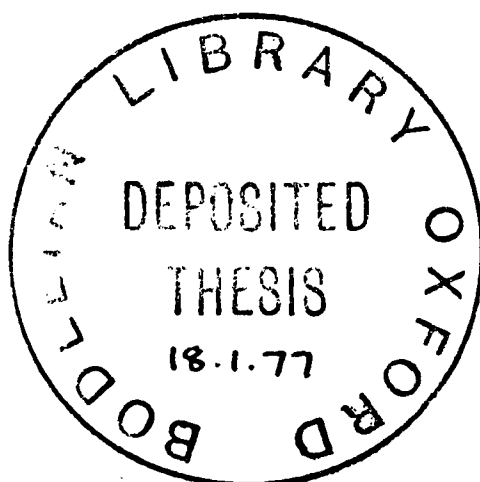


'The Effects of Oral Anticoagulants on the
Synthesis of Glycoproteins.'

A Thesis submitted for the Degree of
Doctor of Philosophy

by

Christopher V. Prowse.
Merton College, Oxford.



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"This apoplexy is, as I take it, a kind of lethargy,
an't please your lordship, a kind of sleeping in the
blood."

Henry IV Part 2

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ABSTRACT

(i)

The coumarin related drugs have been used as oral anticoagulants for many years. These drugs help in the prevention of thrombotic episodes by reducing the activity of certain plasma proteins required for the clotting process. Administration of vitamin K reverses the effects of the coumarin drugs and deficiency of this vitamin results in similar symptoms as are induced by coumarin therapy. It has been proposed, therefore, that vitamin K and the coumarins act at a similar site and affect the plasma activity of the clotting proteins. Factors II, VII, IX and X. Study of the synthesis of these proteins has shown that the site of vitamin K (and coumarin) action is post-ribosomal and that the vitamin might be responsible for the introduction of a 'prosthetic' group into the proteins. This is supported by the finding that the plasma of coumarin treated patients, and cattle, contains a protein with the immunological characteristics of normal prothrombin but with negligible activity in the usual bioassay.

In this study it has been shown that an abnormal prothrombin present in the plasma of patients undergoing anticoagulant therapy with Warfarin may be purified by absorption to barium citrate and subsequent chromatography on DEAE-Sephadex. This barium-absorbable coumarin prothrombin accounts for at least 70% of the immunological

prothrombin in coumarin plasma, i.e. it is the major species of prothrombin during coumarin therapy. The abnormal protein is eluted from DEAE-Sephadex prior to normal prothrombin. Other workers in this field have concentrated on the barium non-absorbable prothrombin from cattle. This form of prothrombin has a lower biological activity than the barium-absorbable protein. Present theory explains this effect in terms of the inhibition, by the coumarin drugs, of the vitamin K-dependant carboxylation of ten glutamic acid residues in the N-terminal portion of the prothrombin with a resultant loss of barium and calcium binding ability.

In order to investigate the reason for the low specific activity of the barium-absorbable prothrombin larger volumes of blood were required than it was feasible to obtain from outpatients. The corresponding protein was therefore isolated from the plasma of cows treated with 'therapeutic' levels of Warfarin. The bovine protein, like the human barium-absorbable prothrombin, has a reduced biological activity. Analysis of acid hydrolysates of this protein showed that it had an identical carbohydrate and amino acid content to normal prothrombin.

Investigation of the activation of the various forms of prothrombin showed that abnormal prothrombin, from both cattle and human patients, gives rise to thrombin which has the same specific activity as normal thrombin and that the

rate of activation of abnormal prothrombin is reduced. Thus abnormal prothrombin does not interact normally with its activating enzyme, Factor Xa, in the presence or absence of Factor V or phospholipid, which act as accelerators of normal activation. Following this it was shown that abnormal prothrombin had a reduced ability to bind calcium; such a defect accounts for the above activation differences. This defect was located in the N-terminal (Fragment 1) portion of the prothrombin molecule. Alkaline hydrolysis of the whole prothrombin molecule and of the isolated Fragment 1 showed that, in contrast to the normal protein, only about 6 of the possible ten glutamic acid residues were carboxylated in the barium-absorbable prothrombin. Detailed analysis of the N-terminal region of this protein was carried out by isolation and characterisation of peptides from this portion of the molecule and confirmed the view that this region is heterogenous in the abnormal protein.

A further bovine plasma protein (Factor X₃) which is affected by the administration of coumarin drugs and by vitamin K has also been partially characterised. This protein contains gamma-carboxy glutamic acid residues and is of similar size to Factors VII, IX and X, but has no clotting activity. Nor has any other activity been associated with Factor X₃.

Factor X is also affected by coumarin therapy. However in this case the protein exists as two forms, even in the vitamin K replete state. Despite this it was shown that these two forms, Factor X_1 and Factor X_2 , differed in the extent of carboxylation. Factor X_1 and X_2 have different specific activities. As in the case of prothrombin this may be explicable in terms of a low gamma-carboxy glutamic acid content resulting in a reduced calcium binding ability.

These results are used in proposing a model of vitamin K action which is discussed in terms of the possible effects of the coumarin drugs of [^]proteins other than those involved in blood coagulation.

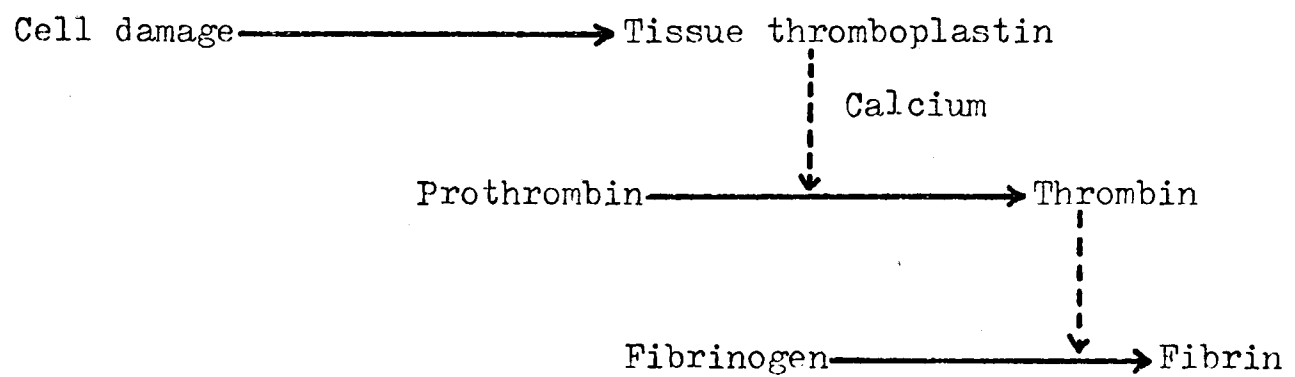
Chapter 1

INTRODUCTION

FIGURE 1•1

The classical four-factor theory of blood clotting.

(Morawitz 1904)



The Quick prothrombin time is based on this scheme.

In this assay thromboplastin substitute and calcium are added to the plasma sample. The resulting formation of thrombin is monitored by the time of appearance of the fibrin clot formed by thrombin cleavage of the fibrinogen in the plasma sample.

(a) Early work on blood coagulation

As long ago as 1770 Hewson showed that the clotting of blood is due to the coagulation of plasma. In 1845 Virchow introduced the concept of thrombosis and embolism and suggested the existence of fibrinogen as a plasma component. At about the same time Buchanan proposed that the conversion of fibrinogen into the fibrin clot was an enzymic process. Experimental support for these hypotheses was first supplied by Schmidt (1872) who separated a 'fibrin ferment' from serum by alcoholic precipitation. These early observations were extended by the demonstration that clotting requires calcium (Arthus and Page 1890), which was required for the formation of thrombin, but not for the subsequent formation of fibrin (Hammerstan 1896) and culminated in the proposal of the so-called classical theory of blood clotting (Morawitz 1905). This is depicted in Figure 1.1 and proposed that cellular damage releases an enzyme (thromboplastin) into the circulation which reacts, in the presence of calcium, with plasma prothrombin to yield a second enzyme, thrombin. This in turn cleaves fibrinogen to fibrin, which enmeshes with the various formed elements of the blood to form the clot. During the nineteenth century it was also shown that blood collected into glass containers will clot in the absence of tissue particles, albeit at a slower rate (Lister 1863, Bordet and Genou 1901), an observation not fully explained

by the above theory.

