No.	134	24	3	1	-	162			
	Exp. No.	132.48	27.13	1.39	0.90	0.09		2.352	3	0.05†
Buginese (Celebes)	Obs. No.	150	11	1	-	-	162			
	Exp. No.	149.26	12.48	0.26	-	-		2.274	3	0.05†
Ternatans (Ternate)	Obs. No.	183	29	1	-	-	213			
	Exp. No.	183.13	28.74	1.13	-	-		0.017	3	0.05†
Galarese (North Halmahera)	Obs. No.	142	38	5	-	-	185			
	Exp. No.	140.11	41.77	3.11	-	-		1.509	3	0.05†

TABLE 4.1. Cont'd

POPULATION		PHENOTYPE 1	2-1	2	1-4	2-4	TOTAL	χ^2	d.f.	p
PAPUA NEW GUINEA										
Eastern Highlanders	Obs. No.	111	33	6	-	-	150			
	Exp. No.	108.38	38.25	3.38	-	-		2.826	1	0.05†
Gulf District	Obs. No.	38	11	-	-	1	50			
	Exp. No.	37.96	10.49	0.71	0.86	0.12		8.386	3	0.05†
Western Highlanders	Obs. No.	34	17	5	-	-	56			
	Exp. No.	32.26	20.49	3.26	-	-		1.626	1	0.05†
Buka Is.	Obs. No.	52	22	2	-	-	76			
	Exp. No.	52.22	21.55	2.22	-	-		0.033	1	0.05†
Goodenough Is.	Obs. No.	104	44	2	-	-	150			
	Exp. No.	105.84	40.32	3.84	-	-		1.250	1	0.05†
AUSTRALIA AND NEW ZEALAND										
Australian Aborigines	Obs. No.	59	34	4	-	-	97			
	Exp. No.	59.55	32.91	4.55	-	-		0.107	1	0.05†
Blood donors	Obs. No.	108	44	11	-	-	163			
	Exp. No.	103.68	52.64	6.68	-	-		4.390	3	0.05†
WESTERN PACIFIC										
New Caledonia	Obs. No.	111	37	2	-	-	150			
	Exp. No.	111.80	35.40	2.80				0.308	1	0.05†

TABLE 4.1. Cont'd

POPULATION		PHENOTYPE					TOTAL	χ^2	d.f.	p
		1	2-1	2	1-4	2-4				
WESTERN PACIFIC										
Guam	Obs. No.	101	36	5	-	-	142			
	Exp. No.	99.73	38.55	3.73	-	-		0.621	3	0.05†
Samoan Is.	Obs. No.	119	27	1	-	-	147			
	Exp. No.	119.43	26.14	1.43	-	-		0.159	3	0.05†
Tokelau Is.	Obs. No.	135	27	-	-	-	162			
	Exp. No.	136.13	24.75	1.13	-	-		1.339	3	0.05†
SOUTH AMERICA										
Pima Indians	Obs. No.	106	47	1	-	-	154			
	Exp. No.	108.90	41.21	3.90	-	-		3.047	3	0.05†
MIDDLE EAST										
Caspian Sea	Obs. No.	44	25	4	-	-	73			
	Exp. No.	43.73	25.54	3.73	-	-		0.033	3	0.05†

* Significant;

† Not significant.

a functionally abnormal product.

3. Gene frequencies

The gene frequencies of the *FXIIIA*1*, *FXIIIA*2* and *FXIIIA*4* alleles for each of the populations studied is given in Table 4.2. Only the *FXIIIA*1* and *FXIIIA*2* alleles are at frequencies that are considered to be polymorphic.

B. The type 1-3 phenotype

1. Electrophoretic studies

After the observation of a two-banded phenotype in a platelet sample from a blood donor in New Zealand (Figure 4.3), other members of the probands family were investigated to see if they showed a similar phenotype. Study of family J showed his wife and son to be of the 1-1 type while his daughter, LJ, had a similar two-banded pattern (Figure 4.4).

Platelet and plasma samples from the proband, JJ and LJ, showed a fast band corresponding to the 1-1 type and a second slower band which was electrophoretically slower than the heterodimer of the 2-1 type but faster than the 2-2 band. No other band was present. The two-banded pattern was revealed by the functional stain and by immuno-fixation.

During storage of platelets suspended in saline at $4^{o}C$ for 14d the second slow band degraded and on subsequent electrophoresis only the normal 1-1 type band was present from the platelets of JJ and LJ. Similarly, plasma samples stored in liquid nitrogen lost the second slow band when freeze-thawed several times. Platelets and plasma samples from controls stored for a similar period of time and treated in a similar manner showed no changes in their banding patterns after electrophoresis.

It is proposed that the pattern seen in the proband is the

TABLE 4.2.

THE GENE FREQUENCIES OF THE ALLELES WITH NORMAL PRODUCTS AT THE FXIII A LOCUS

POPULATION	GENE FREQUENCY			N	S.E.
	*FXIIIA*1*	*FXIIIA*2*	*FXIIIA*4*		
Indonesia					
Yogyakarta	0.952	0.045	0.003*	157	0.012
Lombok	0.919	0.081	-	161	0.015
Bimanese	0.950	0.050	-	161	0.012
Timorese	0.913	0.087	-	161	0.016
Macassarese	0.904	0.093	0.003*	162	0.016
Buginese	0.960	0.040	-	162	0.011
Ternatans	0.927	0.073	-	213	0.013
Galelarese	0.870	0.130	-	185	0.018
Papua New Guinea					
Eastern Highlands	0.850	0.150	-	150	0.021
Gulf District	0.872	0.125	0.003*	51	0.032
Western Highlands	0.759	0.241	-	56	0.040
Buka Is.	0.829	0.171	-	76	0.031
Goodenough Is.	0.840	0.160	-	150	0.021
Australia and New Zealand					
Australian Aborigines	0.784	0.217	-	97	0.030
Blood donors	0.798	0.203	-	163	0.022
Western Pacific					
New Caledonia	0.863	0.137	-	150	0.020
Guam	0.838	0.162	-	142	0.022
Samoan Is.	0.901	0.099	-	147	0.017
Tokelau Is.	0.917	0.083	-	162	0.015
S. America					
Pima Indians	0.841	0.159	-	154	0.021
Middle East					
Caspian Sea	0.774	0.226	-	78	0.035

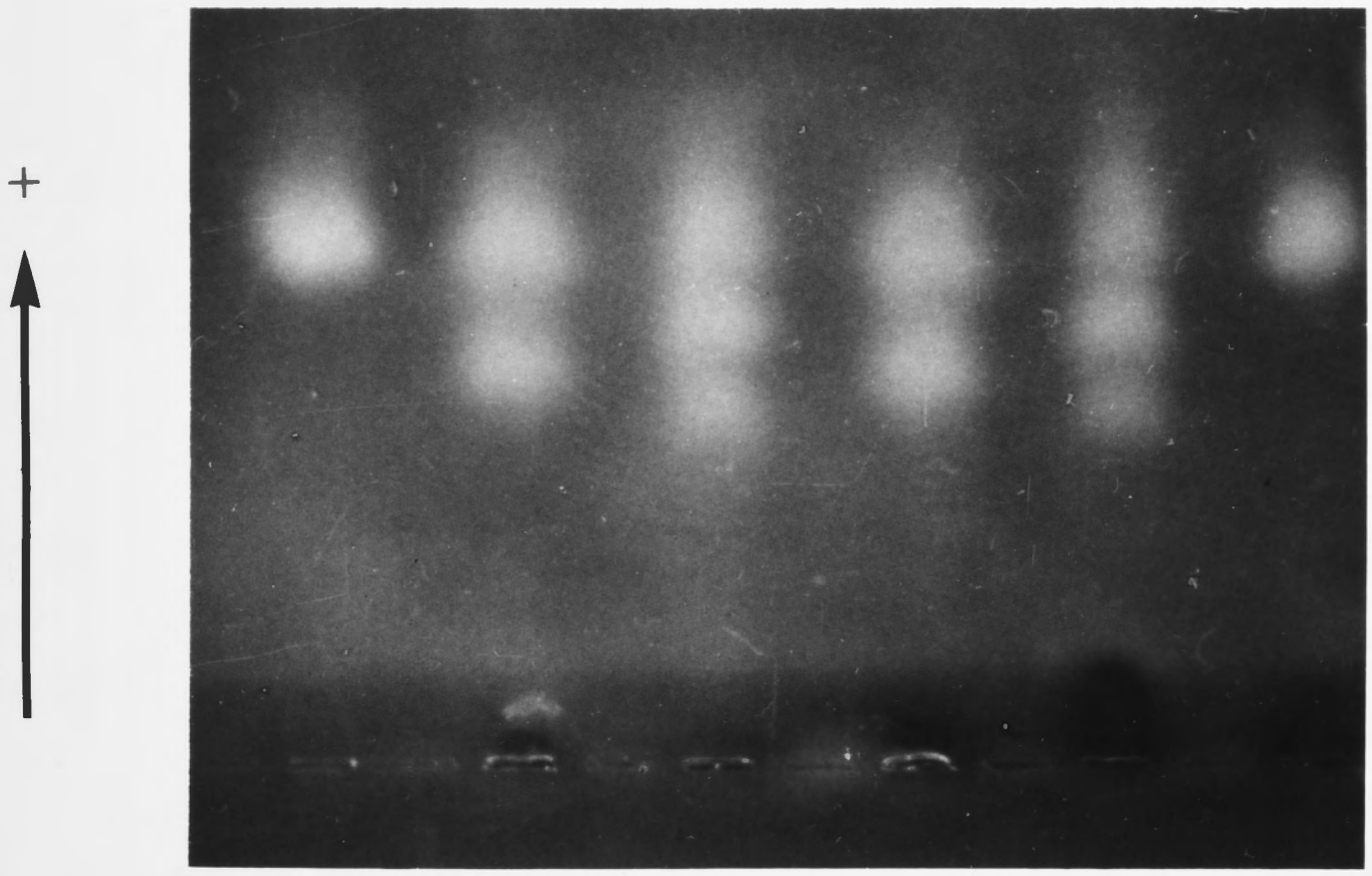
* S.E. = 0.003

Figure 4.3: ELECTROPHORESIS OF FACTOR XIII A SUBUNITS FROM JJ, THE PROBAND (1-3) AND CONTROLS (1-1, 1-2, 2-2):

(a) Localisation of A subunits by the functional stain

(b) Localisation of A subunits by immunofixation

a



b

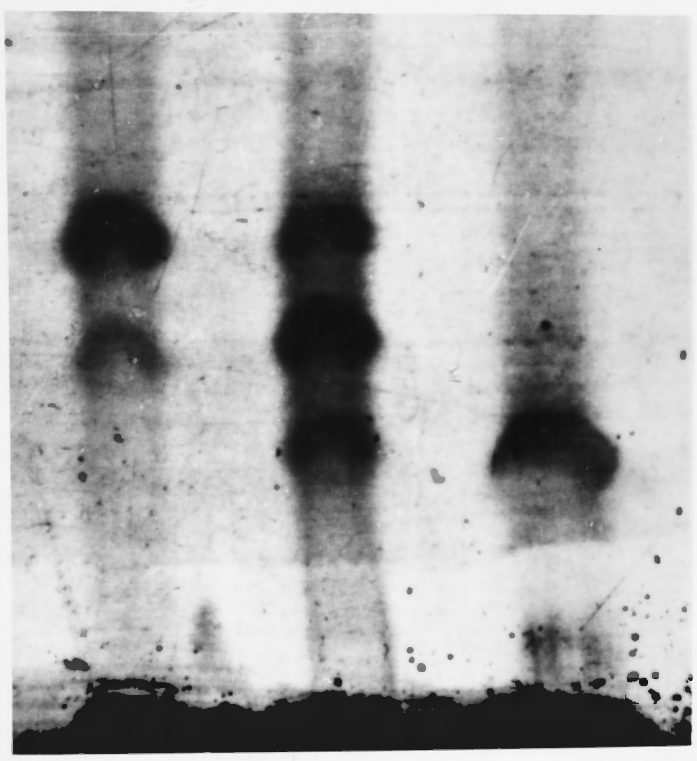
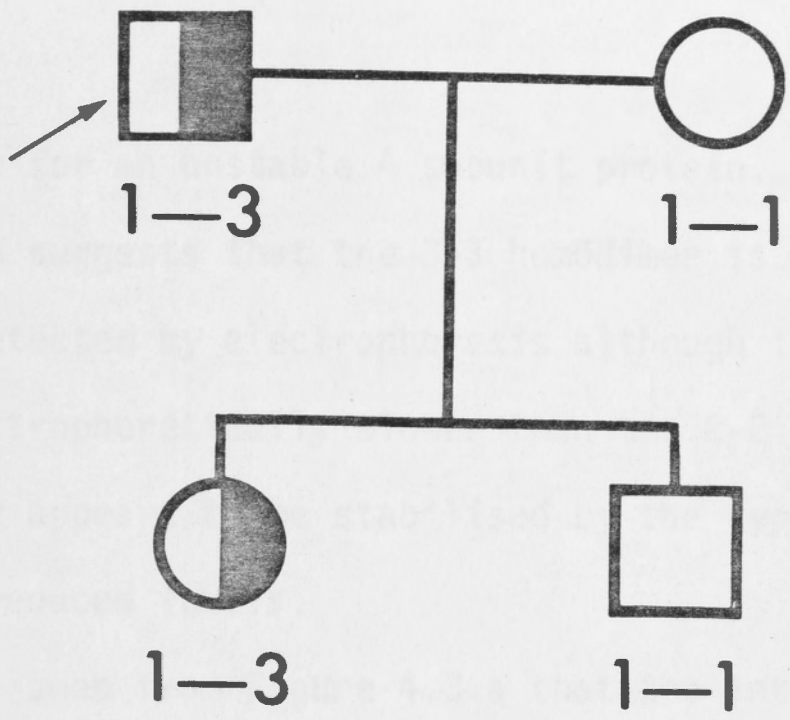
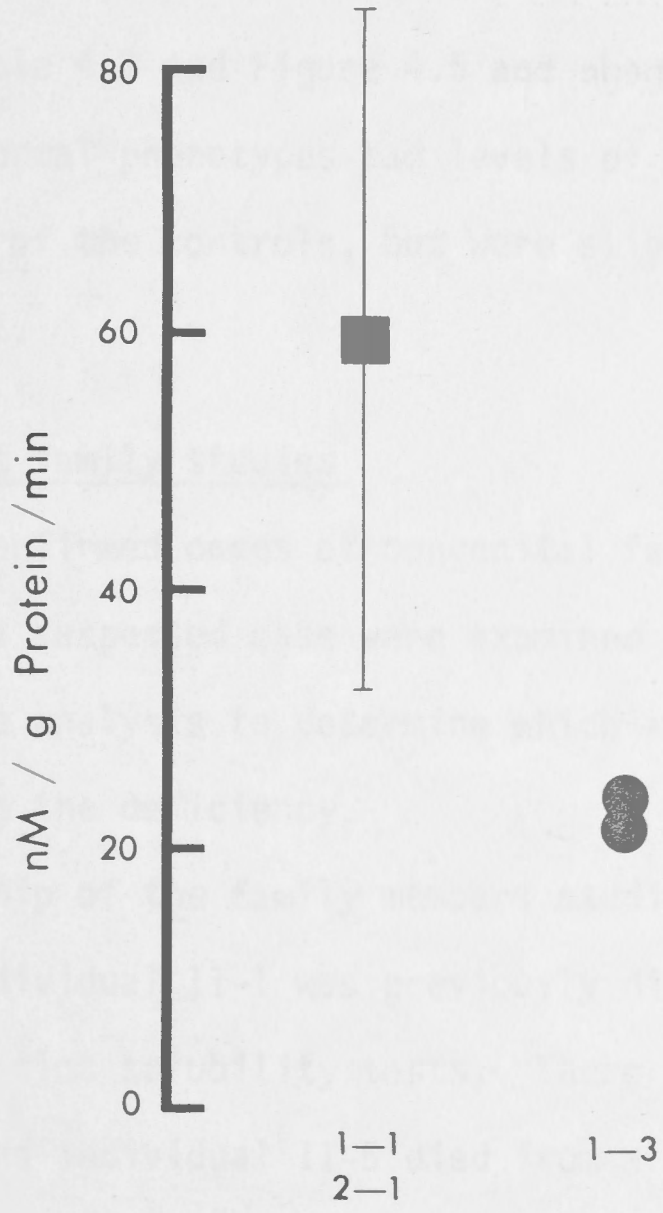


Figure 4.4: PEDIGREE OF FAMILY J WITH THE *FXIIIA*3* ALLELE SHOWING THEIR FACTOR XIII A SUBUNIT GENOTYPE:

Figure 4.5: THE PLASMA FACTOR XIII ACTIVITY OF THE PROBAND AND HIS DAUGHTER (1-3) COMPARED TO THE MEAN ± S.D. OF FIVE CONTROLS



FAMILY J



result of an allele for an unstable A subunit protein, designated 3. This interpretation suggests that the 3-3 homodimer is unstable and is therefore not detected by electrophoresis although it would be expected to be electrophoretically slower than the 2-2 homodimer. The 1-3 heterodimer appears to be stabilised by the Type 1 protein, but is present at reduced levels.