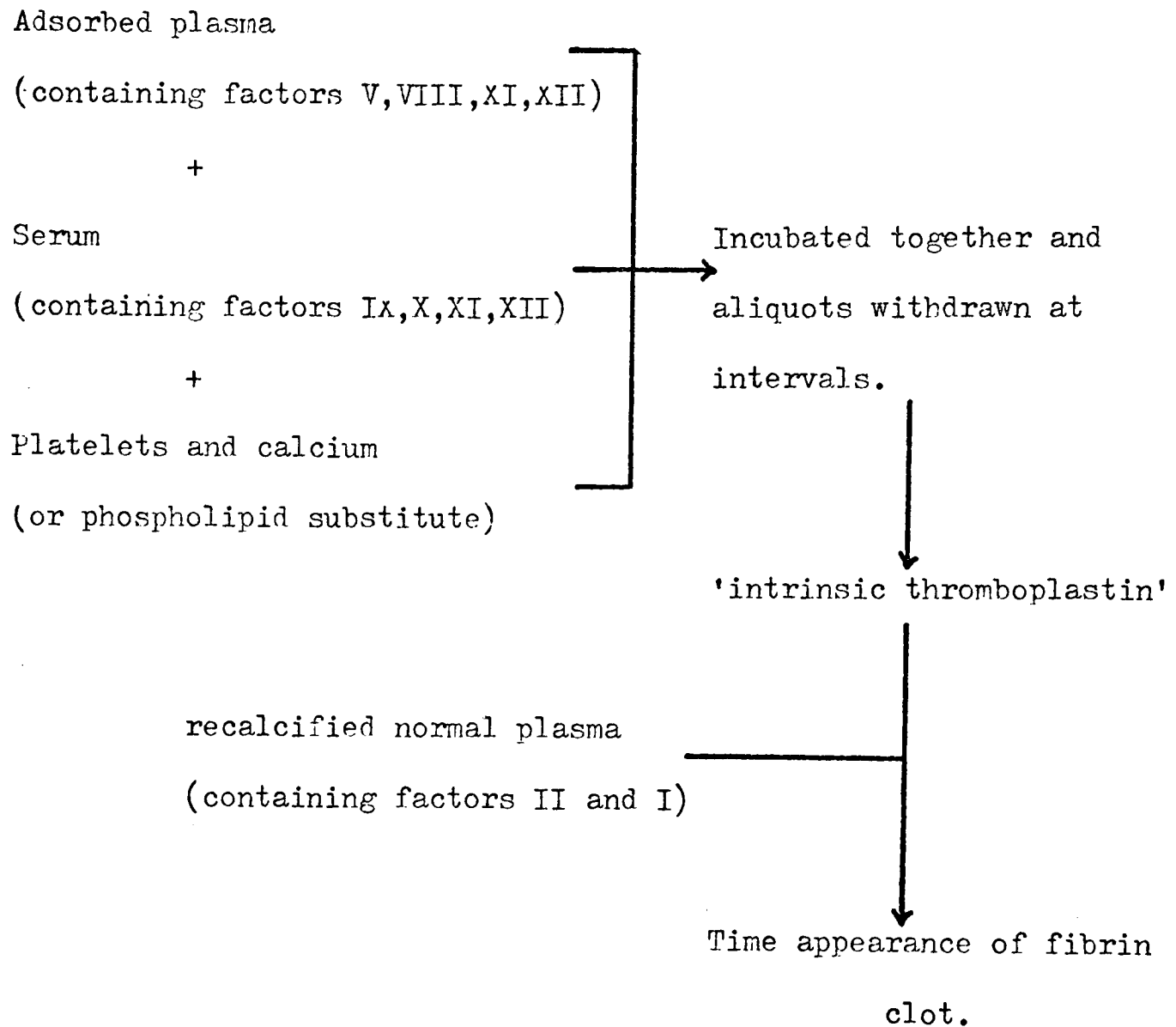
During the early decades of this century the enzymic theory of blood clotting lapsed in favour of a scheme which proposed clot formation by stoichiometric combination of various plasma components (Pickering 1928), but again found favour in the fuller demonstration of the enzymic nature of thrombin by Mellanby (1933) and others. At about this time the introduction of the coumarin drugs allowed treatment of a clinical condition in which clotting occurs spontaneously. This condition is now known as 'hypercoagulability'; no full explanation of the underlying causes of this syndrome is yet known (Wessler 1972). Coumarin drugs act by reducing the plasma activity of prothrombin (and related clotting factors), thereby reducing the risk of thrombosis. However, overdosage readily induces haemorrhage and clinical use therefore requires strict laboratory control. This problem resulted in a dramatic increase in use of the then recently described one-stage prothrombin time assay (Quick 1935). The Quick test is based on the scheme in Figure 1-1 and allows assay of plasma prothrombin activity by providing the other three components in excess. The plasma sample taken for prothrombin estimation usually contains sufficient fibrinogen and is assayed by the addition of excess calcium and a rabbit brain substitute for tissue thromboplastin, the appearance of the fibrin clot being timed.

The widespread use of the Quick prothrombin time (PT) revealed faults in the four factor theory of clotting. It was well known that the PT of plasma increases on ageing; this could be corrected by the addition of fresh coumarin plasma (Quick 1943) or fresh normal plasma absorbed with aluminium hydroxide to remove the prothrombin (Owren 1947). In this paper Owren also describes a patient whose plasma had a prolonged PT, which could be corrected in a similar manner to above. He proposed the term Factor V to describe the factor absent in this deficiency state. Factor V is a labile plasma component which is not absorbed to aluminium hydroxide (or insoluble barium salts) and is unaffected by coumarin therapy. Following the early work of Macfarlane and Barnett (1934), Witts and Hobson (1942) introduced the use of Russell's viper venom as a convenient substitute for brain thromboplastin in the prothrombin assay. However this test apparently overestimated 'prothrombin' during coumarin therapy (Biggs and Macfarlane 1949). The explanation of this phenomenon was found in the work of Owen and Bollman (1948) who showed that the PT of coumarin plasma could be decreased by addition of normal serum but not by coumarin serum or barium sulphate absorbed normal serum. They proposed the inclusion of Factor VII in the clotting scheme as a factor affected by coumarin therapy, absorbable to barium sulphate and present in serum. It is not estimated in the Russell's viper venom ^{test} which bypasses this component.

FIGURE 1.2

The Thromboplastin Generation Test (TGT).

(Biggs and Douglas 1953)



By substituting test adsorbed plasma or serum, defects in one or the other may be detected by the prolongation of the clotting time.

The separation of clotting factors into those present in serum and those in absorbed plasma was utilised in the thromboplastin generation test (TGT - Biggs and Douglas 1953). This test led to the discovery of the serum component, Factor IX, and Factor VIII, present in absorbed plasma (Figure 1-2). Deficiency of these factors results in Haemophilia B and A respectively. Factor IX activity is also reduced in coumarin therapy.

The TGT also led to the discovery of Factors XI and XII, deficiencies of which give rise to abnormalities in both absorbed plasma and serum components. Deficiencies in Factors VII, IX, XI and XII although detected by the TGT have no effect on the PT. In 1956 two patients were seen with an abnormal TGT and PT (Telfer et al 1956, Hougie et al 1957). These patients also had abnormal clotting function in the Russell's viper venom test, and it was shown that this defect was due to absence of Factor X. This protein is also found to be affected by coumarin therapy and to be absorbed to insoluble barium salts. It should be noted that at this time different groups used their own terms for the various clotting factors and that the introduction of the Roman numeral system (Table 1-1, Wright 1959) clarified the situation enormously.

Coumarin therapy reduces the plasma activity of factors II (prothrombin), VII, IX and X. All these

<u>Symbol</u>	<u>Synonyms</u>	<u>Pathway</u>		<u>Present in</u>		<u>affected by coumarins</u>	<u>Int.</u> <u>340,000</u>
		<u>Intrinsic</u>	<u>Extrinsic</u>	<u>Adsorbed plasma</u>	<u>Serum</u>		
I	Fibrinogen	+	+	+	-	-	340,000
II	Prothrombin	+	+	-	-	+	70,000
III	Tissue thromboplastin	-	+			-	200,000 to 300,000
	Tissue factor						
IV	Calcium ions	+	+				
V	Proaccelerin	+	+	+	-	-	300,000 to 400,000
	Accelerator globulin						
VII	Proconvertin	-	+	-	+	+	53,000
	Autoprothrombin I						
VIII	Antihaemophilic globulin	+	-	+	-	-	1,100,000
	Antihaemophilia factor A						
	platelet cofactor 1						
IX	Antihaemophilia factor B	+	-	-	+	+	55,000
	Christmas factor						
	Autoprothrombin II						
X	Stuart-Prover factor	+	+	-	+	+	55,000
	Autoprothrombin III						
Xa	Autoprothrombin C (Thrombokinase)						
XI	Plasma thromboplastin antecedant	+	-	+	+	-	160,000
XII	Hageman factor	+	-	+	+	-	90,000
XIII	Fibrin stabilising factor	+	+	+	+	-	300,000 (plasma) 146,000 (platelets)

TABLE 1-1

The roman numeral system for clotting
factors (Wright 1959) and some
properties of these factors.

proteins are bound to various insoluble inorganic salts, such as barium sulphate and aluminium hydroxide, a property that distinguishes the so-called 'prothrombin complex' group of factors from other clotting factors. This property was used by Seegers et al (1938) in their preparation of 'prothrombin'. This preparation exhibited activity corresponding to all four components of the complex.