It can be seen from Figure 4.3.a that the intensities of the bands produced by the functional stain are approximately equal, while the protein stained after immunofixation showed the 1-1 band to be 3-4 times more intense than the 1-3 band (Figure 4.3.b). This finding suggests that the Type 3 protein may well have increased enzyme activity despite its instability.

2. Factor XIII levels in plasma

The transamidase activity of activated plasma factor XIII was measured in all members of family J and in five controls. These data are presented in Table 4.3 and Figure 4.5 and show that both JJ and LJ, who have the abnormal phenotypes had levels of activity which were lower than the mean of the controls, but were slightly higher than the lowest normal control.

C. Factor XIII deficient family studies

Two families with confirmed cases of congenital factor XIII deficiency and one with a suspected case were examined by electrophoretic and quantitative analysis to determine which allele at the FXIII A locus was causing the deficiency.

Family P: The relationship of the family members studied here is shown in Figure 4.6. Individual II-1 was previously diagnosed as factor XIII deficient by clot solubility tests. There is one miscarriage in the family and individual II-5 died from a Wilm's tumour.

Figure 4.6: PEDIGREE OF FAMILY P WITH THE FACTOR XIII A SUBUNIT GENOTYPES

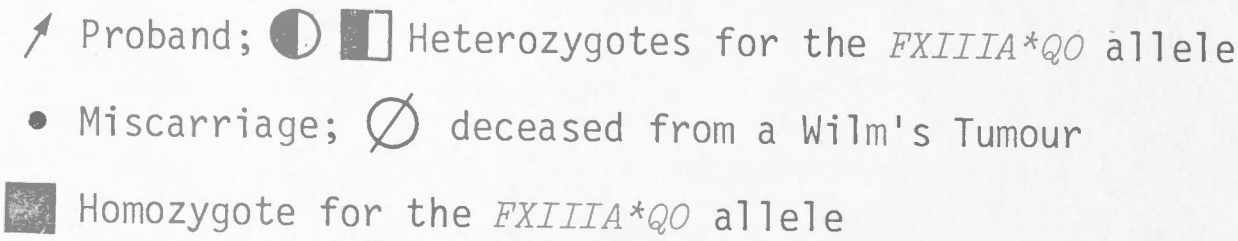
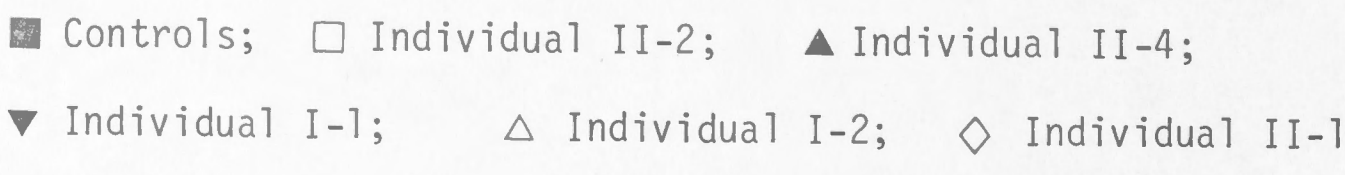
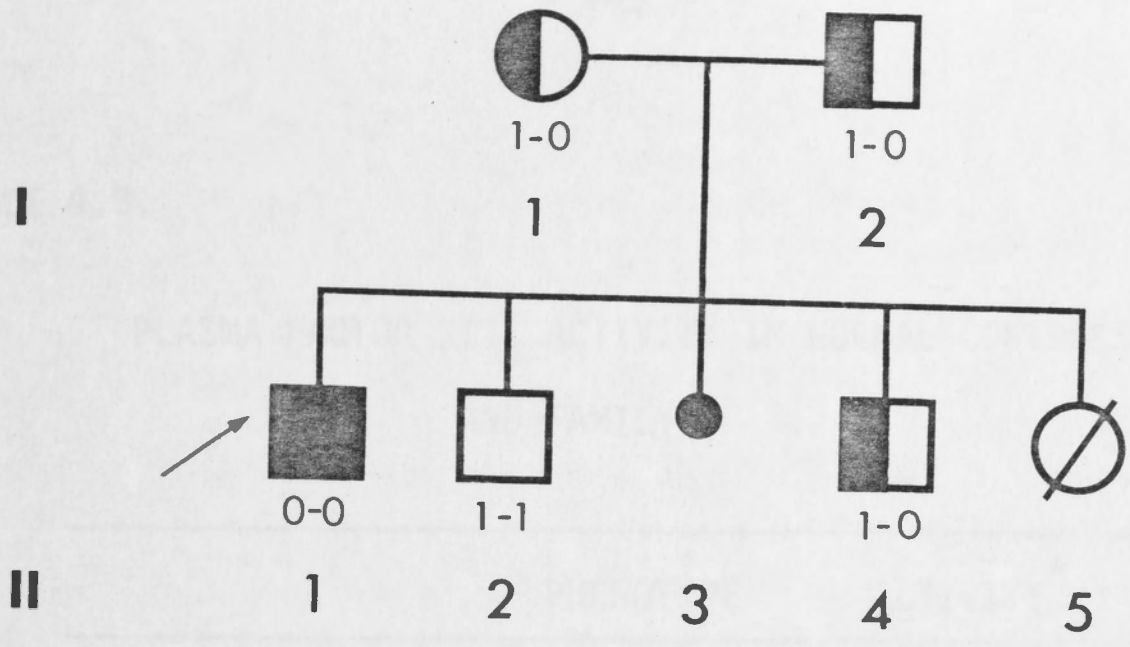


Figure 4.7: THE PLASMA FACTOR XIII ACTIVITY OF MEMBERS OF FAMILY P AND TWO CONTROLS




I
1-0
1-0
1
2
II
0-0
1-1
1-0
1
2
3
4
5

FAMILY P

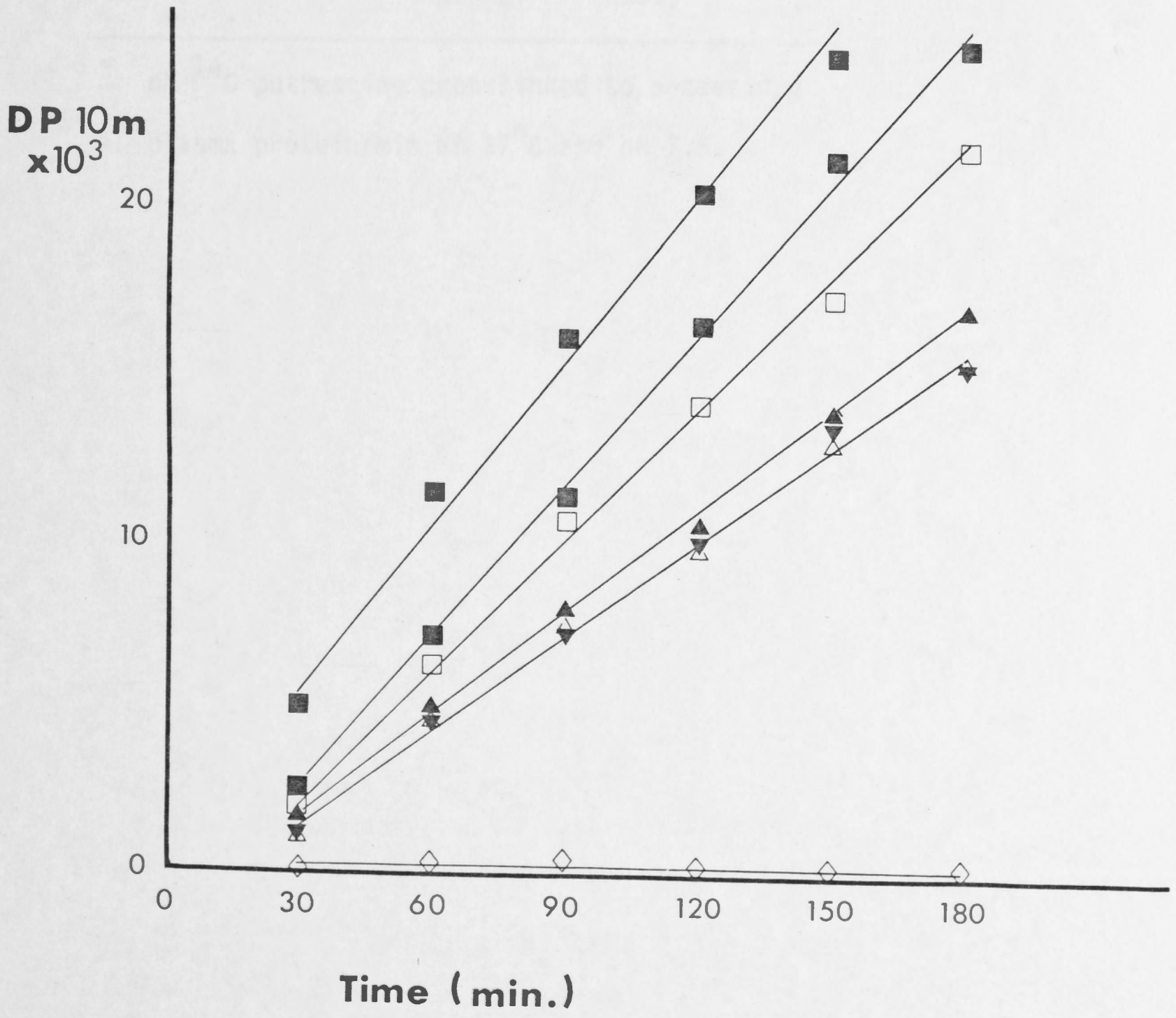

DP 10m
x10³
20
10
0
0
30
60
90
120
150
180
Time (min.)

TABLE 4.3.

PLASMA FACTOR XIII ACTIVITY IN NORMAL CONTROLS AND FAMILY J

	PHENOTYPE	ACTIVITY*
Normal controls	Normal	
Mean of five ± SD		49.4 ± 28.86
Range		19.7 - 95.7
Proband	Variant	22.0
Wife	Normal	86.6
Daughter	Variant	23.4
Son	Normal	39.3

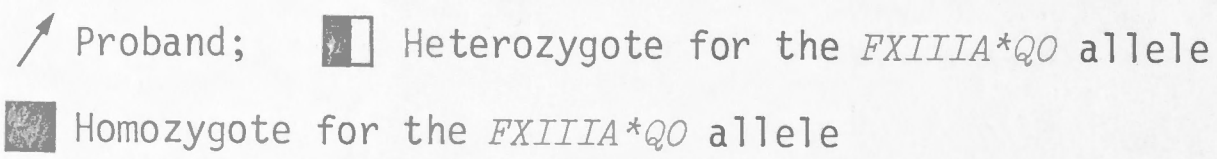
* nM ^{14}C putrescine crosslinked to α-casein/g plasma protein/min at 37°C and pH 7.5.

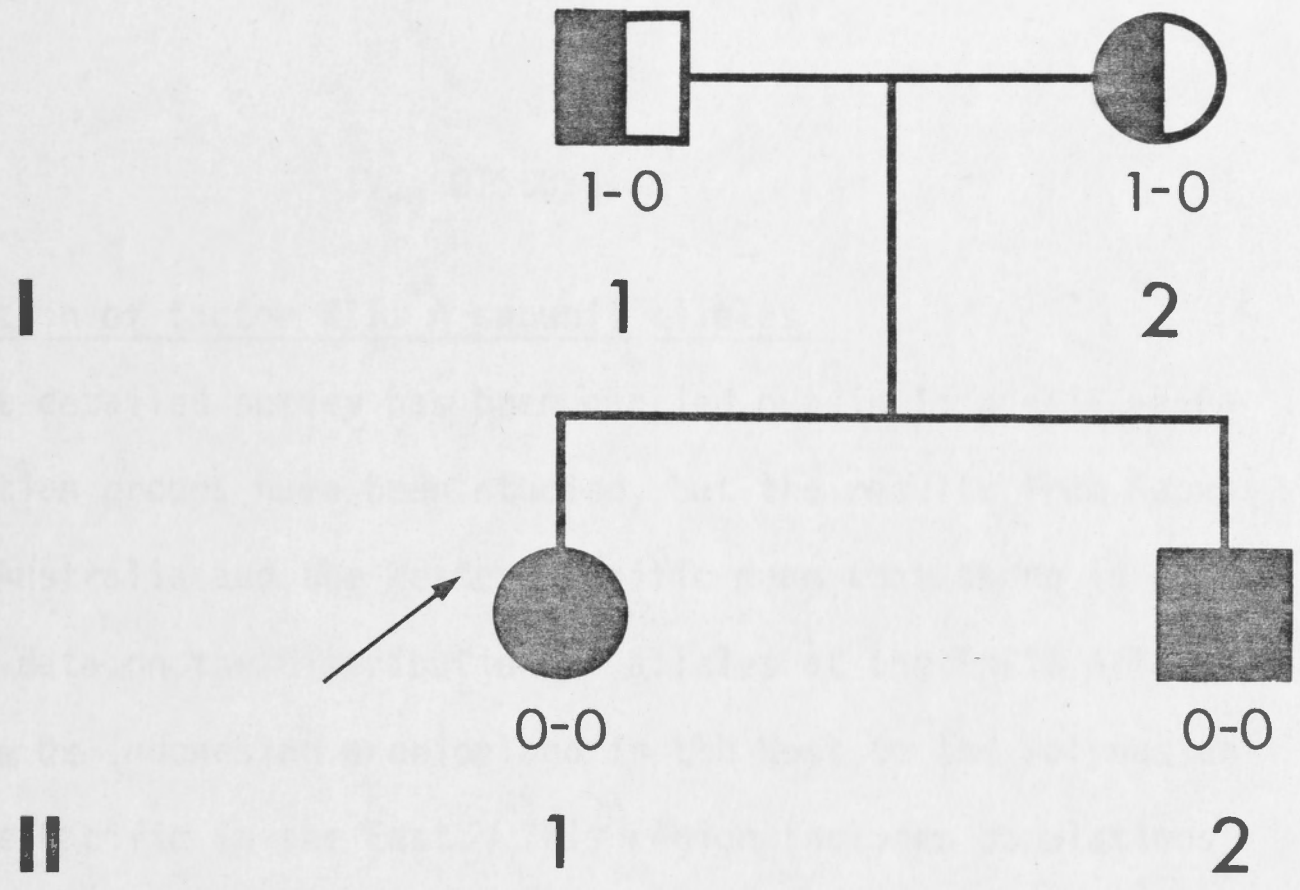
The other members of the family are normal for factor XIII but the mother has Von Recklinghausen's disease. The sample from individual II-1 failed to show any transamidase activity, either after electrophoresis or in the factor XIII assay (Figure 4.7) and no detectable factor XIII A subunit protein was found after immunofixation. In comparison the other family members presented a phenotypic pattern similar to that obtained from a *FXIIIA*1* homozygote, although the band was relatively weak in individuals I-1, I-2 and II-4.

Family E: The relationship of the individuals studied is shown in Figure 4.8. Individuals II-1 and II-2 were previously diagnosed as factor XIII deficient by clot solubility tests and failed to show any detectable transamidase activity either after electrophoresis or during a factor XIII assay. Both parents present 1-1 phenotypes.

Family M: Samples from this family were examined as the child had a spontaneous unexplained umbilical haemorrhage, which is a characteristic of factor XIII deficiency (Chapter 3). However, examination by electrophoresis and factor XIII assay showed all members of the family to be normal for factor XIII activity.