Recognition of the possibility that the prothrombin-complex group of factors could be zymogens, converted to their active forms during the coagulation process (Seegers et al 1945, Spaet and Cintron 1963, Ratnoff and Davie 1962, Esnouf and Williams 1962) and the realisation of two modes of thromboplastin formation corresponding to the PT and TGT assays, led to the proposals, independantly, by Macfarlane (1964) and Davie and Ratnoff (1964) of the cascade or waterfall theory of blood coagulation. This model suggested that Factor X may be activated by an extrinsic or an intrinsic system. In both systems and in the subsequent common pathway of prothrombin activation it was suggested that each factor is a zymogen which is activated by its predecessor in the cascade and activated its successor. In general this theory is still accepted but has required partial modification. It is now thought that the labile factors, V and VIII, do not have enzymic forms but are converted to reactive species and act as accelerators of particular enzymes, factors Xa and IXa respectively, in the

Surface Contact

XII $\xrightarrow{\text{Surface Contact}}$ XIIa $\xrightarrow{\text{Fitzgerald factor}}$ Kallikrein (Fletcher factor)

Fitzgerald factor

Prekallikrein

XI $\xrightarrow{\text{Fitzgerald factor}}$ XIa

intrinsic path

PL

Ca⁺⁺

VIII

X

Xa

extrinsic path

PL

Ca⁺⁺

V

final common path

$\alpha\text{VII} + \text{TF} \rightarrow \beta\text{VII} - \text{TF}$

Tissue Damage

II

IIa

XIII

XIIIa

Fibrinogen $\rightarrow$ Fibrin $\rightarrow$ Insoluble Fibrin

Activator

Plasmin

FDP's

Plasminogen

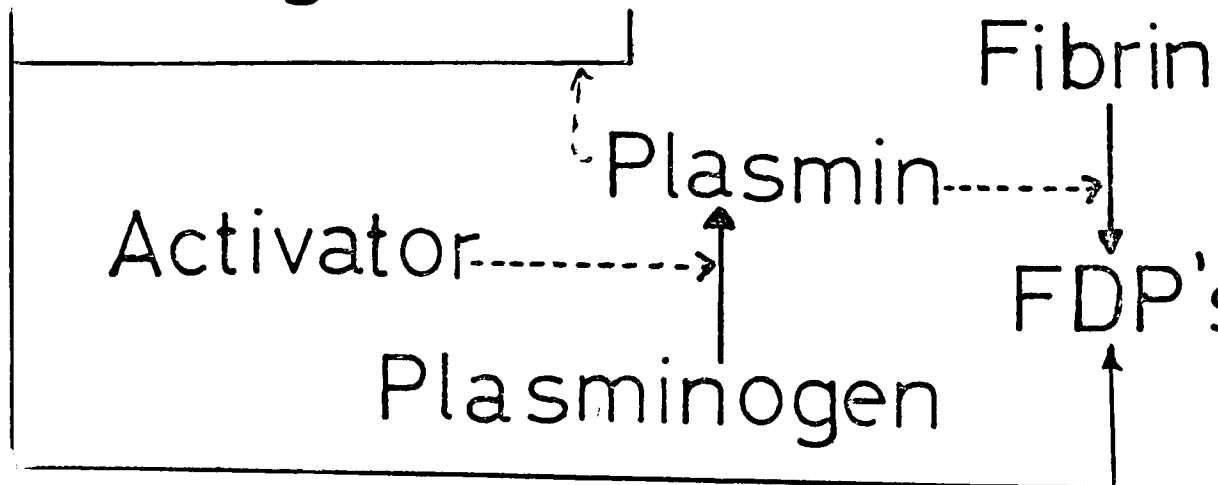


FIGURE 1.3

The cascade model of blood clotting

PL: phospholipid

TF: tissue factor

FDP: fibrin degradation products

—————▶: conversion of precursor molecule

- - - - -▶: enzymic action

subscript a refers to the enzyme form of
the zymogen precursor

(modified from Macfarlane 1964)

cascade process. A modern interpretation of the cascade model is shown in Figure 1.3, which also shows certain feedback pathways (e.g. the activation of Factor V by thrombin). Inspection of this model reveals that the Quick PT measures a combination of II, VII and X activity rather than prothrombin activity alone.

For fuller reviews of the blood clotting field I refer you to Esnouf and Macfarlane (1968), Nemerson and Pitlick (1972) and Davie and Kirby (1973).

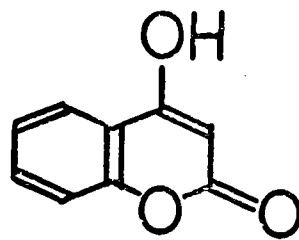
(b) The coumarins and Vitamin K

In the early twenties a Canadian vet reported that cows in his area were dying from a haemorrhagic disorder which at first was diagnosed as a form of Blackleg. However it was soon shown that the disorder was due to spoilt feed and that transfusion of whole blood would save the cattle (Schofield 1922). Roderick (1931) showed that a prothrombin-rich acetone fraction of plasma would replace the whole blood as the prophylactic in the sick cattle. This work was taken up by Link who confirmed that cattle fed spoilt sweet clover had prolonged prothrombin times and devised a bioassay in which rabbits were fed experimentally spoilt clover. Using this assay Campbell and Link (1941) were able to isolate the haemorrhagic agent which was characterised as 3,3'-methylene-bis(4-hydroxycoumarin). This compound was the basis of the subsequent development of the oral anticoagulant drugs. The structure of a few of these

FIGURE 1.4

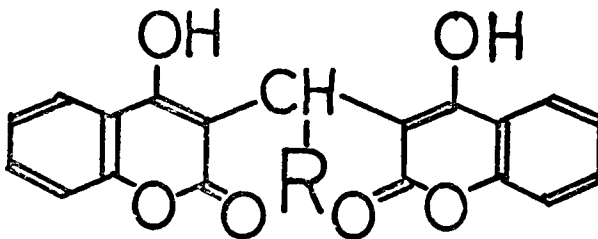
Oral Anticoagulants

- a. 4-hydroxy
coumarin

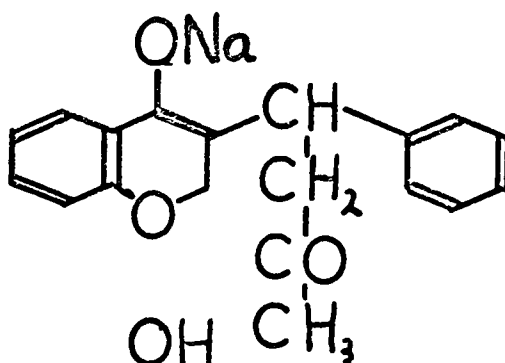


- b. Dicoumarol (R=H)

Tromexan ($R = \text{COOC}_2\text{H}_5$)

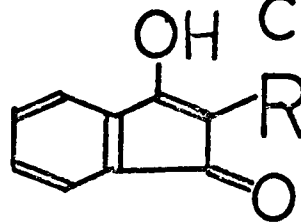


- c. Sodium
Warfarin



- ## d. Indandione

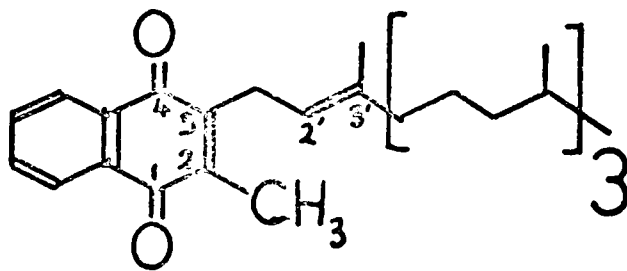
(R=C₆H₅: Phenindione)



K-Vitamins

- a. Vitamin K₁

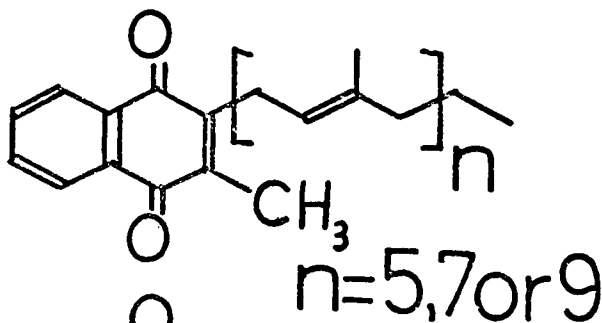
'Phylloquinone'
from plants.



- b. Vitamin K₂

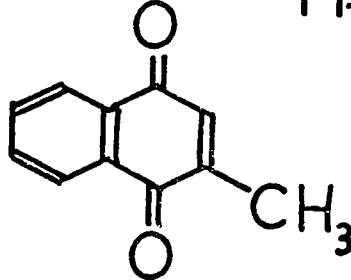
'Menquinone'

from animals and micro-organisms.



- c. Vitamin K₃

'Menadione'
synthetic.



coumarins are shown in Figure 1-4, together with the indanedione structure, also an active anticoagulant. These drugs all have a similar effect but differ in the degree and speed of effect. This early work was reviewed by Link (1944), at which time he pointed out the similarity of the effects of the coumarin drugs and Vitamin K deficiency.

Vitamin K had been discovered by Dam during experiments on the fat-soluble vitamins. When chicks were fed a fat free diet the birds quickly developed severe haemorrhage, together with degeneration of certain mucous membranes. The effects were not due to deficiency of vitamins A, D or E so Dam proposed a 'Koagulation Vitamin' (Dam and Schonheyder 1934). The haemorrhagic effect induced by deficiency of this vitamin was shown to result from a prolonged prothrombin time (Dam and Schonheyder 1936a) and using this as an assay Vitamin K was found in plant (e.g. alfalfa) and animal (e.g. a lipid fraction of hog liver) sources (Dam and Schonheyder 1936b). Vitamin K was also shown to be produced by bacteria as putrefying fish meal was a good source, whereas the fresh meal was not (Almquist and Stockstad 1935). Bacterial synthesis is probably the major source of Vitamin K in animals as is shown by the induction of Vitamin K deficiency in man by prolonged courses of antibiotics which deplete the gut flora (Quick and Collentine 1950). Apart from this, Vitamin K deficiency is rare in man, except in conditions

in which fat absorption is impaired, e.g. obstructive jaundice. Chickens on the other hand have a poor gut flora which is readily depleted and were therefore a fortunate choice for Dam's early studies (Green 1966).

Further work by Dam led to the isolation of Vitamin K from both plant (K_1) and animal/bacterial (K_2) sources. The structures were shown to be as depicted in Figure 1-4 (Dam et al 1939). A number of Vitamin K analogs were synthesised and many of them are effective in curing Vitamin K deficiency (Fieser et al 1941). Two worthy of note are Vitamin K_3 , or menadione, which has no hydrocarbon at the 3-position, as this is water soluble and requires longer for action than the fat soluble forms; and K_1 -epoxide in which oxygen is introduced across the 2,3 double bond of the naphthoquinone structure. This epoxide has since shown to play an important part in the mechanism of Vitamin K action.

(c) Metabolic effects of Vitamin K and the coumarins

Link (1944) showed that Vitamin K rapidly reversed the effects of Warfarin. Chandreshkar et al (1965) subsequently demonstrated that if labelled Vitamin K_1 is given to Warfarin treated rats the resultant shortening in the prothrombin time is proportional to the amount of vitamin sequestered in the liver. At that time this was taken as

demonstrating that Vitamin K and Warfarin were in competition at a liver site required for the formation of plasma prothrombin activity.

Barnhart and Anderson (1962) showed by an immuno-fluorescent technique that Vitamin K does not control the release of whole rat prothrombin from the liver. The later discovery that Vitamin K has no effect on the degradation rate of prothrombin indicates that it has some effect on the synthesis of prothrombin. Martius (1966) suggested three possibilities as to the site of action:

1. Vitamin K introduces a prosthetic group into the coagulation factor;
2. Vitamin K is required for the formation of the coagulation factor;
3. Vitamin K has a general effect on protein synthesis which is most effectively observed in the clotting system.

Martius felt that the last proposal the most likely explanation as Vitamin K had been shown to participate in the respiratory chain of bacteria and might therefore be presumed to have a general effect on energy supply within the cell.

R.E. Olson and co-workers (1964) suggested that Vitamin K might act as a gene effector of mRNA synthesis, in line with the then developing theory of the operon.

This theory corresponds to the second of Martius's proposals and would explain the apparently high specificity of Vitamin K action. However there is little experimental evidence in favour of this theory and further studies led to the retraction of this proposal in favour of a site of action at the stage of initiation of peptide assembly (Olson 1968, Olson 1969). Subsequent work in Olson's laboratory, using both in vivo and in vitro systems, has shown that Vitamin K has no general effect on protein synthesis (Berry et al 1971) and led to the hypothesis that Vitamin K interacts with a regulator protein, the ribosome and mRNA to promote the formation of polysomes specific for prothrombin synthesis, even in the presence of cycloheximide (Johnston and Olson 1972). Recently Nardacci et al (1975) have used an heterologous system from rabbit reticulocytes to synthesise prothrombin from rat liver mRNA; this system shows the production of an incomplete prothrombin in the absence of Vitamin K. This abnormal prothrombin is degraded by *Echis carinatus* venom, but not by the normal physiological prothrombin activator. These properties correspond to those of the abnormal prothrombin produced in the absence of Vitamin K as discussed below. This evidence together with that presented below has led to the demise of this theory in favour of one proposing post-ribosomal modification as the site of Vitamin K action.

The proposals of Martius, listed above, required a simpler system than the whole animal as an experimental

model and led to the development of in vitro systems such as isolated perfused liver, liver slices and microsomal preparations. Preliminary work was mainly concerned with Factor VII as this activity can be detected at very low levels and small changes monitored (Prydz 1965, Babior 1966). However there are dangers in this as VII is readily activated to a more active form. This resulted in confusion between the activity and the amount of the enzyme.

This confusion was overcome by J.P. Olson et al (1966) using an isolated perfused liver system. This preparation exhibited synthesis of the Vitamin K-dependant clotting factors II, VII and X and also Factor V which is independent of Vitamin K. The system was Warfarin sensitive in a similar way to the whole animal, as shown by the response of II, VII and X but not of V. Warfarin inhibition of the system was released by Vitamin K even in the presence of sufficient puromycin to completely inhibit Factor V release. Similar effects were found by Hill et al (1968) using a rat liver microsomal preparation isolated from rats given either Actinomycin D (to inhibit transcription) or cycloheximide (to inhibit translation) just before slaughter. These authors therefore proposed that Vitamin K acted at some post-ribosomal stage of protein synthesis, a conclusion supported by Babior (1966), Suttie (1967) and Pereira and Couri (1971). Suttie also provided evidence for this mechanism operating in vivo (Shah and Suttie 1971). Working with rats made deficient in Vitamin K by using the diet of

Mameesh and Connor-Johnson (1959) they showed that administration of Vitamin K caused a rapid release of prothrombin into the plasma. In the presence of cycloheximide the extent of the release was only slightly reduced. By administering labelled amino acids immediately prior to the cycloheximide it was shown that the released prothrombin was not labelled, whereas an earlier administration of amino acids does result in labelled prothrombin.

At the time the present study was started the nature of the post ribosomal modification was not known. Johnson et al (1971) had shown the presence of an abnormal plasma protein, which absorbed to barium sulphate, in Vitamin K deficient rats. Using labelled sugars and amino acids together with the technique of gel electrophoresis they apparently showed that administration of Vitamin K resulted in the conversion of the abnormal protein to prothrombin with the concomitant incorporation of sugar residues. Thus Vitamin K appeared to be involved in the glycosylation of prothrombin. Similar conclusions were reached by Pereira (1970) and Pereira and Couri (1971). In view of more recent experiments the identity of the abnormal proteins in these studies is not at all clear.

Vitamin K may also be characterised by following the fate of the vitamin rather than studying its effects. Martius (1967) suggested that all the various forms of the vitamin had a common derivative in the liver. Using labelled Vitamin K₁ Matshiner et al (1970) showed that during Warfarin treatment of rats up to half the vitamin

was present in the liver as the 2,3 epoxide, whereas in untreated rats the level of this metabolite was barely detectable. This group also found that whereas the epoxide normally has Vitamin K activity it would not release warfarin inhibition, in contrast with the unoxidised vitamin (Bell and Matshiner 1970). Following on from this they proposed that coumarins inhibit the reduction of the epoxide. Thus the reduced vitamin would act in the completion step of prothrombin (and other vitamin K dependant proteins) during which process it is oxidised; coumarin drugs inhibit the reduction of the epoxide and thus reduce the availability of reduced vitamin for the completion step. This is supported by observations on the oxidised-reduced vitamin ratio in coumarin treated and untreated rats (Bell et al 1972, Sadowski and Suttie 1974).

(d) The present study

Initially prothrombin was recognised only by its plasma activity. The work of Seegers et al (1945) led to a substantially purified prothrombin. Although considered pure at the time this preparation has since been shown to contain all four Vitamin K-dependant clotting factors. Seegers argued that all these activities were derived from a single prothrombin species. However the amino acid composition of prothrombin (Magnusson 1971) and Factor X (Fujikawa et al 1972) differ, as do their tryptic peptide

maps (Aronson et al 1969). In the case of the bovine proteins these two clotting factors are readily separable by ion exchange chromatography (Esnouf et al 1973). The clotting factors may also be distinguished by the use of specific antisera (Denson 1967) and specific activators (e.g. Esnouf and Williams 1962, Jobin and Esnouf 1966). Thus distinct proteins are present in plasma corresponding to the activities measured by the various clotting assays.