Figure 4.8: PEDIGREE OF FAMILY E WITH FACTOR XIII A SUBUNIT GENOTYPES





FAMILY E

IV. DISCUSSION

A. Distribution of factor XIII A subunit alleles

The most detailed survey has been carried out in Indonesia where eight population groups have been studied, but the results from Papua New Guinea, Australia and the Western Pacific mean that there is now considerable data on the distribution of alleles at the FXIII A locus in the area from the Indonesian archipelago in the West to the Polynesian region of the Pacific in the East. This region includes populations recognised as being Mongoloid, Melanesian, Micronesian, Polynesian and Caucasian.

1. The *FXIIIA*1* and *FXIIIA*2* alleles

All of the populations studied were polymorphic for the *FXIIIA*1* and *FXIIIA*2* alleles with the frequency of the *FXIIIA*1* allele ranging from 0.759 to 0.960. The values obtained for Australian and New Zealand blood donors were in good agreement with those published previously for Caucasian populations (Board, 1979 and Kreckel *et al.* 1982).

The frequencies of the two alleles were variable between the population groups studied in Indonesia, but there was no obvious cline in frequency in a geographical direction. The frequency of the *FXIIIA*1* was higher than that found in Indians (Board and Coggan, 1981) although the Indians studied were from Fiji, but was closer to the frequency reported for Japanese (Nishigaki *et al.* 1981).

The *FXIIIA*1* allele had a lower frequency in the Melanesian populations of Papua New Guinea than in the Indonesian populations. Melanesians from New Caledonia had a similar *FXIIIA*1* frequency to the Papua New Guinea populations as did Micronesians from Guam. Board and Coggan (1981) reported a very high frequency of the *FXIIIA*1* allele in Polynesians from Cook Island, 0.98. The two Polynesian populations

studied here, Samoan and Tokelau Islanders, showed a lower frequency, 0.901 and 0.917 respectively. Although these values are higher than those for the Melanesian populations studied here, they are of a similar magnitude to that published previously for Loyalty Island Melanesians, 0.925 (Board and Coggan, 1981).

In addition to the relatively detailed survey of the Western Pacific region populations from the Caspian Littoral in Iran and of South American Indians were shown to be polymorphic for the *FXIIIA*1* and *FXIIIA*2* alleles.

2. The *FXIIIA*4* allele

Board and Coggan (1981) reported the presence of the *FXIIIA*4* allele in the Melanesian populations of Fiji, but failed to find any evidence of the presence of the allele in other Pacific populations. Although the survey carried out here failed to detect the *FXIIIA*4* allele in four Pacific Island populations, the allele was detected in Melanesians from Papua New Guinea and the Yogyakarta and Macassarese of Indonesia. While these latter two populations are not Melanesian they have been shown to contain some alleles which are considered to be Melanesian markers (Sofro, 1982). Therefore, the data suggests that the *FXIIIA*4* allele may well be a Melanesian marker gene.

The *FXIIIA*4* allele does not appear to be polymorphic in any of the populations where it has been found. Although the frequency in the Gulf District population is 0.01, the sample size is too small for this to be considered a reliable estimate.

B. <u>The type 1-3 phenotype</u>

The two-banded electrophoretic pattern observed in both the proband and his daughter suggests that they are heterozygous for an allele for a normal A subunit and an allele for an abnormal A subunit, which has been termed type 3 in accordance with the previously defined nomen-

clature (Board, 1979). Since the A subunit of factor XIII is a dimeric molecule, heterozygotes for electrophoretically different allelic products normally present a three-banded phenotype. In this case the absence of a third electrophoretically slow, enzymatically active, component representing the expected 3-3 homodimer, implies that the *FXIIIA*3* allele product is either inactive or unstable. Immunological investigation of these subjects by immunofixation of the electrophoretically separated A subunits failed to provide any evidence of an inactive 3-3 homodimer, suggesting that the *FXIIIA*3* allele product is either unstable as a homodimer, or is both inactive and not recognised by the anti A antiserum. However, since there was a rapid breakdown of the 1-3 heterodimer during storage at $4^{o}C$, it seems most likely that the Type 3 protein is, in fact, unstable (the data presented in Chapter 5 appears to confirm this hypothesis).

Despite the apparent instability of the Type 3 protein, the relatively high transamidase staining intensity of the 1-3 heterodimer in comparison with its proportionally low total protein content, as evaluated by immunofixation, suggests that the Type 3 protein may, in fact, have an elevated activity.

The subjects studied here do not suffer from the clinical features of factor XIII deficiency but, because of the apparent instability of the 3-3 homodimers, it is likely that no stable product would be found in individuals homozygous for this allele, or heterozygous for the *FXIIIA*3* allele and the previously described nullallele *FXIIIA*QO* (Board *et al.* 1980). This identification of the *FXIIIA*3* allele clearly indicates that there are multiple alleles at the FXIIIA structural locus which can lead to factor XIII deficiency in man and that can only be differentiated by electrophoretic examination of samples from the parents of deficient individuals (see section C).

It is not possible to detect heterozygotes for the *FXIIIA*QO* allele by electrophoresis as these individuals show a phenotype identical to that of homozygotes for the normal alleles. However, the two-banded nature of heterozygotes for the *FXIIIA*3* allele suggests that they should be easily identified in population surveys and therefore that an estimate of the frequency of this allele could be made. In 2,938 individuals studied here and 1940 individuals studied previously, only one example of a 1-3 phenotype has been found, therefore suggesting that the *FXIIIA*3* allele is relatively rare. Still, it is possible that the instability of the 1-3 heterodimer might cause this band to be lost under certain storage conditions and after several freeze-thaw episodes. In fact, during the population survey carried out here, two samples from the Samoan collection showed a two-banded pattern similar to that of a 1-3 heterozygote albeit with a weak slower band, but when the samples were rechecked this band was absent and the samples gave a 1-1 phenotype. It may, therefore, be that the *FXIIIA*3* allele is only detectable in relatively fresh samples.

C. Factor XIII deficiency

As mentioned above it appears that there are multiple alleles at the FXIII A structural locus which can lead to factor XIII deficiency in man. Therefore, studies by the electrophoretic technique described by Board (1979) and used here, of the parents and other family members of those cases reported previously, should reveal the relative importance of the *FXIIIA*QO*, *FXIIIA*3* and other possible alleles with unstable or inactive products (Ukita *et al.* 1976 and Forman *et al.* 1977) in the aetiology of factor XIII deficiency.

Unfortunately, due to the absence of any reported cases of factor XIII deficiency in Australia, it was only possible to study two additional families with cases of factor XIII deficiency here. In both family

P and family E the results of transamidase activity measurement and immunofixation after electrophoresis suggest the factor XIII deficiency is a result of the null allele being present in the homozygous state. Therefore, in the three families where these studies have been carried out, the *FXIIIA*QO* allele appears to be the causal agent. However, there is no proof of identity of the null alleles in these three families.

In previously studied cases of factor XIII deficiency, the absence of active A subunits has indicated that heterozygotes could be readily identified for genetic counselling because of their considerably reduced transamidase activity (Kitchens and Newcombe, 1979 and Board *et al.* 1980). Although the levels of activity in the two heterozygotes for the *FXIIIA*3* allele were lower than the mean for the controls, they were slightly higher than the lowest control samples. The occurrence of an unstable A subunit with an increased activity in the family reported here, suggests that heterozygote detection by enzyme assay may miss some individuals who are potential parents of deficient children. Heterozygote detection by enzyme assay, combined with electrophoretic studies similar to those utilised here, should not miss any of these heterozygotes. Although, it is possible that a 2-3 heterozygote will be difficult to distinguish from a 2-2 homozygote as the 2-3 heterodimer should migrate close to the 2-2 homodimer. However, the relatively low frequency of the *FXIIIA*2* allele in most populations indicates that the *FXIIIA*3* allele is much more likely to be found in combination with the *FXIIIA*1* allele.

V. CONCLUSIONS

1. The previously reported polymorphism at the structural locus for factor XIII A subunits has been extended in its distribution to Indonesia, Papua New Guinea and several Island groups in the Western Pacific as well as to the Pima Indians of South America and the Caspian Littoral.

2. The *FXIIIA*4* allele has been identified in two Indonesian and one Papua New Guinean population in addition to the Melanesians of Fiji reported previously. The data suggests that this allele may be a Melanesian marker.

3. A new allele with an unstable product, *FXIIIA*3*, has been identified. This allele in the homozygous state or in the heterozygous state with the previously described null allele, *FXIIIA*QO*, is likely to result in congenital factor XIII deficiency. This represents the first clear evidence of genetic heterogeneity of congenital factor XIII deficiency.

4. The occurrence of an unstable A subunit protein with an apparently raised activity casts doubt on the ability of an enzyme assay to identify all potential parents of factor XIII deficient children. It is, therefore, recommended that the electrophoretic technique used here is employed in conjunction with an enzyme assay for the identification of heterozygotes for alleles capable of resulting in factor XIII deficiency.

5. The presence of another autosomal allele, *FXIIIA*3*, capable of causing factor XIII deficiency lends further evidence to this disorder being an autosomal recessive disease.

Chapter 5

I. INTRODUCTION

The general reaction and reaction mechanism of transglutaminases was established in the early 1970's (Folk, 1969; Chung *et al.* 1970; Chung and Folk, 1972; Folk and Chung, 1973 and Folk and Finlayson, 1977; also see Chapter 3.IV and Figures 3.2 and 3.3). Specificity studies with glutamine-containing polypeptides, glucagon (Loewy *et al.* 1966) and the acetylated B chain of oxidised insulin (Chung and Folk, 1972) indicated that transglutaminases are specific for the carboxamide groups of glutamine.

Factor XIII catalyses a calcium-dependent acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutamine are the acyl donors. Primary amine groups in a variety of compounds function as acyl acceptors, with the subsequent formation of monosubstituted γ-amides of peptide bound glutamic acid. The acyl receptors of physiological substrates are the ε-amino groups of peptide bound lysine, thus yielding γ-glutamyl-ε-lysyl crosslinks (Folk and Finlayson, 1977).

A variety of substrates, which include the acetylated B chain of oxidised insulin, α-casein, β-casein, γ-casein, k-casein and fibrin as the acyl acceptors and several synthetic amines, in particular ^{14}C methylamine as well as ^{14}C histamine and ^{14}C putrescine as the acyl donors, have been used to confirm that the factor XIII catalysed reaction conforms to the general transglutaminase reaction mechanism (Chung and Folk, 1972; Lorand *et al.* 1972 and Folk and Finlayson, 1977).

The plasma factor XIII catalysed reaction also conforms to Michaelis-Menten kinetics with the various substrates (Lorand *et al.* 1972 and Folk and Finlayson, 1977). Therefore, the Michaelis constant (K_m) has been calculated for a number of these substrates (Table 5.1) as well as a maximum velocity (V_{max}) of 142 n moles of ^{14}C methylamine incorporated into acetylated B chain of oxidised insulin/min/mg of enzyme (Chung, 1975).

All the kinetic studies of factor XIII have been carried out on the purified enzyme from pooled samples. Board (1979) demonstrated that the A subunits of factor XIII, which are the enzymatically active subunits, are genetically polymorphic. Therefore, the previous studies were almost certainly carried out on factor XIII which had a heterogeneous genetic origin. Consequently, it was considered important to attempt to evaluate any possible differences in the biochemical characteristics of the two common types of A subunit, as it is possible that selectable differences may exist between them.

The number of plasma proteins which are potential substrates for factor XIII, for example, fibronectin (Mosher, 1975), α_2-macroglobulin (Mosher, 1976a), fibrin (Lorand *et al.* 1968; Matacic and Loewy, 1968 and Pisano *et al.* 1968) and α_2-plasmin inhibitor (Sakata and Aoki, 1980) mean that the purification of factor XIII is essential for the determination of accurate kinetic values. Fortunately, the gene frequencies of the two common alleles, *FXIIIA*1* and *FXIIIA*2* in the Australian population are such (see Table 3.5) that it was feasible to obtain sufficient plasma from homozygotes for each allele to allow the purification of each type of A subunit. However, the frequency of Type 2 homozygotes is such that the quantity of this protein available limited the number of comparative studies which could be carried out.

TABLE 5.1.

THE K_m VALUES OF PLASMA FACTOR XIII FOR A VARIETY OF SUBSTRATES (Lorand *et al.* 1972 and Folk and Finlayson, 1977)

Substrate	K_m mM
acetylated B chain of oxidised insulin	2.08*
α_{1S}-casein	0.07*
β- casein	0.03*
γ- casein	0.02*
k- casein	0.09*
fibrin (human)	0.04*
^{14}C methylamine	0.62*
^{14}C histamine	0.27†
^{14}C putrescine	0.15†

* at pH 7.5 and 25°C;

† at pH 7.5 and 37°C.

It was decided to use an assay system based on the incorporation of ^{14}C putrescine into a variety of acyl acceptors to compare the K_m's and V_{max}'s of the two genetic forms for each of the substrates. Because fibronectin has been shown to be a substrate for factor XIII (Mosher, 1975) it was planned to compare the reactions of the two types of A subunits with the genetically variant forms of plasma fibronectin. However, an electrophoretic screening program of plasma fibronectin (see Chapter 2) did not show any detectable variation. Therefore, the experiments were only carried out on the normal and dimethyl forms of plasma fibronectin.

In addition, the assay based on the incorporation of ^{14}C putrescine into dimethylcasein was used to determine pH and heat stability curves for the two types of A subunit.

The observation that an apparently unstable factor XIII A subunit, the Type 3 (Castle *et al.* 1981 and Chapter 4), was present in some individuals indicated that some of the biochemical properties of this subunit are different to those of the two common types of A subunits. The rarity of *FXIIIA*3* allele (Chapter 4), has restricted its biochemical characterisation. Homozygotes for *FXIIIA*3* have yet to be detected and it was not possible to obtain a large enough quantity of plasma or platelets from the two known heterozygotes to purify the Type 3 A subunits in sufficient yield to allow extensive kinetic characterisation. Even if extensive quantities of plasma of this type were available, it is unlikely that pure Type 3 homodimers could be prepared since none are found in the 1-3 samples studied so far (Chapter 4). Consequently, the studies reported here were restricted to plasma and samples from one heterozygote were compared with plasma samples from homozygotes for each of the Type 1 and Type 2 proteins for the effects of pH and temperature on the rate of incorporation of ^{14}C putrescine into dimethylcasein.

II. MATERIALS AND METHODS

A. Protein measurement

The method of Lowry *et al.* (1951) was used for studies on factor XIII, while the technique of Bradford (1976) was utilised for the measurement of fibronectin concentrations. Bovine serum albumin was used as a standard in each procedure.

B. SDS electrophoresis

SDS polyacrylamide electrophoresis in the absence of β-mercapto-ethanol, according to the system of Laemmli (1970) with the apparatus O'Farrell (1975) was used to compare the molecular weights of the Type 1 and Type 2 A subunits of plasma factor XIII. After electrophoresis gels were stained in 0.2% (w/v) Coomassie blue R solution (45% (v/v) methanol; 10% (v/v) acetic acid; 45% (v/v) water) for 1-3 hours. Gels were then placed in a minimum of 500 ml of destain solution (25% (v/v) ethanol; 10% (v/v) acetic acid; 65% (v/v) water).

C. Agarose gel electrophoresis

The electrophoretic system of Board (1979) was used to determine the factor XIII A subunit phenotypes of plasma samples and to check the purification fractions for enzyme activity. The specific fluor-escent stain was the same as that described in Chapter 4.

D. Plasma fibronectin purification

Plasma fibronectin was purified by the method described in Chapter 2, except that the fibronectin was dialysed against distilled water to remove salts. The fibronectin was then lyophilised and stored at -20^{o}C until use. The fibronectin obtained by this procedure was essentially pure and therefore suitable as a substrate in factor XIII assays (see Chapter 2).

E. Preparation of N,N-Dimethyl proteins

The preparation of N,N-dimethylcasein from casein powder (Sigma) and N,N-dimethylfibronectin from plasma fibronectin was by the method of Lin *et al.* (1969). The lyophilised samples were stored at -20°C until use.