In 1963 Hemker et al concluded that patients on coumarin drugs possessed a plasma inhibitor of prothrombin activation. It was thought that this might be a precursor of prothrombin and it was named PIVKA (for Protein Induced by Vitamin K Absence/Antagonists) prothrombin. The first physical proof of such a molecule arose from immunological studies on coumarin patients (Nilehn and Ganrot 1968, Ganrot and Nilehn 1968, Josso et al 1968). Coumarin plasma was found to contain an inactive prothrombin with normal immunological reactivity, but with a reduced ability to bind calcium ions. It was not absorbed to barium sulphate and was present unchanged in serum.

Similar results were obtained for bovine material (Stenflo and Ganrot 1972) and abnormal bovine prothrombin, that did not bind to insoluble barium salts, was isolated by Stenflo (1972) and Nelsestuen and Suttie (1972b). Analysis of this material yielded no obvious differences in the structure apart from the reduced calcium binding

ability (Nelsestuen and Suttie 1972c, Stenflo and Ganrot 1973) and the low physiological activity. These abnormalities were shown not to be due to an abnormal arrangement of the intrachain disulphide bonds (Nelsestuen and Suttie 1972c). Abnormal proteins have also been demonstrated for factors IX and X during coumarin treatment of cattle (Reekers et al 1973) and human patients (Prydz and Gladhaug 1971, Larrieu and Meyer 1970); there is also some evidence for a similar effect for Factor VII (Howarth et al 1974). Preliminary work has also been carried out on an abnormal prothrombin from coumarin plasma of cattle (Malhotra and Carter 1971) and humans (Morrison and Esnouf 1973) that does absorb to insoluble barium salts. These proteins also have reduced activity in physiological systems.

These abnormal proteins would appear to be forms of the various clotting factors that are incomplete in some way. Therefore study of the structure of such molecules should yield information on the mode of action of Vitamin K and coumarin drugs. It seems likely that completion involves the addition of some prosthetic group (in line with Martius's first proposal). As discussed above Pereira and Couri (1971), Johnston et al (1971) and, in this laboratory, Morrison and Esnouf (1973) proposed that the abnormality was due to failure to achieve normal glycosylation of the protein. However partial deglycosylation has been shown

to have little effect on the activity (Nelsestuen and Suttie 1971) or the calcium binding ability (Stenflo 1970) of bovine prothrombin.

In this study the abnormal proteins have been further characterised in the hope of identifying the prosthetic group, its effect on activity and perhaps its mode of attachment. The majority of the work has been carried out on bovine prothrombin; firstly because of the larger volumes of blood available from these animals and secondly due to the fact that this clotting factor is present in plasma at a higher concentration than the other Vitamin K-dependant factors. Parallel studies were carried out on prothrombin obtained from human coumarin patients in order to test the results of the bovine studies in the clinical situation. Certain other Vitamin K-dependant proteins encountered during the course of the study have also been characterised.

Chapter 2

ISOLATION OF NORMAL PROTHROMBIN AND ITS ACTIVATION PRODUCTS

(a) Introduction

Barium sulphate absorbs all four Vitamin K dependant clotting factors from oxalated plasma and has been used by many investigators as a first step in their purification. However, subsequent elution from this salt does not give very good yields of prothrombin (Esnouf et al 1973). Other barium salts also absorb prothrombin; the citrate is particularly useful as it will absorb the proteins from citrated plasma (the sulphate will not) and can therefore be used on out-dated bank plasma and since it is more soluble than the sulphate, the proteins may be recovered by redissolving the absorbant. Absorption and elution with citrate were therefore carried out essentially as described by Ingwall and Scheraga (1969). Different methods of separating the protein and the absorbant have been used by other authors (Dowex 50 - Tishkoff et al 1969, ammonium sulphate - Aronson and Menache 1966, 0.1 M EDTA - Kisiel and Hanahan 1973). Citrate has been used here as it is a convenient buffer for the next stage of purification which is chromatography on DEAE-Sephadex.

The method used also yields pure bovine Factor X, which has been used in the activation experiments (Chapter 4), in studies of Factor X itself and in the isolation of prothrombin activation products.

Isolation of the activation products of prothrombin has been described by Heldebrant and Mann (1973), who

utilised DEAE-cellulose and sulphoethyl (SE)-Sephadex. Better separation of the pro-pieces has been achieved using DEAE-Sephadex (Jesty and Esnouf 1973). The method used here was a modification of the latter method, together with the use of sulphopropyl(SP)-Sephadex to separate the less acidic proteins. Recently Owen et al (1974) have separated the various products in one step on a column of QAE-Sephadex.

Activation products prepared in this study were compared with those obtained by other groups by amino acid and N-terminal analysis and by measurement of molecular weights. Having shown that our products were not distinguishable from those of other authors, they were then used to localise the defect caused by coumarin administration and to characterise the products formed during the activation experiments. The method devised for the purification of prothrombin (and Factor X) has also been used, with minor modifications, in the isolation of abnormal prothrombins that do absorb to barium citrate.

(b) Experimental

1. Purification of normal bovine prothrombin and Factor X.

Although larger volumes have been tried it was found convenient to carry out preparations on 50 litre lots of blood. 50 l of blood was collected, at the slaughterhouse, into 0.1 volume of 4% trisodium citrate, 0.5% benzamidine hydrochloride (BZ) in plastic buckets, and the froth

removed. As soon as possible (usually about 30 minutes), the plasma was separated on a Westphalia continuous flow centrifuge (Model SA00H 205 - Westphalia Separator Ltd., Habig House, Wolverton, Bucks.) operated in the separator mode (blank vanes at the top) with a 38mm centripetal pump and a 36mm ring dam. The bowl was rotated at 10,000 rpm and blood run through the machine at 180 l/hr (back pressure approx. 23psi). This usually yielded about 25 l of plasma, which was contained in three plastic buckets and 600g of barium chloride, dissolved in a minimum volume of water (approx. 1500mls), was slowly added and stirred for 20 minutes. The resultant barium citrate precipitate was collected on the Westphalia, operated in the clarifier mode (blank vanes at the bottom) with a 38mm pump and 27mm ring dam, at 10,000 rpm. The feed was partially blocked in order to maintain the flow at 70 l/hr (back pressure about 20psi). Barium sulphate is not ejected cleanly from the bowl during desludging at this stage (Esnouf et al 1973), the citrate, however, is a good deal softer and the bowl can be almost filled before desludging. One or two drops of caproic alcohol were added to disperse the foam on the barium citrate suspension which was then recycled through the Westphalia and washed with ice-cold water. Two further washes were carried out on an MSE-Mistral 6L centrifuge fitted with a 4x1250 ml swing-out head, at 2,000 rpm.

Protein was eluted from the barium citrate with 2 l 0.1M citrate/NaOH pH6.9, 10mM BZ, the precipitate being

well broken up to aid rapid dissolution (buffers are described throughout this study with the molarity referring to the first named component, the second being used to maintain the stated pH). 132g of ammonium sulphate was then added and the mixture stirred for 20 minutes. Barium sulphate precipitated (the ionic strength was sufficient to prevent reabsorption of the protein) and was spun off on the Mistral centrifuge at 5,000 rpm for 30 minutes, using the 6x750 ml angle head.

The clear yellow supernatant was dialysed for 8 hours against 10 l of distilled water and then pumped onto a (5x12)cm column of DEAE-Sephadex A-50 (pre-equilibrated in 50mM citrate/NaOH pH6.9, 1mM BZ) at 200ml/hr, overnight. The column was washed with 50mM citrate/NaOH pH6.9, 1mM BZ and the proteins then eluted with a (2x1500ml) linear gradient from 50 to 400mM citrate/NaOH pH6.9, 1mM BZ at 100 ml/hr. 25 ml fractions were collected using an LKB Ultrorac fraction collector, model 7000. The absorbance of the column effluent was monitored during the run with an LKB Uvicord Mark II, but the fractions were read individually, after the run, on a Pye-Unicam SP-500 ultraviolet spectrophotometer at 280nm.

2. Purification of human prothrombin.

In early preparations the method of Morrison and Esnouf (1973) was used. Subsequently the method described below was used as the product was similar and the technique

somewhat simpler. The method is described for 1 l of out-dated bank ACD-plasma, but is quite suitable for preparations from single donors (about 30mls of plasma).

The method was a scaled down version of the bovine purification. On receipt 1g BZ was added to 1 l ACD plasma and 30g barium chloride, in 100mls of water, slowly added. The purification was then carried out as described for the bovine material, except that all separations were carried out on the Mistral centrifuge; the washes and the eluting buffer were each of 100 mls and 6.6g of ammonium sulphate was required. Dialysis was carried out against 2 l of 50mM citrate/NaOH pH6.9, 1mM BZ using a hollow fibre beaker (BioRad Laboratories, Bromley, Kent) with a nominal cut off of 30,000 daltons. The dialysed sample was then concentrated to 30 mls on the same device and applied to a (1.5x25)cm column of DEAE-Sephadex A-50 (pre-equilibrated in the above buffer) at 15 ml/hr. After washing, the column was developed with a (2x200 ml) linear gradient from 50 to 300mM citrate/NaOH pH6.9, 1mM BZ at 15ml/hr. 5 ml fractions were collected.