F. Factor XIII assay

Thrombin activated plasma factor XIII activity was determined by a modification of the procedure described by Dvilansky *et al.* (1970). The standard enzyme activation mixture which consisted of 25µl 0.1M $CaCl_2$ (in 0.3M Tris.HCl pH 7.5), 25µl 0.2M dithiothreitol (DTT) (neutralised), 25µl enzyme, 50µl 0.3M Tris.HCl pH 7.5 and 25µl thrombin (50 units/ml physiological saline) was incubated at 37°C for 30 minutes, at which time 100µl of a solution containing 3 parts 10mg/ml dimethylcasein (in 0.3M Tris.HCl pH 7.5) to one part 10mM ^{14}C putrescine dihydrochloride (New England Nuclear 0.5 m Ci/mM) was added. The reaction was carried out at 37°C and the rate was determined by transferring 25µl aliquots of the reaction mixture onto 2cm Whatman 3MM chromatography paper discs which were immediately quenched in 5% TCA (w/v) and processed as previously described by Mans and Novelli (1961). Aliquots were removed at time zero and after 30 minutes and the reaction rates were calculated by the subtraction of the zero time value from the value at 30 minutes. Previous pilot studies indicated that the reaction rate was linear for at least 150 minutes.

The dimethylcasein was replaced by α-casein, casein powder, fibronectin and dimethylfibronectin for the determination of the K_m and V_{max} for these substrates.

Inactivation of plasma factor XIII by iodoacetamide was examined by adding various levels of iodoacetamide, 0.1 to 3 µM, to the initial activation mixture.

For those assays where plasma samples were studied, the plasma samples were heated at 56^{o}C for 3 minutes and centrifuged at 2000g for 5 minutes to remove fibrinogen.

G. pH curves

Solutions containing 200µl of 0.2M DTT (neutralised), 200µl of 10mM ^{14}C putrescine hydrochloride, 600µl of 10mg/ml dimethylcasein (or 10mg/ml fibronectin) and 400µl of buffer (0.3M Tris, 0.3M imidazol, 0.3M glycine) were prepared at pH's between pH 6 and pH 11.5.

An activation mixture of 25µl 0.1M $CaCl_2$, 25µl thrombin and 25µl enzyme was incubated at 37^{o}C for 30 minutes, after which 175µl of each buffered pH solution was added. The reaction rate was determined as described above.

H. Heat stability curves

Enzyme samples were either incubated at a range of temperatures for 10 minutes before use or incubated at one temperature for 90 minutes. In the latter procedure 30µl aliquots were taken every 15 minutes and placed on ice until use. The assay was as described above.

I. Origin of plasma samples

Plasma was obtained by the procedure described in Chapter 2. Each plasma sample was typed by agarose electrophoresis and stained for factor XIII activity. Type 1 samples and Type 2 samples were both pooled and stored overnight at 4^{o}C before purification.

J. Plasma factor XIII purification

Plasma factor XIII was purified by a modification of the procedure described by Lorand and Gotah (1970). The protein fraction precipitated between 16 and 20% of saturation with ammonium sulphate

(4^{o}C) was taken. The precipitates were collected by centrifugation at 10,800g for 20 minutes and dissolved in a 50mM Tris.HCl buffer, pH 7.0, containing 1mM EDTA and 0.1M NaCl. The solution, containing factor XIII, was heated to 56^{o}C, by immersion in an 80^{o}C waterbath, for 3 minutes to remove fibrinogen. The solution was centrifuged as above and the precipitate discarded after squeezing to remove any trapped soluble enzyme.

Saturated ammonium sulphate was added to the supernatant to a final saturation of 30%. After 1 hour at 4^{o}C the enzyme solution was centrifuged as described above and the supernatant was discarded. The precipitate was dissolved in a minimal volume of 50mM Tris.HCl buffer, pH 7.5, containing 1mM EDTA and dialysed overnight against 1000 volumes of the same buffer.

After centrifugation, the dialysed enzyme was applied to a DEAE-cellulose column equilibrated against 50mM Tris.HCl buffer, pH 7.5 containing 1mM EDTA (DE52, 2.0 x 80 cm) and the column was washed with 720 ml of equilibration buffer. The column was then developed with a linear gradient (total volume, 600 ml), of 0 to 0.3M NaCl, in the equilibration buffer.

Fractions of 10ml were collected and tested for protein at $O.D_{280}$. Tubes with protein were tested for factor XIII activity by the electrophoretic system described above and protein content by SDS polyacrylamide electrophoresis.

Tubes with pure factor XIII, as judged by SDS polyacrylamide electrophoresis were pooled and saturated ammonium sulphate was added to a final saturation of 36%. After 1 hour at 4^{o}C, the solution was centrifuged as above and the supernatant discarded. The precipitate was dissolved in a minimal amount of 50mM Tris.HCl buffer, pH 7.5 containing 1mM EDTA and 50mM NaCl and dialysed overnight against 5000

volumes of the same buffer. The enzyme solution was stored in 1 ml aliquots at $4^{o}C$.

III. RESULTS

A. Plasma factor XIII purification

The results of the isolation of plasma factor XIII are summarised in Tables 5.2 and 5.3. The two types of A subunit behaved in a similar manner in the purification procedure with the final enzyme preparations having very similar specific activities. The specific activities for the Type 1 and Type 2 subunits were 98.22 and 94.00 units/mg respectively (units of activity are defined in Table 5.2). The behaviour of the subunits on the DEAE chromatography column is shown in Figure 5.1.

SDS polyacrylamide electrophoresis showed that the preparations of both types of subunits were clear of any major contaminants (Figure 5.2). The three bands in each preparation represent the A and B subunits of plasma factor XIII. Because factor XIII has been shown to migrate on SDS gels to positions which give anomalous molecular weights, compared to those obtained by sedimentation equilibrium (Schwartz *et al.* 1973), SDS gels were used to compare molecular weights only. It can be seen from Figure 5.2 that there is no obvious difference in the molecular weights of the Type 1 and Type 2 A subunits.

B. The factor XIII assay

The Ca^{2+} requirement for the activation of factor XIII are different to that for the catalytic reaction of the enzyme (Chung and Folk, 1972 and Cooke, 1974) and therefore the assay system used was modified from that described in Chapter 4 to allow the enzyme to be activated before the reaction was started. The use of an activation mixture also eliminated the lag-phase in the reaction rate described in Chapter 4 and thus allowed the reaction rate to be calculated from

TABLE 5.2.

PURIFICATION OF FACTOR XIII FROM HUMAN PLASMA: TYPE 1 A SUBUNITS

Procedure	Volume ml	Total Activity units*	Total Protein mg	Specific Activity units/mg†	Purification Factor	Yield %
Citrated Plasma	2000	2424.0	101000.00	0.02	1	100.0
Ammonium sulphate (16-20%)	100	2152.8	1380.00	1.56	60	88.8
Heat treatment and ammonium sulphate (30%)	12	1404.4	24.96	64.23	2635	57.9
DEAE chromatography and ammonium sulphate (36%)	3	957.6	9.75	98.22	4087	39.3

* 1 unit of activity = 1 nM of ^{14}C putrescine incorporated into dimethylcasein/min

† Specific activity is per mg of plasma factor XIII zymogen

TABLE 5.3.

PURIFICATION OF FACTOR XIII FROM HUMAN PLASMA: TYPE 2 A SUBUNITS

Procedure	Volume ml	Total Activity units*	Total Protein mg	Specific Activity units/mg†	Purification Factor	Yield %
Citrated plasma	1000	1856.0	58000.00	0.03	1	100.0
Ammonium sulphate (16-20%)	50	1103.6	680.00	1.62	51	59.4
Heat Treatment and ammonium sulphate (30%)	18	777.6	31.68	24.55	767	41.6
DEAE chromatography and ammonium sulphate (36%)	2	413.6	4.40	94.00	2938	22.3

* 1 unit of activity = 1ng of ^{14}C putrescine incorporated into dimethylcasein/min

† Specific activity is per mg of plasma factor XIII zymogen

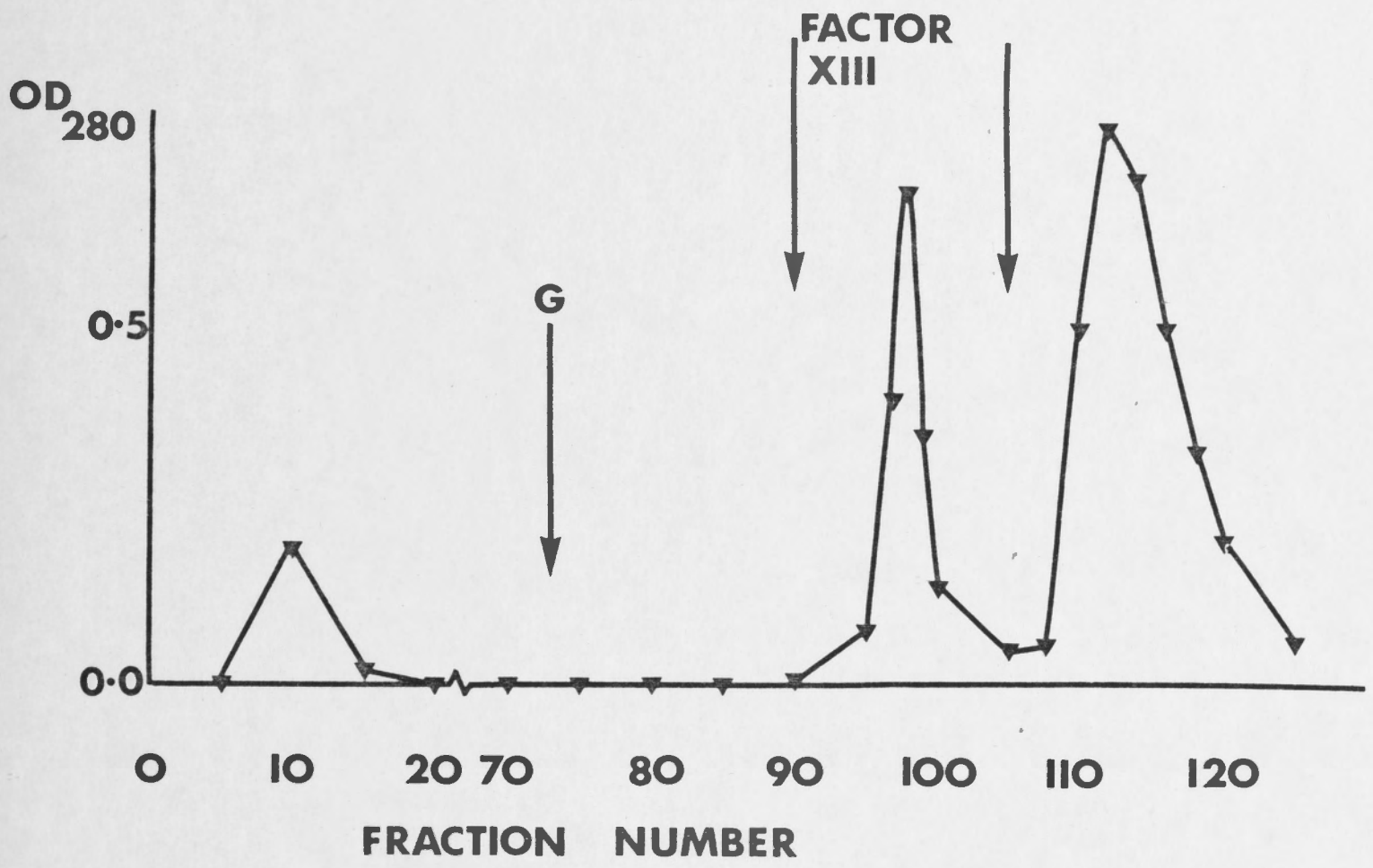
Figure 5.1: THE BEHAVIOUR OF THE TYPE 1 AND TYPE 2 A SUBUNITS OF PLASMA FACTOR XIII ON A DEAE CELLULOSE CHROMATOGRAPHY COLUMN

a) Type 1 A subunits

b) Type 2 A subunits

G = salt gradient applied; fraction volume was 10 ml.

a


FACTOR
XIII
OD 280
0·5
G
0·0
0
10
20
70
80
90
100
110
120
FRACTION NUMBER

b

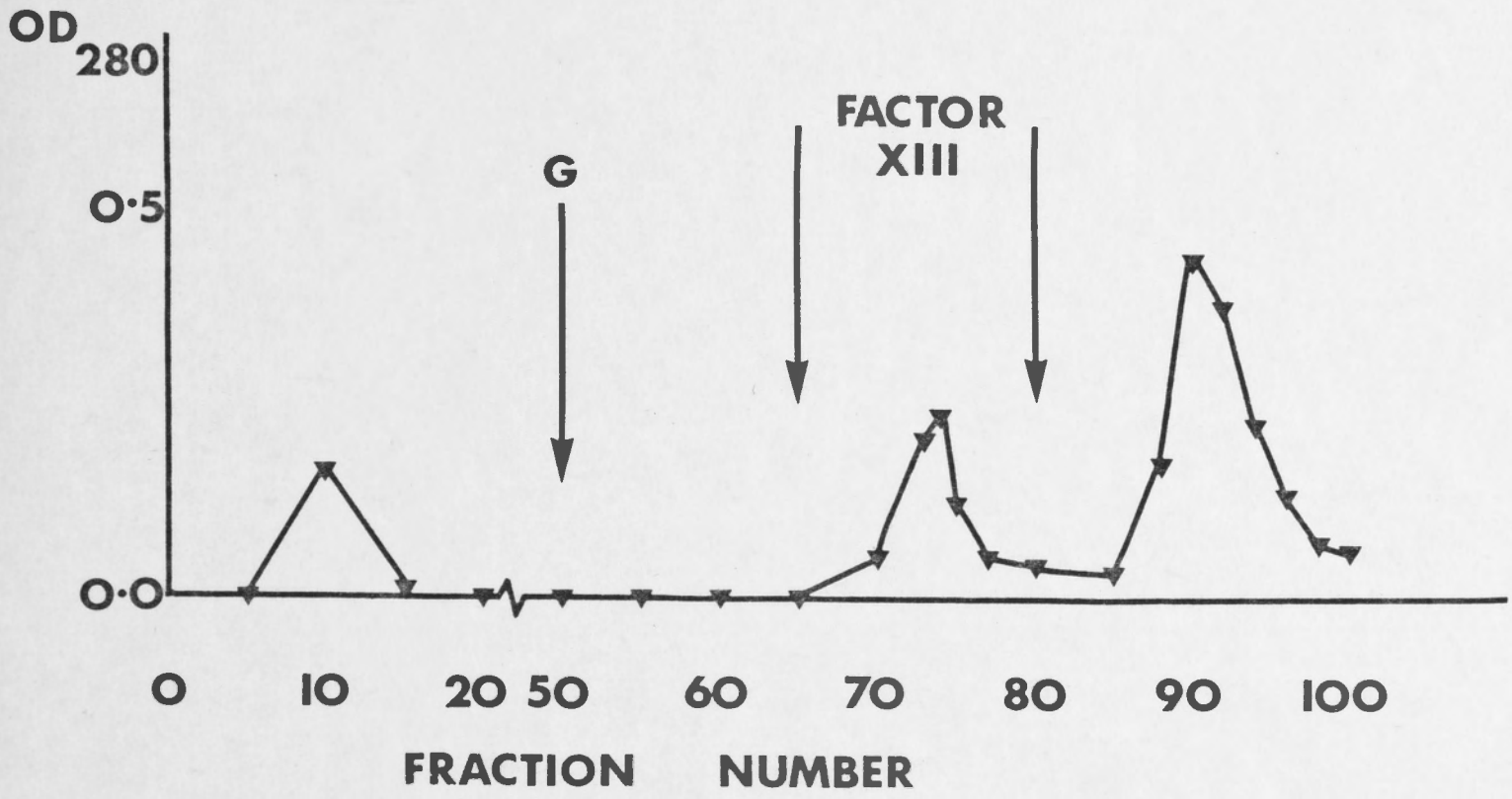
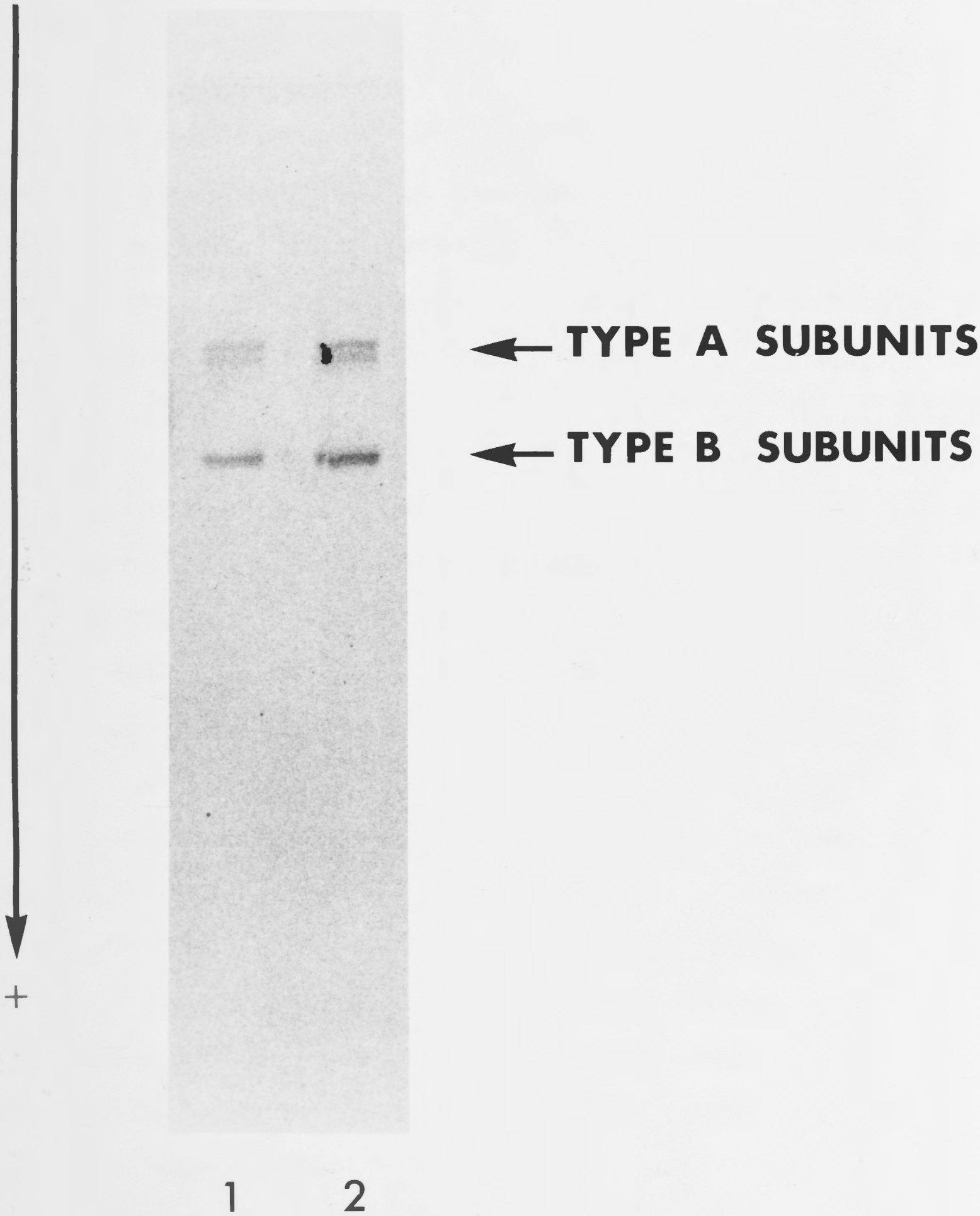

OD 280
0·5
G
FACTOR
XIII
0·0
0
10
20
50
60
70
80
90
100
FRACTION NUMBER

Figure 5.2: SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED FACTOR XIII FROM PLASMA

1 = Type 1 A subunits; 2 = Type 2 A subunits


TYPE A SUBUNITS
TYPE B SUBUNITS
+
1
2

readings taken at time zero and after 30 minutes.

All the results described below are the average values from duplicate samples which were used in all experiments.

C. Purified factor XIII

1. Substrate studies

The kinetic constants obtained for the Type 1 and Type 2 A subunits of plasma factor XIII for α-casein, casein powder, dimethylcasein, fibronectin, dimethylfibronectin, ^{14}C putrescine and Ca^{2+} are given in Table 5.4. The kinetic constants were obtained from the plot of the equation described by Hanes (1932):

$$\frac{S}{v} = \frac{K_m}{V} + \frac{1}{V} \cdot S$$

as plotted by Dixon and Webb (1966). For V_{max} the values from the plots were corrected for Zymogen concentration. The plots are shown in Figures 5.3-5.9.

2. Inactivation by iodoacetamide

Figure 5.10 illustrates that the effect of iodoacetamide inactivation was the same for both the Type 1 and Type 2 A subunits.

3. pH curve

The pH curves for the two types of A subunits with dimethylcasein as the acyl acceptor are shown in Figure 5.11. All the points are calculated as a percentage of the enzyme activity at pH 8.32. There was a small, but repeatable difference between the pH optima of the enzyme preparations in three separate experiments. The pH optima for the Type 1 and Type 2 A subunits were approximately pH 8.74 and pH 8.32 respectively.

If fibronectin was used as the acyl acceptor the pH optima were approximately pH 8.34 for both types of A subunits (Figure 5.12).

4. Heat stability

The heat stability curves from 20^{o}-$70^{o}C$ for the two enzyme

TABLE 5.4.

KINETIC CONSTANTS FOR THE TYPE 1 AND TYPE 2 A SUBUNITS OF PLASMA FACTOR XIII

Substrate	K_m*		V_{max}†	
	Type 1	Type 2	Type 1	Type 2
α-casein	0.72	0.70	85.23	84.72
casein powder	0.58	0.54	86.34	85.29
dimethylcasein	0.95	1.00	90.03	90.82
fibronectin	0.54	0.55	49.48	42.84
dimethylfibronectin	0.41	0.41	48.68	49.38
^{14}C putrescine††	0.45	0.45	88.80	85.10
Ca^{2+} ††	0.10	0.10	-	-

* Km measured in mg/ml except for ^{14}C putrescine and Ca^{2+} which are mM

† V_{max} measured in nM ^{14}C putrescine incorporated/min/mg zymogen

†† dimethylcasein was the acyl acceptor

Figures 5.3-5.6: PLOTS OF s AGAINST s/v FOR THE INCORPORATION OF ^{14}C PUTRESCINE INTO VARYING CONCENTRATIONS OF:

5.3: α-casein

5.4: casein powder

5.5: dimethylcasein

5.6: fibronectin

▽ = Type 1 A subunits

○ = Type 2 A subunits

Figure 5.3

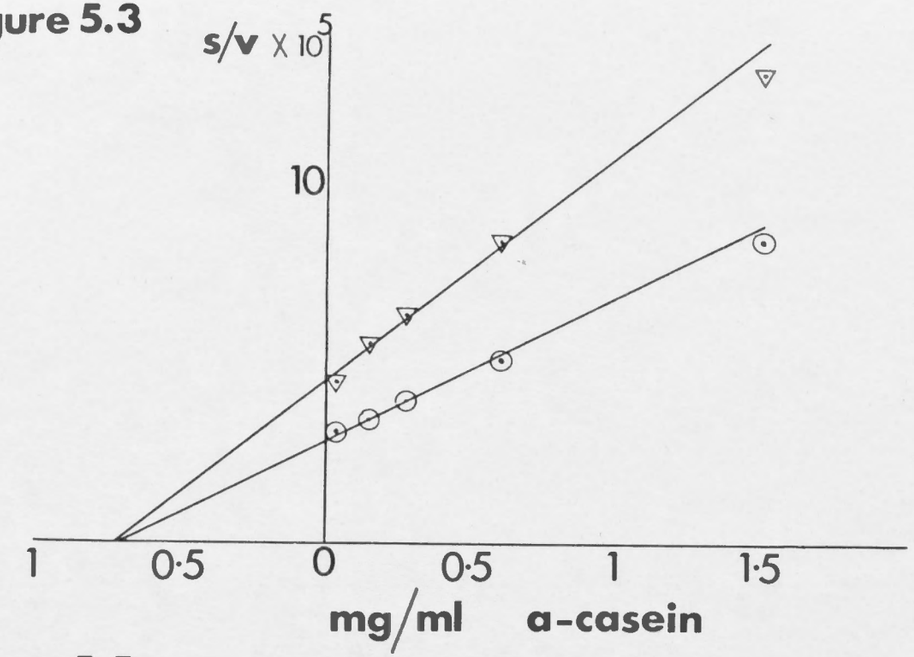


Figure 5.4

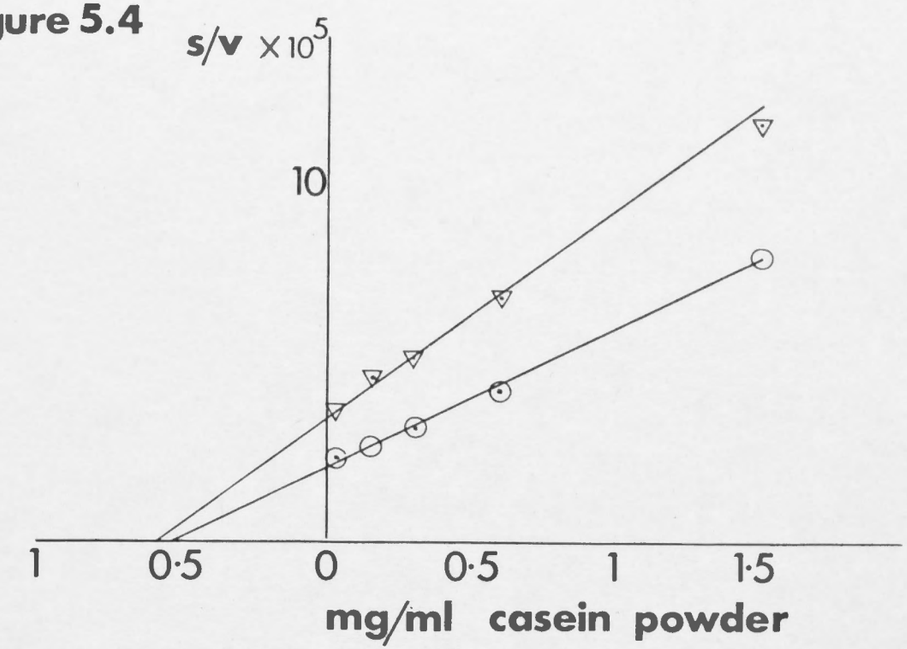


Figure 5.5

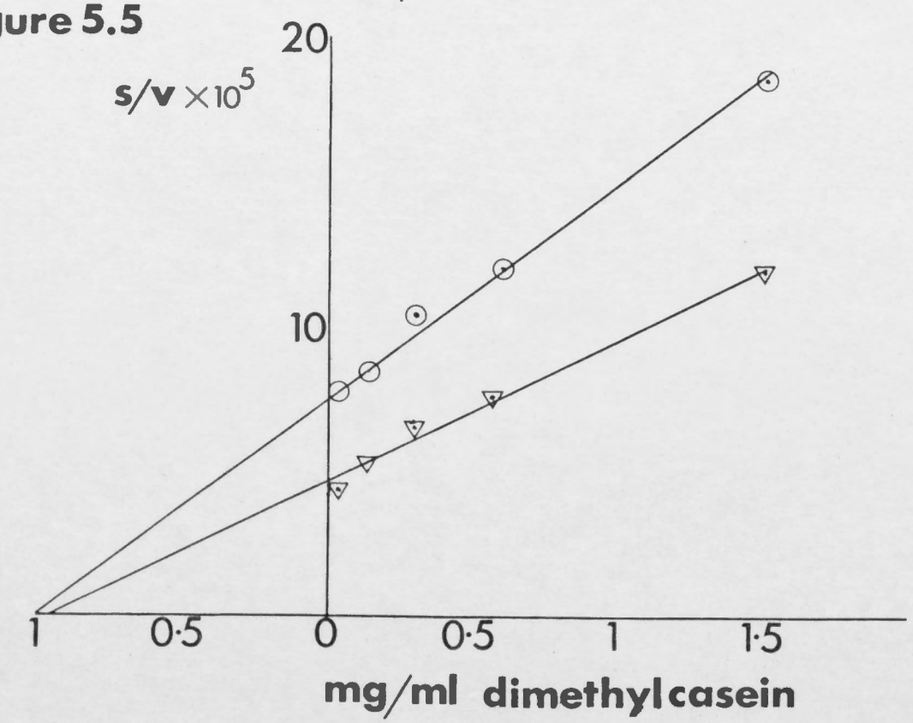


Figure 5.6

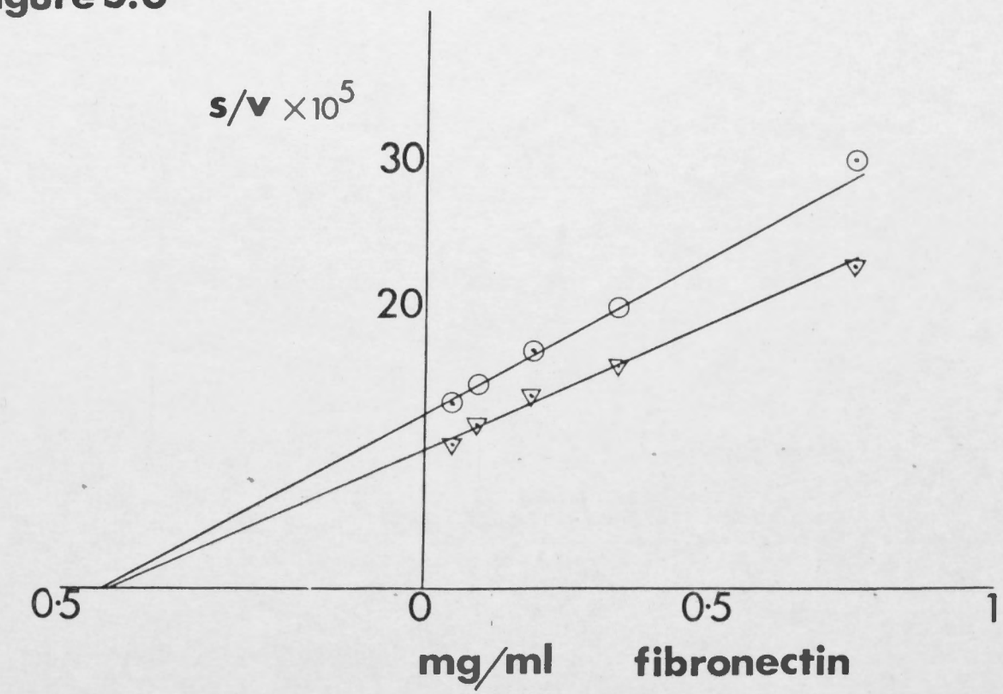


Figure 5.7: A PLOT OF s AGAINST s/v FOR THE INCORPORATION OF ^{14}C PUTRESCINE INTO VARYING CONCENTRATIONS OF DIMETHYL-FIBRONECTIN

Figure 5.8: A PLOT OF s AGAINST s/v FOR THE INCORPORATION OF VARYING CONCENTRATIONS OF ^{14}C PUTRESCINE INTO DIMETHYL-CASEIN

Figure 5.9: A PLOT OF s AGAINST s/v FOR THE INCORPORATION OF ^{14}C PUTRESCINE INTO DIMETHYLCASEIN AT VARYING Ca^{2+} CONCENTRATIONS