3. Clotting assays.

In this study one stage assays, based on snake venom activators, have been used. Prothrombin was assayed using the venom of the Australian tiger snake (*Notechis scutatus Occidentalis*). This venom has the same cofactor requirements of Factor V, phospholipid and calcium (Jobin and Esnouf 1967)

and results in similar products (J. Jesty - D.Phil. thesis 1972) as the physiological activator of prothrombin, Factor Xa. Similarly, Factor X was assayed with venom from *Vipera Russellii Russellii* (Russell's viper). This also gives rise to products identical with those obtained with physiological activators (Radcliffe and Barton 1973). Thrombin was assayed by its action on fibrinogen or benzoyl arginine ethyl ester (see Chapter 4).

The details of these assays are described in brief below:

a. Tiger snake venom (TSV) assay of prothrombin.

(Jobin and Esnouf 1966).

1. 0.1ml barium sulphate absorbed bovine plasma.
(oxalated normal plasma was absorbed twice with 5% barium sulphate, for 20 minutes each time).
2. 0.1ml TSV/Inosithin in 20mM Tris/HCl pH7.5.
 - final concentration of crude venom 80ug/ml
 - final concentration of phospholipid 1.4mg/ml.
 - this is a 1 in 35 dilution of a stock solution prepared as follows:

500mg of Inosithin

is dissolved in 10mls diethyl ether and then emulsified with 10mls 10mM Tris/HCl pH8.4 and the ether blown off with oxygen-free nitrogen at 37°C.

3. 0.1ml prothrombin containing sample appropriately diluted in 20mM Tris/HCl pH7.5, 1% bovine serum albumin.
4. 0.1ml 25mM CaCl_2 in 20mM Tris/HCl pH7.5.

The assay was carried out at 37°C and 30 seconds was allowed before the final addition of calcium to allow for mixing and calcium-free hydrolysis of the prothrombin. The time to clot after the addition of calcium was compared with the times for five dilutions of normal plasma (usually in the 10 to 1000 fold dilution range).

b. Russell's viper venom (RVV) assay of Factor X.

(Denson 1961)

1. 0.1ml X-deficient bovine plasma available
(prepared by charcoal absorption) commercially
2. 0.1ml RVV/Cephalin reagent from Diagnos-
tic Reagents
Ltd., Thame.
3. 0.1ml Factor X containing sample diluted
as for TSV assay.

After 30 seconds

4. 0.1ml 25mM CaCl_2 in 20mM Tris/HCl pH7.5.

The assay was carried out and calibrated as described for the TSV assay. Factor Xa was assayed by substituting 0.1ml 1.4mg/ml Inosithin solution for the RVV/Cephalin reagent.

c. Thrombin assays.

The assay of thrombin is complicated by its adsorption to glass; containers for dilution and assay were therefore plastic, unused glass or nitric acid washed glass.

1. 0.1ml sample, appropriately diluted in 20mM Tris/HCl pH7.5.
2. 0.1ml 20mM Tris/HCl pH7.5.
3. 0.1ml 25mM CaCl_2 in the same buffer (to improve detection of the end point).
4. 0.1ml barium sulphate absorbed oxalated plasma.

Clot formation was timed from the addition of the absorbed plasma. The calibration plot was obtained using a commercial preparation of thrombin of known activity.

Alternatively the assay was carried out by mixing 0.2ml of sample with 0.2ml fibrinogen (10 mg/ml Kabi grade L fibrinogen in water) at 37°C and timing clot appearance; or by assay for esterase activity as described in Chapter 4.

Since the introduction of the Quick prothrombin time, many clotting assays have been devised which depend on the timing of the formation of the fibrin clot. Tests for specific clotting factors depend on the use of either specifically deficient (genetic or artificial) plasmas or specific activators (physiological or artificial). For

factors higher up the cascade than thrombin these may be one-stage, in which the time to clot after the addition of the activator is observed, or two-stage, in which activation of the required factor is allowed to proceed to plateau in the absence of the succeeding factors and then assayed by the addition of these components. The latter are generally preferred in assaying the latent activity of zymogens. Active enzymes, such as thrombin, may also be assayed by their activity against certain esters.

Such tests may be used to assay both plasmas and purified factors. However, plasma samples will contain both inhibitors and accelerators of the cascade mechanism. This means that the activity of a purified factor and plasma (standard) may give dilution curves of different slopes. In general, however, the clotting assays are found to be accurate to within 10%. Furthermore the accuracy of the clotting assays can be improved by the inclusion of 1% albumin in the diluent buffer. Quantitation of these assays is carried out utilising a log-log plot of clotting time versus dilution, using dilutions of normal plasma as standards (usually five dilutions in the 10-1000 fold range). Due to the nature of this plot accurate clotting times are required and therefore important assays were performed at different dilutions, in duplicate or triplicate.

4. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS).

Throughout this study the purity of proteins and the apparent molecular weight of proteins in activation mixtures has been obtained by the technique of electrophoresis in polyacrylamide gels, in the presence of SDS.

Reynolds and Tanford (1970) have shown that the amount of SDS bound to a carbohydrate-free protein is a function of the length of the molecule. When reduced with 2-mercaptoethanol all secondary structure is lost and the protein becomes fully extended due to the mutual repulsion between the negative groups of the bound SDS. Thus on electrophoresis through a polyacrylamide gel proteins will separate on the basis of their molecular weight and the degree of cross-linking of the gel, all molecules having the same charge/mass ratio (Shapiro et al 1967). Glycoproteins are less mobile in this method due to the lower affinity of sugar residues for SDS (Bretscher 1971, Segrest and Jackson 1972) and the projection of these residues from the otherwise linear molecule; their molecular weight is thus overestimated.

The method used here is adapted from that of Weber and Osborn (1969), whereby the molecular weights of polypeptides may be determined by comparison with standards of known molecular weight, utilising a plot of log molecular weight versus relative mobility (with respect to tracking

dye). On occasion the discontinuous buffer system of Laemmli (1970) has been used. This method supposedly gives sharper bands; however the method of Weber and Osborn was found to be nearly as good, except for smaller species of protein, simpler to perform and was not susceptible to isotachophoresis in the upper portion of the gel (which effectively reduces the length of the gel).

Method based on Weber and Osborn (1969).

7½% polyacrylamide gels containing 1% SDS were prepared from the following mixture:

45mls solution A	0.2M phosphoric acid/NaOH pH7.1
30mls solution B	- 22.2g acrylamide (recrystallised from chloroform: 125g/l)
	- 0.6g N,N' methylene bis-acrylamide (recrystallised from acetone: 10g/l)
10mls	10% SDS
4.5 mls solution C	1.5% ammonium persulphate
0.15 mls	tetramethylethylenediamine (TEMED).

Fifteen glass gel tubes (15cm long, i.d.5mm) were placed in a section of plastic piping (14cm long) with a rubber bung in one end. The gel solution was poured into the pipe until about 2cm below the top of the tubes and 0.1ml n-butanol layered on top of each gel. This method allows the even polymerisation of the gels and ensures that the gels have a uniform length and flat tops, thereby

permitting comparison of mobilities between gels.

Samples containing 20-60ug of protein in a maximum volume of 0.1 ml were added to an equal volume of a sample solution containing 0.2M phosphoric acid/NaOH pH7.1, 8M urea, 1% SDS with or without 1% 2-mercaptoethanol. A drop of 0.05% bromophenol blue was added to each sample as a tracking dye. The tank buffer was 0.1M phosphoric acid/NaOH pH7.1, 0.1% SDS. Samples were layered on top of the gels, beneath the surface of this buffer and electrophoresed at 4mA per gel overnight (or at 12mA per gel for about 6 hours). The gels were then reamed out of the glass tubes, cut at the centre of the tracking dye band and fixed in 25% isopropanol, 10% acetic acid for 24 hours (Fairbanks et al 1971).

Gels were stained with 0.1% Coomassie Brilliant Blue in 25% isopropanol, 10% acetic acid for 2 hours and then destained in 14% methanol, 7% acetic acid. On storage in 7% acetic acid the remaining background gradually cleared.

The gels may alternatively be stained for carbohydrate-containing bands (rather than the protein stain described above) by the method of Fairbanks et al (1971). After fixing as for the protein stain this procedure may be summarised as follows:

TABLE 2.1
Standard proteins for molecular weight
determination by SDS-gel analysis.

<u>Sample</u>	<u>Mol. Wt.*</u>	<u>Source</u>	<u>Supplier</u>
1. Fibrinogen	333,000	Human	1
alpha chain	71,000		
beta chain	56,500		
gamma chain	49,000		
2. Immunoglobulin	150,000	Bovine plasma	2
IgG			
H chain	50,000		
L chain	23,500		
3. Phosphorylase A	92,500	Rabbit muscle	3
4. Transferrin	76,000	Human	3
5. Serum albumin	68,000	Bovine	2
6. Ovalbumin ^e	43,500	Hen	3
7. Aldolase	40,000	Rabbit muscle	3
8. Pepsin	35,000	Porcine	3
9. alpha-	25,700	Bovine pancreas	3
chymotrypsinogen			
10. Myoglobin	17,200	Horse	4
11. alpha-	14,300	Bovine	5
lactalbumin			
12. Lysozyme	14,300	Hen	4
13. Ribonuclease	13,700	Bovine	3

*Darnall and Klotz (1972).