Figure 5.10: FACTOR XIIIa INACTIVATION BY IODOACETAMIDE

FOR FIGURES 5.7-5.10 ▽ = Type 1 A subunits

○ = Type 2 A sububits

Figure 5.7

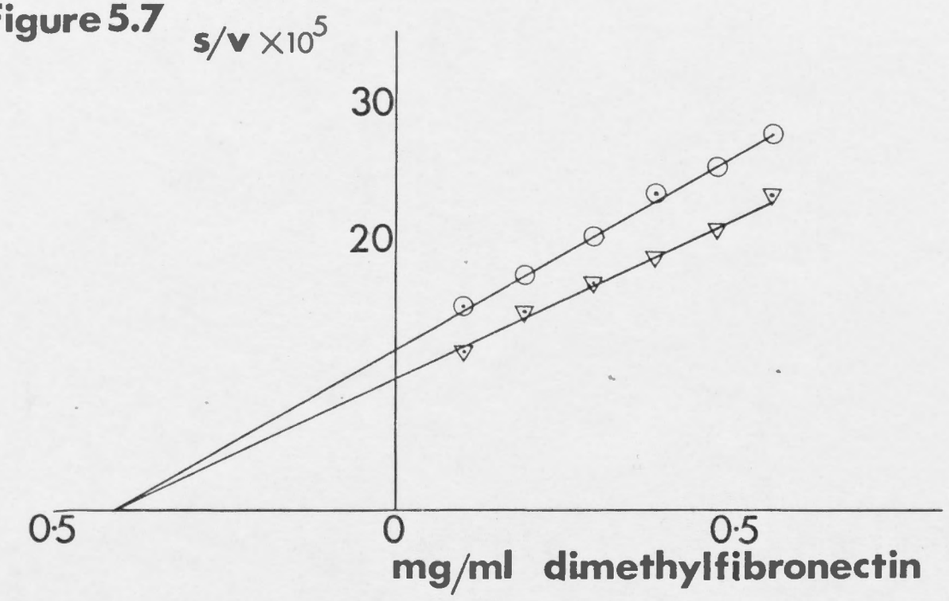


Figure 5.8

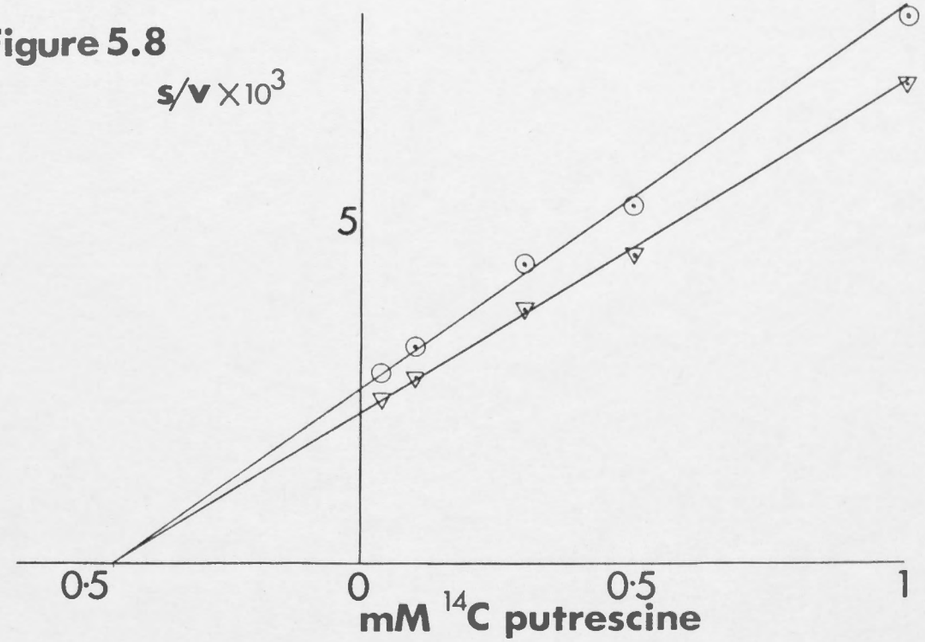


Figure 5.9

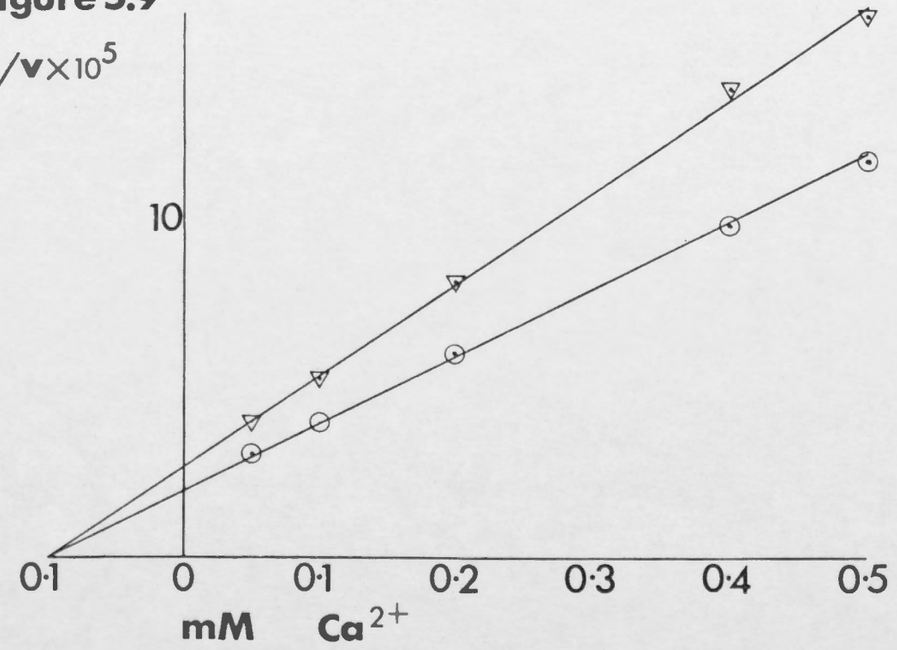


Figure 5.10

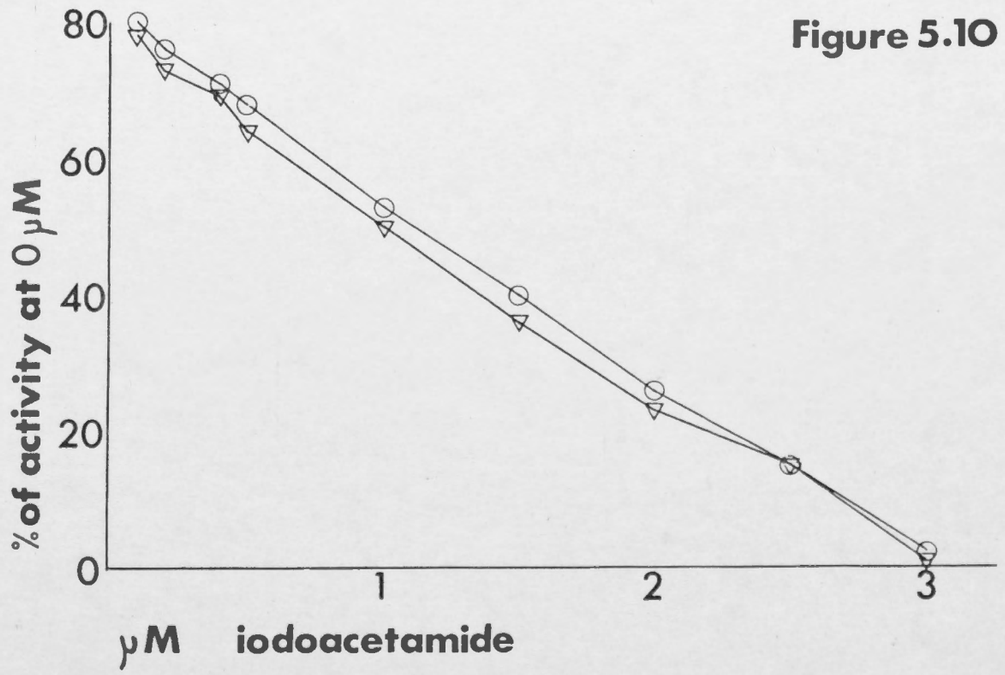
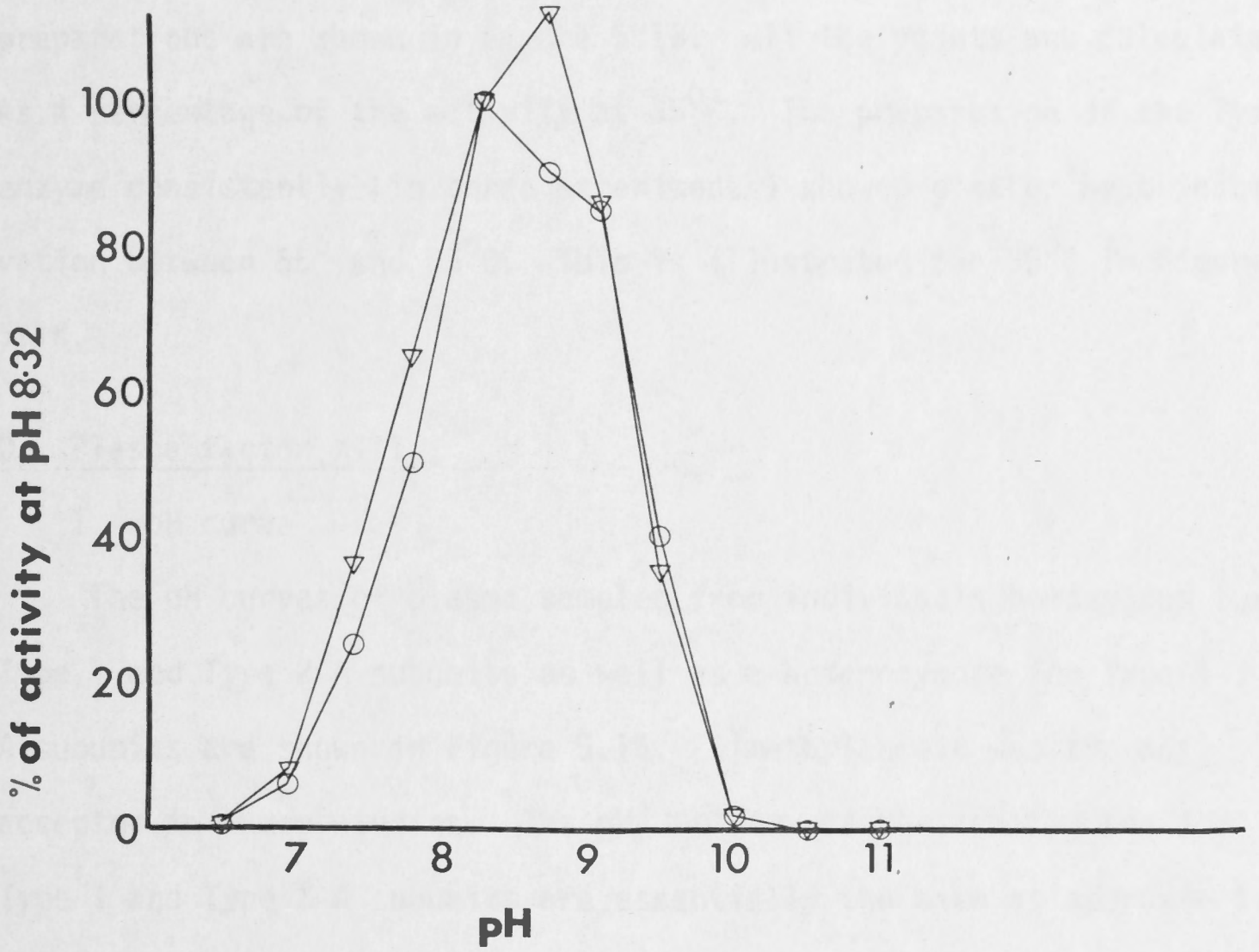


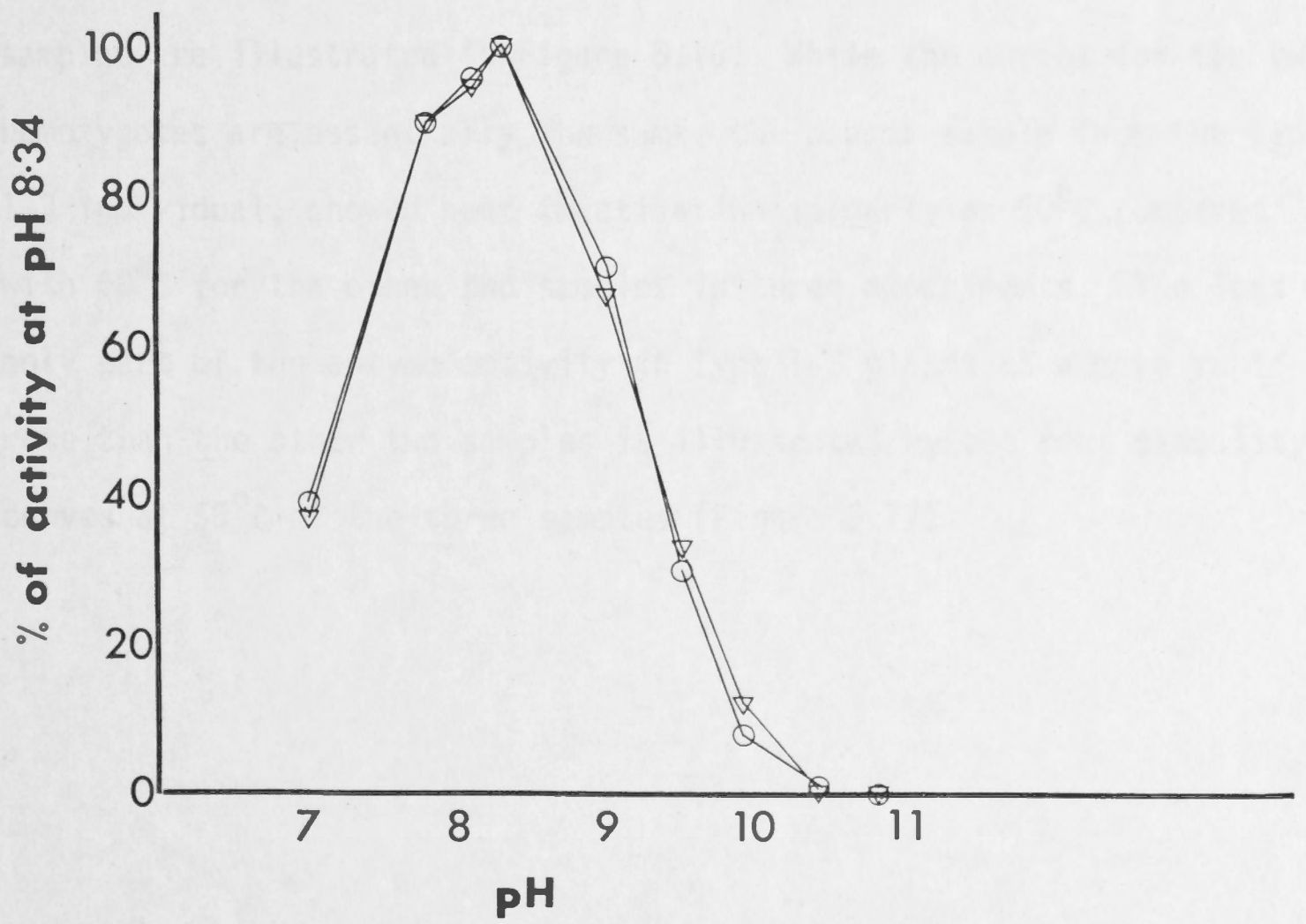
Figure 5.11: THE pH CURVES FOR PURIFIED SAMPLES OF THE TWO COMMON TYPES OF A SUBUNIT WITH DIMETHYLCASEIN AS THE ACYL ACCEPTOR

▽= Type 1 A subunits; O = Type 2 A subunits

Figure 5.12: THE pH CURVES FOR PURIFIED SAMPLES OF THE TWO COMMON TYPES OF A SUBUNIT WITH FIBRONECTIN AS THE ACYL ACCEPTOR

▽ = Type 1 A subunits; O = Type 2 A subunits


% of activity at pH 8·32
100
80
60
40
20
0
7
8
9
10
11
pH


% of activity at pH 8·34
100
80
60
40
20
0
7
8
9
10
11
pH

preparations are shown in Figure 5.13. All the points are calculated as a percentage of the activity at 35°C. The preparation of the Type 2 enzyme consistently (in three experiments) showed greater heat inactivation between 55° and 65°C. This is illustrated for 55°C in Figure 5.14.

D. Plasma factor XIII

1. pH curve

The pH curves of plasma samples from individuals homozygous for Type 1 and Type 2 A subunits as well as a heterozygote for Type 1-3 A subunits are shown in Figure 5.15. Dimethylcasein was the acyl acceptor in these studies. The pH optima of the homozygotes for Type 1 and Type 2 A subunits are essentially the same at approximately pH 8.38. The Type 1-3 plasma, however, showed two pH peaks at pH 8.38 and pH 9.10 in three separate experiments.

2. Heat stability

The heat stability curves from 40°-65°C for the above three samples are illustrated in Figure 5.16. While the curves for the two homozygotes are essentially the same, the plasma sample from the Type 1-3 individual, showed heat inactivation as early as 50°C compared with 60°C for the other two samples in three experiments. The loss of only part of the enzyme activity in Type 1-3 plasma at a more rapid rate than the other two samples is illustrated by the heat stability curves at 55°C of the three samples (Figure 5.17).

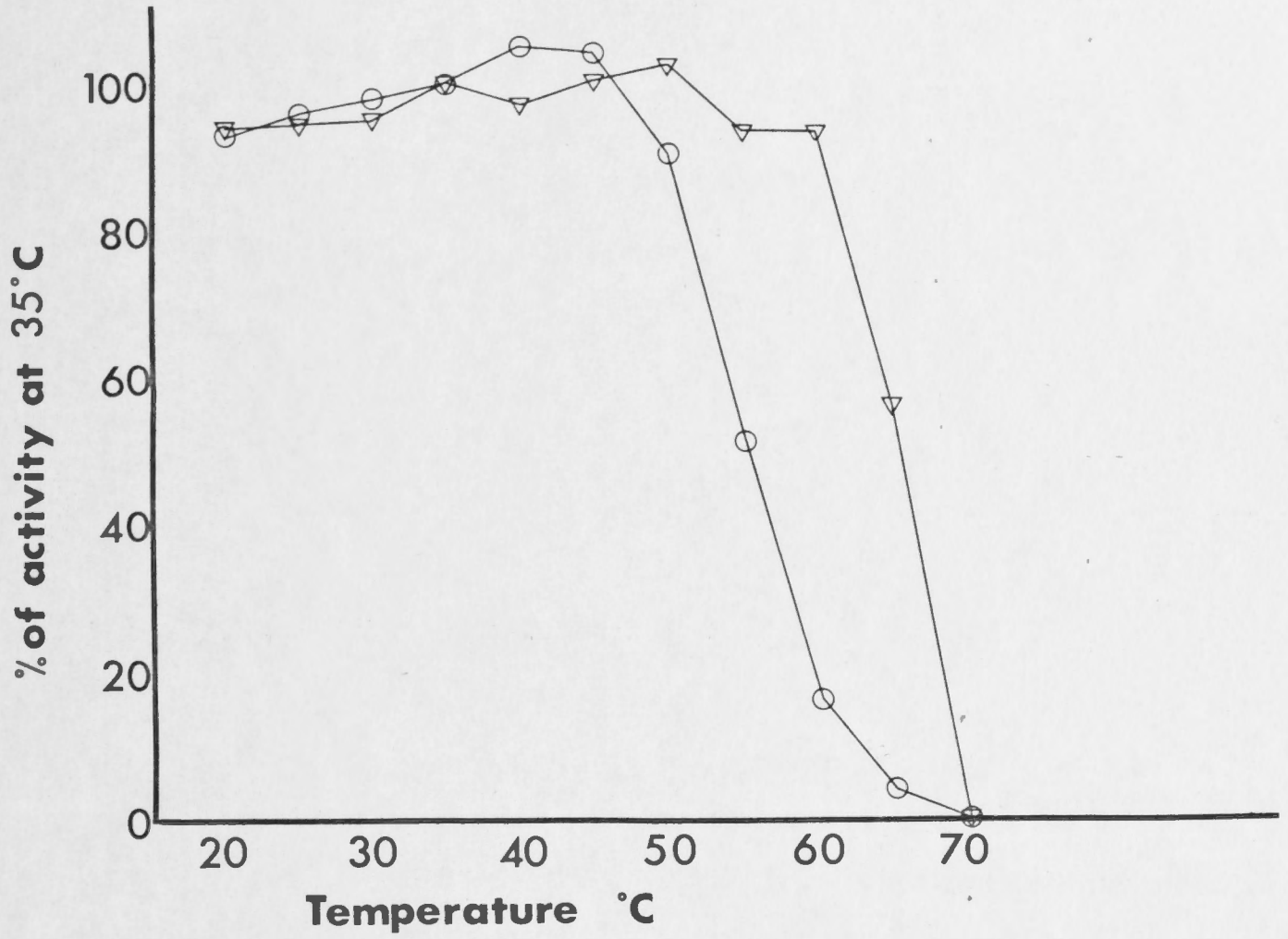
Figure 5.13: THE EFFECT OF TEMPERATURE ON THE ENZYME ACTIVITY OF PURIFIED SAMPLES OF THE TYPE 1 AND TYPE 2 A SUBUNITS OF FACTOR XIII

The A subunits were incubated for 10 minutes at the specific temperatures before the assays were started.

▽= Type 1 A subunits; O = Type 2 A subunits

Figure 5.14: THE INACTIVATION OF PURIFIED SAMPLES OF FACTOR XIII TYPE 1 and TYPE 2 A SUBUNITS AT $55^{o}C$

▽ = Type 1 A subunits; O = Type 2 A subunits


% of activity at 35°C
100
80
60
40
20
0
20
30
40
50
60
70
Temperature °C

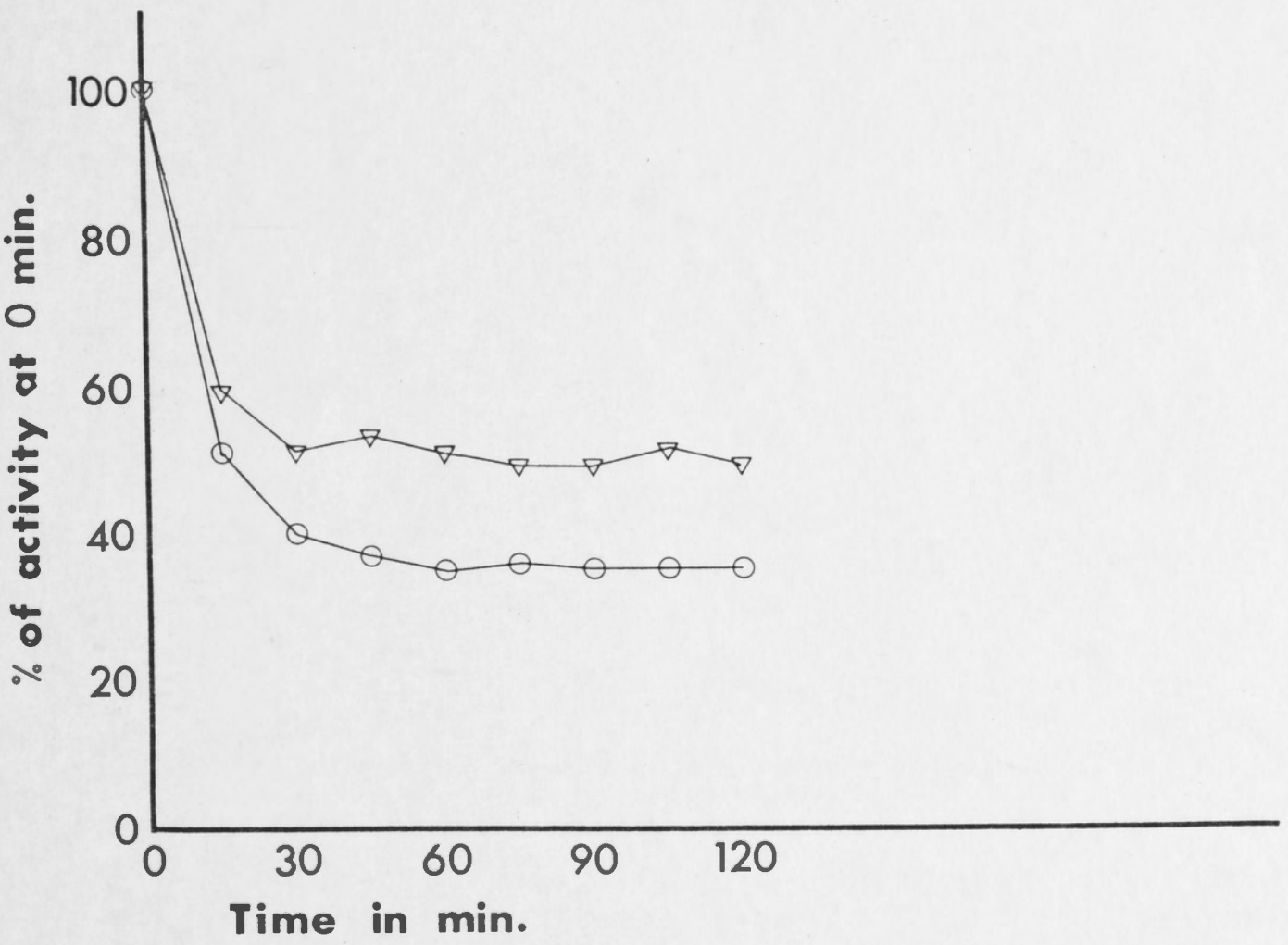

% of activity at 0 min.
100
80
60
40
20
0
0
30
60
90
120
Time in min.

Figure 5.15: THE pH CURVES OF UNPURIFIED PLASMA SAMPLES OF TYPE 1, TYPE 2 AND TYPE 1-3 A SUBUNITS OF FACTOR XIII

▽ = Type 1 A subunits; ○ = Type 2 A subunits;

□ = Type 1-3 A subunits.

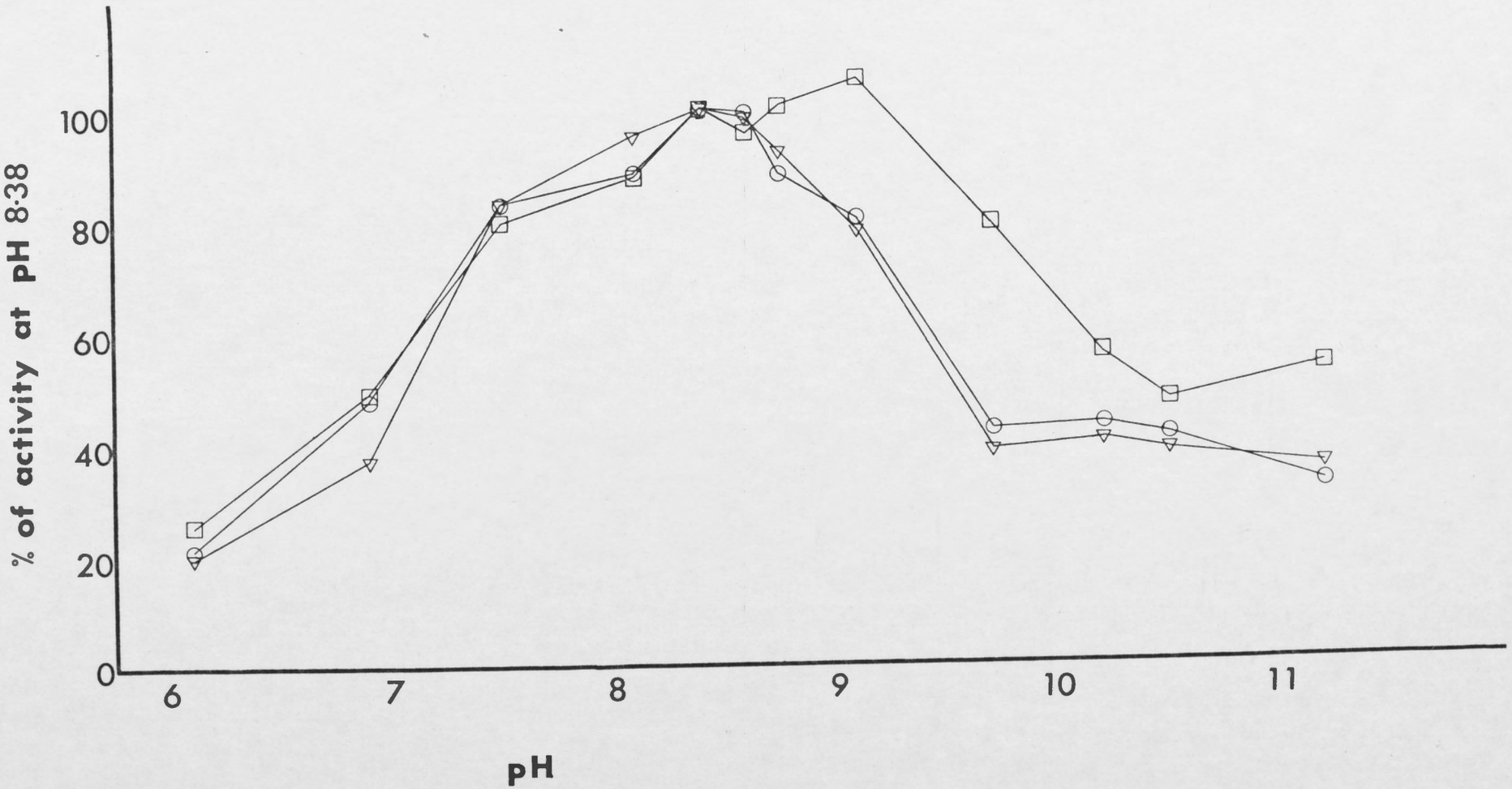

% of activity at pH 8·38
100
80
60
40
20
0
6
7
8
9
10
11
pH

Figure 5.16: THE EFFECT OF TEMPERATURE ON UNPURIFIED PLASMA SAMPLES OF TYPE 1, TYPE 2 AND TYPE 1-3 A SUBUNITS OF FACTOR XIII

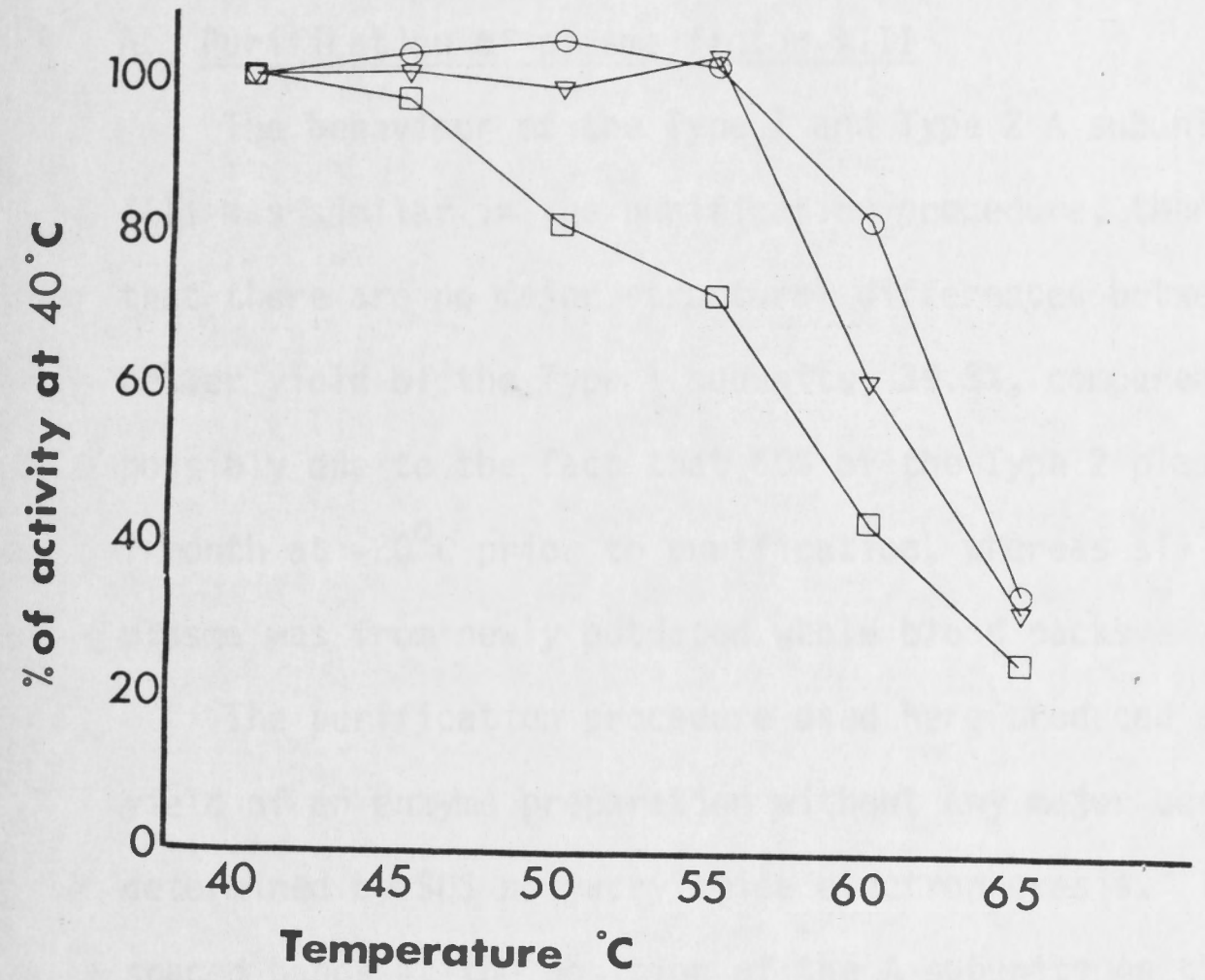
▽ = Type 1 A subunits; O = Type 2 A subunits;