Suppliers:

1. Kabi Pharmaceuticals Ltd.
2. Armour Pharmaceutical Co. Ltd.
3. Sigma London Chemical Co. Ltd.
4. Koch Light Laboratories Ltd.
5. Purified in this laboratory.

each gel was washed in at least 50 mls of:

1. 10% isopropanol, 10% acetic acid, 8 hours.
2. 10% acetic acid, overnight.
3. 10% acetic acid, 8 hours.

then at least 80 mls per gel of each of the following:

4. 0.5% periodic acid, 2 hours.
5. 0.5% sodium arsenite, 5% acetic acid, 1 hour.
6. 0.1% sodium arsenite, 5% acetic acid, 20 mins
(repeated x 2).
7. 5% acetic acid, 20 mins.

Gels were then transferred to 10 mls of Schiff reagent overnight and then destained in a large bath of 0.1% sodium metabisulphite, 0.01 N HCl for several hours with changes until the wash solution was no longer pink to formaldehyde solution. The gels were stored in 7% acetic acid and scanned as soon as possible as they faded fairly rapidly.

Gels stained by either method were scanned on a Joyce-Loebl double-beam recording microdensitometer mark IIIC (Joyce, Loebl and Co.Ltd., Gateshead, England). The relative mobilities of each band were calculated relative to the tracking dye and compared, on a plot of mobility against log molecular weight, with the mobilities of proteins of known size. Standard molecular weights were obtained from Darnall and Klotz (1972) and are listed in Table 2.1. When necessary individual bands were quantitated by tracing scans onto thick paper, cutting out and weighing.

The alternative procedure of Laemmli (1970) as modified by Owen et al (1973) is briefly described below:

solution A: 30% acrylamide

solution B: 2.7% bis-acrylamide

solutions C_1 : 1M Tris;

C_2 : 1M Tris/HCl pH 6.8;

C_3 : 1M Tris/HCl pH 8.8

solution D: 1.92M Glycine.

Also 10% SDS, 1.5% ammonium persulphate and TEMED.

1. Tray buffer: 0.025M Tris/glycine pH 8.3, 0.1% SDS

Mix 50 mls C_1 , 200 mls D and 20 mls 10% SDS to 2 l.

2. Separating gel:

10% acrylamide, 0.27% bis, 0.375M Tris/HCl pH 8.8,

0.1% TEMED, 0.1% persulphate, 0.1% SDS.

Mix 33.3 mls A, 10 mls B, 37.5 mls C_3 , 1 ml 10% SDS

6.7 mls persulphate and 0.1 ml TEMED to 100 mls

and the solution briefly degassed.

3. Stacking gel:

3% acrylamide, 0.08% bis, 0.125M Tris/HCl pH 6.8,

0.1% SDS, 0.1% persulphate, 0.1 TEMED.

Mix 1 ml A, 0.3 mls B, 1.25 mls C_2 , 0.1 ml 10% SDS,

0.7 mls persulphate and 0.01 mls TEMED to 10 mls

and degas.

4. Sample mixture:

0.125M Tris/HCl pH 6.8, 4% SDS, 20% glycerol,

10% 2-mercaptoethanol, 0.002% bromophenol blue.

An equal volume of sample solution was added to the 0.1 to 0.2 ml sample containing up to 50ug of protein. Smaller volumes than this required extra tracking dye. The sample was applied to gels consisting of 1cm of stacking gel formed over 8cm of separating gel and run at 4mA per gel for about 6 hours and then treated in the same way as Weber and Osborn gels.

5. Thin layer gel filtration in guanidinium hydrochloride (GuHCl).

Andrews (1964) has shown that the position at which a globular protein elutes from a gel filtration column depends on the volume available to it in the column. If the elution volume, v_e , of a series of globular proteins of known molecular weight are determined, the molecular weight of an unknown may be determined from a calibration curve of v_e versus log molecular weight. This is only possible if the protein is not excluded from the matrix, does not interact with the column material, is present as a single molecular species and has the same volume/weight characteristics as the standards.

Glycoproteins act anomalously in two ways in such systems. Firstly, some show a specific interaction with dextran matrices and are therefore retarded in their elution from columns such as those of the Sephadex type (Agrawal and Goldstein 1965). This may be avoided by the use of alternative supports, such as polyacrylamide. Secondly,

TABLE 2.2

Standard proteins for thin layer gel
filtration in guanidinium hydrochloride.

<u>Protein</u>	<u>Source</u>	<u>Molecular weight</u>
1. Serum albumin	Bovine	68,000
2. Transferrin	Human	76,000
3. Ovalbumin	Hen	43,500
4. alpha- chymotrypsinogen	Bovine pancreas	25,700
5. Myoglobin	Horse	17,000
6. Pepsin	Porcine	35,000

the presence of an appreciable proportion of sugar residues in the glycoprotein results in a greater binding of water than in a similar sized molecule containing no carbohydrate. Thus the effective volume occupied by the molecule is increased, v_e , is decreased and the apparent molecular weight is high (Andrews 1965).

These effects are not simple and will vary according to which particular sugar residues are present (Gibbons 1965). Anomalies due to differences in the shape and the extent of aggregation of molecules may be eliminated by using reduced and carboxymethylated proteins in denaturing solvents, such as 6M GuHCl (Fish et al 1969, 1970). This method will not totally eliminate the anomalous behaviour of glycoproteins for the reasons discussed in the section on the SDS-gel method.

The standard proteins used in this technique are shown in Table 2.2. These proteins, together with the unknown samples, were prepared as 5mg/ml solutions in 6M GuHCl, 0.2M Tris/HCl pH8.0. Reduction was accomplished by the addition of 2-mercaptoethanol to a final concentration of 5% (v/v) and incubating for 1 hour. The reduced proteins were then carboxymethylated in the presence of 0.1M iodoacetic acid (recrystallised from ethanol). To restrict alkylation to the sulphhydryl groups the mixture was maintained at pH8.0 by the addition of 1M Tris. After a few minutes the mixture was allowed to stand in the dark

for 15 minutes at room temperature and then unreacted acid removed by the addition of a second aliquot of mercapto-ethanol.

Thin layer gel filtration is analogous to column gel filtration (Radola 1968), and although less accurate is easier to carry out and requires less protein. Rather than measuring the elution volume of the samples, the mobility is compared with that of dextran blue (to mark the excluded volume) and DNP-alanine (to ^{approximately} mark the included volume).

Gel filtration was carried out in 6M GuHCl using Sepharose 6B as a support which gave an exclusion limit of about 80,000. The Sepharose, in 6M GuHCl was spread on a grease-free glass plate and rolled out to give an even layer of about 0.5mm. The plate was placed in a Desaga high voltage electrophoresis apparatus with the air space minimised to reduce evaporation and the apparatus tilted to an angle of about 10° . The filter bed was connected to the buffer compartments with muslin wicks and 6M GuHCl, 0.2M Tris/HCl pH8.0 run through the bed overnight. 50ug of each protein, together with the markers, was applied to the top end of the plate, at 2cm intervals, together with the standards and chromatography allowed to proceed until the dextran blue marker reached the lower wick (about 3 hours). The plate was then returned to the horizontal and a print of the run made by pressing a sheet of Whatman

3MM paper on the plate, the marker positions being recorded by pricking. The protein was stained in 0.1% Lissamine green, 5% acetic acid, washed in 5% acetic acid and dried. The relative mobilities of the spots were calculated and compared with the plot of relative mobility versus $\log$ molecular weight for the standard proteins.

6. Amino Acid Analysis

Approximately 1mg samples of protein were prepared for hydrolysis by precipitation with trichloroacetic acid, followed by ethanol and ether washing. Alternatively they were rotary evaporated after extensive dialysis against 0.1M ammonium bicarbonate. Both procedures were carried out in screw-cap vials (Soveril) capable of withstanding 5 atmospheres pressure. The samples were finally dried in a vacuum dessicator over phosphorus pentoxide. 0.3 ml of 6N HCl was then added, the protein dissolved and the solution deoxygenated by flushing with a slow stream of nitrogen. This reduces oxidation of the tyrosine and sugar residues during hydrolysis. Hydrolysis was carried out at 110°C for 24, and in some cases 48, hours, after which the sample was rotary evaporated and vacuum dessicated to remove the acid. For analysis the samples were taken up in 0.2N sodium citrate/HCl pH2.2. If necessary humin was removed by filtration through small glass sintered filters on a bench centrifuge. Analysis for cysteine

was carried out after oxidation to cysteic acid as described by Hirs (1967).