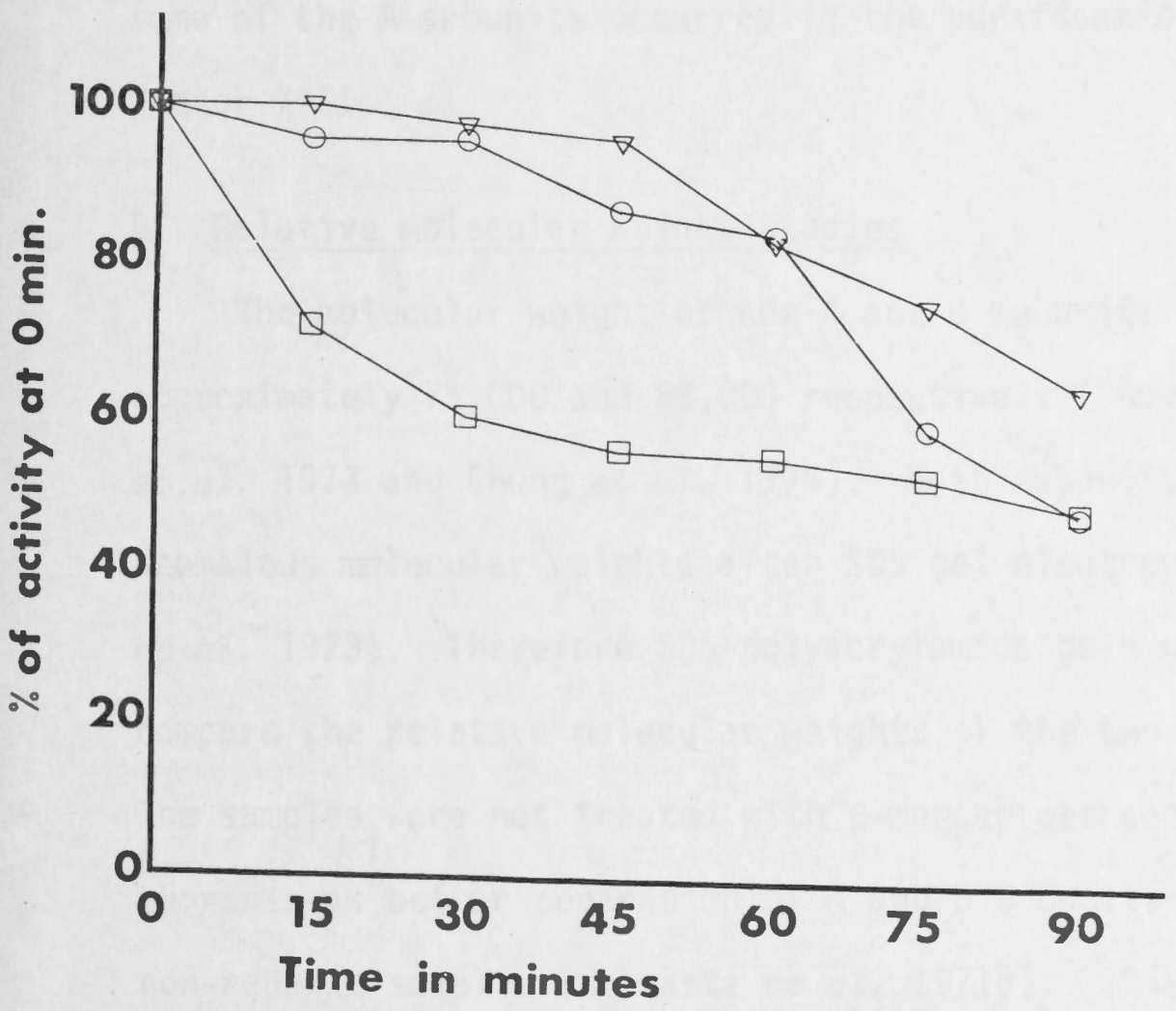
□ = Type 1-3 A subunits

Figure 5.17: THE INACTIVATION OF UNPURIFIED PLASMA SAMPLES OF TYPE 1 TYPE 2 and TYPE 1-3 A SUBUNITS OF FACTOR XIII AT $55^{o}C$.

▽ = Type 1 A subunits O = Type 2 A subunits;

□ = Type 1-3 A subunits


100
80
60
40
20
0
% of activity at 40°C
40
45
50
55
60
65
Temperature °C


100
80
60
40
20
0
% of activity at 0 min.
0
15
30
45
60
75
90
Time in minutes

IV. DISCUSSION

A. Purification of plasma factor XIII

The behaviour of the Type 1 and Type 2 A subunits of plasma factor XIII was similar in the purification procedure, therefore suggesting that there are no major structural differences between them. The better yield of the Type 1 subunits, 39.3%, compared to 22.3%, was possibly due to the fact that 60% of the Type 2 plasma was stored for 1 month at -20^{o}C prior to purification, whereas all of the Type 1 plasma was from newly outdated whole blood packs.

The purification procedure used here produced a relatively high yield of an enzyme preparation without any major contaminants, as determined by SDS polyacrylamide electrophoresis. The two closely spaced bands at the position of the A subunits on the polyacrylamide gel (Figure 5.2) are thought to represent the normal A subunits and activated A subunits with a 4000 M.W. peptide cleaved off. The activation of some of the A subunits occurred in the purification of both types of factor XIII.

B. Relative molecular weight studies

The molecular weight of the A and B subunits has been shown to be approximately 75,000 and 88,000 respectively (Chung, 1972; Schwartz *et al*. 1973 and Chung *et al*. 1974). Both subunits are known to give anomalous molecular weights after SDS gel electrophoresis (Schwartz *et al*. 1973). Therefore SDS polyacrylamide gels were only used to compare the relative molecular weights of the two types of A subunit. The samples were not treated with β-mercaptoethanol prior to electrophoresis as better separation of A and B subunits is obtained from non-reduced samples (Schwartz *et al*. 1971a). This finding was confirmed by studies not shown here.

It can be seen from Figure 5.2 that there was no detectable mobility difference between the two enzyme preparations. This result indicates that the molecular weights of the Type 1 and Type 2 A subunits are essentially the same. The occurrence of similar molecular weights for the two types of A subunit suggests that the difference in the alleles for each subunit has arisen by point mutation, leading to a single amino acid substitution in the subunits themselves. A frame shift mutation or an unequal crossing over event resulting in a deletion or insertion would be expected to cause changes in the subunits which would result in detectable molecular weight differences.

C. A comparison of the Type 1 and Type 2 A subunits

1. Substrate studies

If the initial rate of an enzyme reaction is measured at a series of substrate concentrations, it is possible to obtain values for V_{max} the maximum rate attained by the reaction at saturating substrate concentrations and K_m, the Michaelis constant, which is the concentration when the rate is half the maximum attainable rate.

The K_m and V_{max} values for the purified Type 1 and Type 2 A subunits, for the substrates tested, α-casein, casein powder, dimethylcasein, fibronectin, dimethylfibronectin and Ca^{2+}, were similar for each substrate. These results indicate that the mutational event that gave rise to the two forms of A subunits does not effect their reaction kinetics at pH 7.5 and 37°C. Therefore, in these conditions the probable amino acid substituion does not effect the active site, the substrate binding sites or the conformation of factor XIIIa. Board *et al.* (1980) have shown that the reaction rates for the incorporation of ^{14}C putrescine into α-casein are the same for unpurified plasma samples of both types of enzyme. The possibility that the mutation occurred in the part of the gene coding for the 4000 M.W. peptide lost in the activation process was ruled out by Board (1979).

The N'N-dimethyl forms of both casein and fibronectin were used in these studies as the ε-amino groups of lysine residues of these modified proteins are blocked. Therefore, they should not be cross-linked to themselves by the action of factor XIII, which could occur when the unmodified proteins are used as substrates.

The K_m value for Ca^{2+} of 0.10mM is in reasonable agreement with the value of Cooke (1974) of 0.17mM, while the K_m value for ^{14}C putrescine of 0.45mM is three times that of the value of 0.15mM obtained by Lorand *et al.* (1972). However, the latter value was obtained from plasma samples which contain several other possible substrates for factor XIII.

2. Inactivation by iodoacetamide

Chung *et al.* (1974) examined the effects of low levels of ^{14}C iodoacetamide on plasma and platelet factor XIII. In both cases it was shown that the loss of catalytic activity of factor XIII, in the presence of Ca^{2+}, was accompanied by the incorporation of ^{14}C iodoacetamide at the sulphydryl group of a cysteine residue. The addition of iodoacetamide to the enzyme activation mixture produced similar results for both forms of the plasma enzyme (Figure 5.10). This result indicates that iodoacetamide is able to bind to the active site of both forms of the enzyme in a similar manner at pH 7.5 and 37°C, which suggests that the conformation of the A subunits around the active site is similar in both types of A subunit.

3. pH

An enzyme is only active as a catalyst within a relatively narrow range of pH and usually the enzyme is most active at a specific pH, called the optimum pH. Two explanations are possible for this phenomenon (Wynn, 1973). The first considers that at pH values removed from the optimum the enzyme starts to lose the tertiary structure necessary for the conformation of the active site. The

second, and more probable explanation in most cases, is that the pH affects the ionisation of acidic and basic groups at the active centre of the enzyme.

The purified preparations of the two types of A subunit showed a slight, but consistent, difference in their pH optima when dimethylcasein was used as a substrate. The pH optima were, on three occasions, approximately pH 8.74 and pH 8.32 for the Type 1 and Type 2 A subunits respectively. When fibronectin was substituted for dimethylcasein, the pH optimum for the Type 1 A subunits dropped to the same value as that for the Type 2 A subunits, approximately pH 8.34. When unpurified plasma factor XIII was used with dimethylcasein as a substrate the pH optima were similar for the two types of A subunit at approximately pH 8.38.

The reason that the value for the purified Type 1 A subunits, with dimethylcasein as a substrate, differs from all the other values is difficult to explain exactly. It is possible that the charge difference of the Type 1 enzyme, compared to the Type 2 enzyme apparent during electrophoresis at a pH around 8.6 (Chapter 4) results in either the active site or the substrate binding site of the Type 1 form having a greater affinity for dimethylcasein at pH 8.74. The fact that this difference was not evident when unpurified plasma samples were used suggests that plasma factors interact with factor XIII in such a manner as to negate the difference between the two forms of A subunits.

Both the Type 1 and Type 2 enzyme preparations only showed significant activity in the range, pH 6.5-10.0. In contrast the plasma samples show significant enzyme activity over a more extended range, pH 6.0-11.0. Whether this is due to plasma components interacting with factor XIII either to maintain the tertiary structure of

the enzyme or to inhibit the ionisation of the basic and acidic groups of the enzyme, at the extreme pH values requires further study to determine.

4. Heat stability

At a specific temperature the energy of the system is sufficient to cause the breakdown of hydrogen bonding and other forces which maintain the tertiary structure of an enzyme. At this temperature the enzyme starts to lose its activity and eventually becomes completely inactive.

The more rapid heat inactivation of the Type 2 enzyme between 55^{o}-65^{o}C observed in these studies indicates that the Type 2 A subunits are more unstable and lose their tertiary structure at a lower temperature than the Type 1 A subunits. However, the fact that plasma samples of the two forms do not show any differences in their heat profiles suggests that the environment provided by plasma and the interaction of the factor XIII molecule with other plasma components protects the tertiary structure of the Type 2 A subunits from heat denaturation at lower temperatures. It has been suggested that factor XIII circulates in plasma bound to both fibrinogen (Greenberg and Shuman, 1981 and 1982) and fibronectin (McDonagh, 1981). However, in the present studies fibrinogen was removed from the plasma samples before the assays were carried out.

D. Plasma factor XIII studies: The Type 3 A subunit

1. pH

The pH curve of the plasma sample from the Type 1-3 individual shows two peaks of activity at pH 8.38 and pH 9.10. The biphasic curve shown by this sample is what would be expected if the Type 1 and Type 3 A subunits have distinct pH optima. As the optimum pH for the Type 1 A subunits was pH 8.38 it is reasonable to conclude that the

lower value in the heterozygous sample represents the peak activity of the Type 1 subunits. Therefore, the pH optimum of the Type 3 subunits for dimethylcasein at 37^{o}C is approximately pH 9.10.

2. Heat stability

The electrophoretic studies of Type 1-3 A subunits from platelets suggested that the Type 3 A subunits were unstable when compared to the common Type 1 and Type 2 subunits (Chapter 4). Therefore, plasma samples from individuals homozygous for the latter two forms of factor XIII were compared with a Type 1-3 plasma sample for the effects of heat denaturation.

Both the Type 1 and Type 2 samples showed no loss of enzyme activity when incubated at temperatures up to 55^{o}C for 10 minutes prior to enzyme assay. By comparison, the Type 1-3 sample lost approximately 20% and 30% of its enzyme activity at 50^{o} and 55^{o}C respectively (Figure 5.16). However, at 65^{o}C all three samples showed similar losses in activity, although the Type 1-3 sample still showed the largest reduction in activity. These results suggest that the Type 3 A subunits are denatured at 50^{o}C and above, and that the majority of the enzyme activity lost at 65^{o}C in the Type 1-3 sample is due to the inactivation of Type 1 A subunits.

The results of prolonged incubation of all three samples at 55^{o}C (Figure 5.17) support this idea. In the first 30 minutes approximately 40% of the enzyme activity of the Type 1-3 sample is lost compared to less than 5% in the other two samples. Therefore, the Type 3 A subunits, which appear to account for between approximately 25%-50% of the enzyme activity of Type 1-3 plasma (Chapter 4), are largely inactivated before the Type 1 and Type 2 A subunits show any loss of activity at 55^{o}C.

The results of the heat stability studies, therefore, appear to confirm the hypothesis proposed in Chapter 4 that the *FXIIIA*3*

allele codes for subunits with a tertiary structure which is unstable in the plasma environment. It is, therefore, possible that the presence of this allele in the homozygous state or, in combination with the *FXIIIA*QO* allele, may result in congenital factor XIII deficiency.

E. Fibronectin as a substrate for factor XIII

Mosher (1975) showed that fibronectin was a physiological substrate for factor XIII. The results shown here confirm this finding by demonstrating the factor XIII catalysed incorporation of ^{14}C putrescine into the plasma fibronectin molecule. The fact that the dimethylation of plasma fibronectin had no effect on the V_{max} of the reaction suggests that, in the assay conditions used here, fibronectin is not cross-linked to itself by factor XIII, as the dimethylation process blocks the ε-amino groups of lysine residues on the protein thereby preventing the formation of fibronectin multimers.

V. CONCLUSIONS

1. The results given and discussed above indicate that there are no differences in the substrate specificities or the iodoacetamide inactivation at pH 7.5 and 37°C between the commonly found Type 1 and Type 2 A subunits of factor XIII. However, the pH optimum of the purified Type 1 enzyme for dimethylcasein is slightly higher than that of the Type 2 enzyme. Also, the purified Type 2 A subunits appear to be inactivated at a lower temperature than the Type 1 A subunits. As neither of these differences were apparent in plasma samples, it is uncertain if there are any physiologically significant differences between the two common types of A subunits.

2. The Type 3 A subunits are unstable compared to the two common forms of the subunit and have a higher pH optima for dimethylcasein. As both these differences are present in plasma samples they are probably physiologically significant and, therefore, the Type 3 A subunits may be subject to different selection pressures than the Type 1 and Type 2 A subunits.

EXTENT OF PUBLICATION

The following papers arising from the material presented in this thesis and also arising from concurrent work in other fields have been published during the authors candidature.

Castle, S., Board, P.G. and Anderson, R.A.M. (1981). Genetic heterogeneity of factor XIII deficiency: First description of unstable A subunits. Brit. J. Haematol. 48: 337-342.

Castle, S.L. and Board, P.G. (1982). Electrophoretic investigation of formaldehyde dehydrogenase from human tissues. Hum. Hered. 32: 222-224.

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