Amino acid analysis was carried out according to Moore and Stein (1954) utilising an accelerated two column methodology permitting complete analysis in about $3\frac{1}{2}$ hours. The apparatus was built in this laboratory and was based on the Technicon system. The basic residues were separated on a (0.9x8)cm column at 55°C with 0.35N sodium citrate/HCl pH5.28 at a pressure of 20psi, using Aminex A5 resin (BioRad Laboratories, Bromley, Kent), while the acidic and neutral amino acids were resolved on a (0.9x60)cm Aminex A4 column with 0.164N sodium citrate/HCl pH3.40 and 0.2N sodium citrate/HCl pH4.30, at 85psi and 55°C . Methanol was included in the buffer to achieve better resolution of serine and threonine; and thiodiglycol~~4~~ to protect methionine (and cysteine) against oxidation. Peaks were quantitated by the dot counting method and by comparison with a standard amino acid mixture.

7. N-terminal analysis

N-terminal analysis was carried out by the dansyl procedure. In this method the protein is treated with ^{methyl} diamino naphthelene sulphonyl chloride (DNS-C1) under conditions which restrict reaction to the N-terminal alpha amino group, the epsilon amino group of lysine and the hydroxyl moiety of tyrosine. After hydrolysis the

fluorescently labelled amino acids were identified by thin layer chromatography on double-sided polyamide sheets, the reverse side being used to run suitable standard amino acids.

The method of dansylation used was essentially that of Woods and Wang (1967) on a micro scale. Separation of DNS-amino acids was achieved on the polyamide sheets using the following solvents:

1st dimension 1. 1.5% formic acid (Woods and Wang 1967)

2nd dimension 2. Toluene:acetic acid, 9:1.

(modified from Woods and
Wang 1967)

3. Ethyl acetate:methanol:acetic acid
20:1:1

(Crowshaw et al 1967)

4. 0.05M Na_3PO_4 in 25% ethanol
(Hartley 1970).

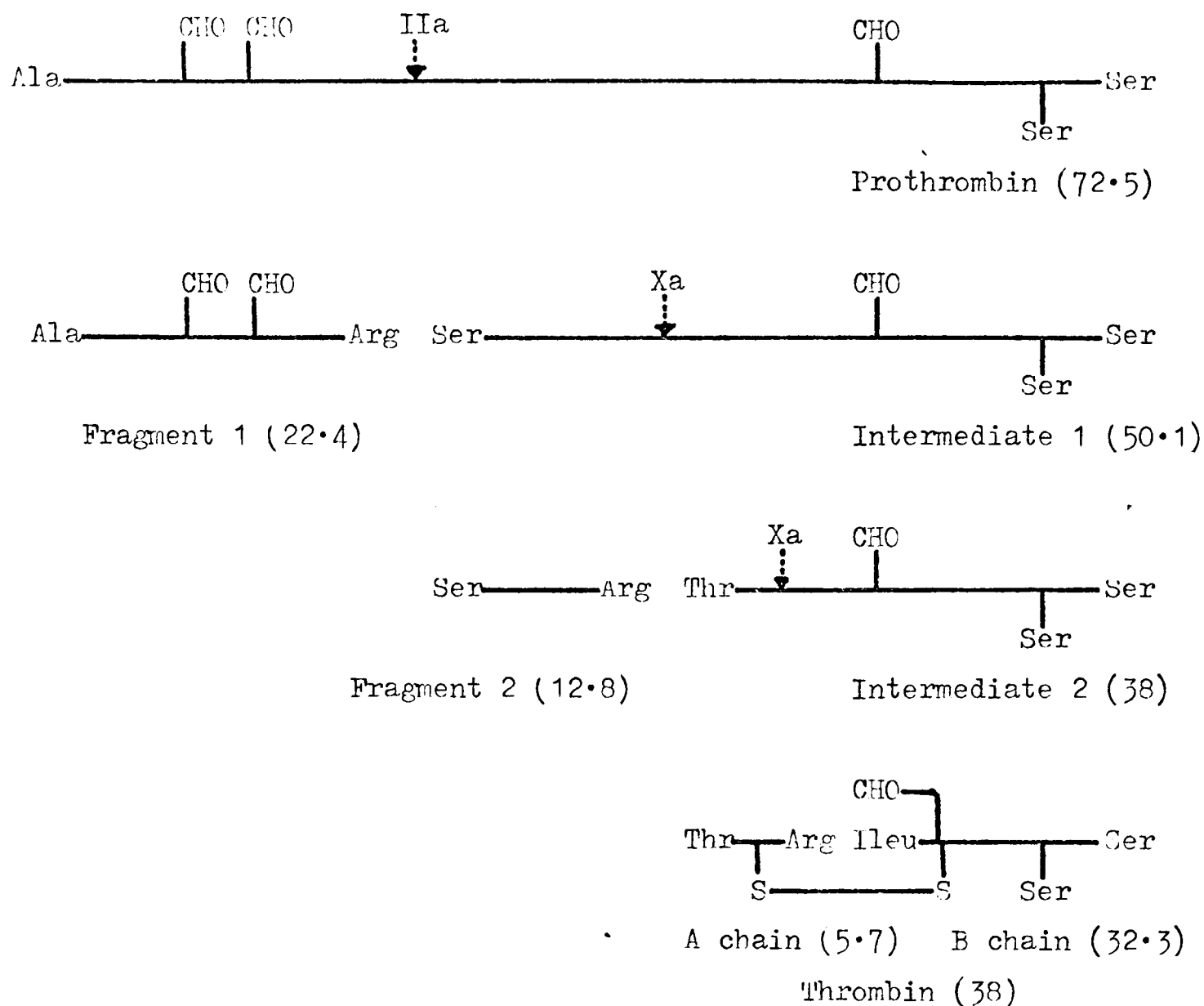
Spots were detected under ultraviolet light.

Separation was evident after solvent 2; solvents 3 and 4 were used for further resolution when necessary. Final identification with the standards on the reverse side of the sheet was carried out by pricking the sheet.

8. Activation of prothrombin and separation of the products

If Vitamin K introduces a prosthetic group into prothrombin this may be localised by study of the

FIGURE 2•1



adapted from Owen et al (1974)

Line diagram of prothrombin activation showing molecular weight ($\times 10^{-3}$) and location of products. The location of carbohydrate side chains, terminal amino acid residues and the serine active site are also shown.

In rapid activations (or those in which thrombin is inhibited) the **Xa** cleavage occurs first yielding Fragment 1•2 (35) which may then be subsequently cleaved by thrombin to Fragment 1 and Fragment 2 (Esmon et al 1974).

activation products of prothrombin. Activation is normally achieved by the 'prothrombinase complex' (Jobin and Esnouf 1967) which consists of Factor Xa, calcium, phospholipid and Factor V. However the only enzymic activity in this complex is due to Factor Xa, other components acting as accelerators of the proteolysis. In this preparation of the activation products Factor Xa and calcium, with or without phospholipid, have been used as the acceleration caused by Factor V is so great that halting of the reaction at a selected extent of activation is only achieved with difficulty. By using a somewhat slower activation method, e.g. with Factor Xa and calcium, the process may be terminated at a predetermined point.

The work of Mann and his colleagues (Heldebrant and Mann 1973, Heldebrant et al 1973a) and others (Stenn and Blout 1972, Owen et al 1974) has demonstrated the existence of two intermediates and two by-products in the production of thrombin from prothrombin. The location and size of these components of the prothrombin molecule is shown in Figure 2-1.

Prothrombin was activated using the same conditions as for the kinetic experiments described in Chapter 4. The formation of thrombin was followed by its esterase or clotting activity and by SDS-gel analysis. On reaching a suitable distribution of the activation products the activation was essentially stopped by the addition of

0.1M EDTA/NaOH pH7.5 to a final 10mM. This removes the calcium from the 'prothrombinase complex' and stops any phospholipid enhancement of activation. In later experiments a Factor-Xa-Sepharose column (see Appendix 1 for synthesis) was used for activation so as to conserve Factor Xa. In this case prothrombin was flowed through the column in 50mM Tris/HCl pH8.0, 5mM CaCl_2 and 0.1M EDTA/NaOH pH7.5 added to the effluent to a final 10mM. The rate of flow through the column may be varied to give different distributions of the activation products.

Activation mixtures prepared by either of these methods were then separated into their components as follows:

1. The mixture was rapidly concentrated to 2 or 3mg/ml in an Amicon ultrafiltration device fitted with a UM-10 membrane.
2. The concentrate was applied to a (1.5x20)cm column of SP-Sephadex C-50, pre-equilibrated in 50mM HCl/Tris pH7.5 at 30 ml/hr. This binds the thrombin and some of the Intermediate 2, all other components being eluted in the dead volume. This column was developed with a (2x200ml) linear gradient from 0 to 0.35M NaCl in 50mM HCl/Tris pH7.0 at 20 ml/hr. 5 ml fractions were collected.
3. The SP column breakthrough was immediately applied to a (1.5x15)cm DEAE-Sephadex A50 column, pre-equilibrated with 50mM HCl/Tris pH7.5, at 20 ml/hr. The various intermediates, fragments and Factor Xa

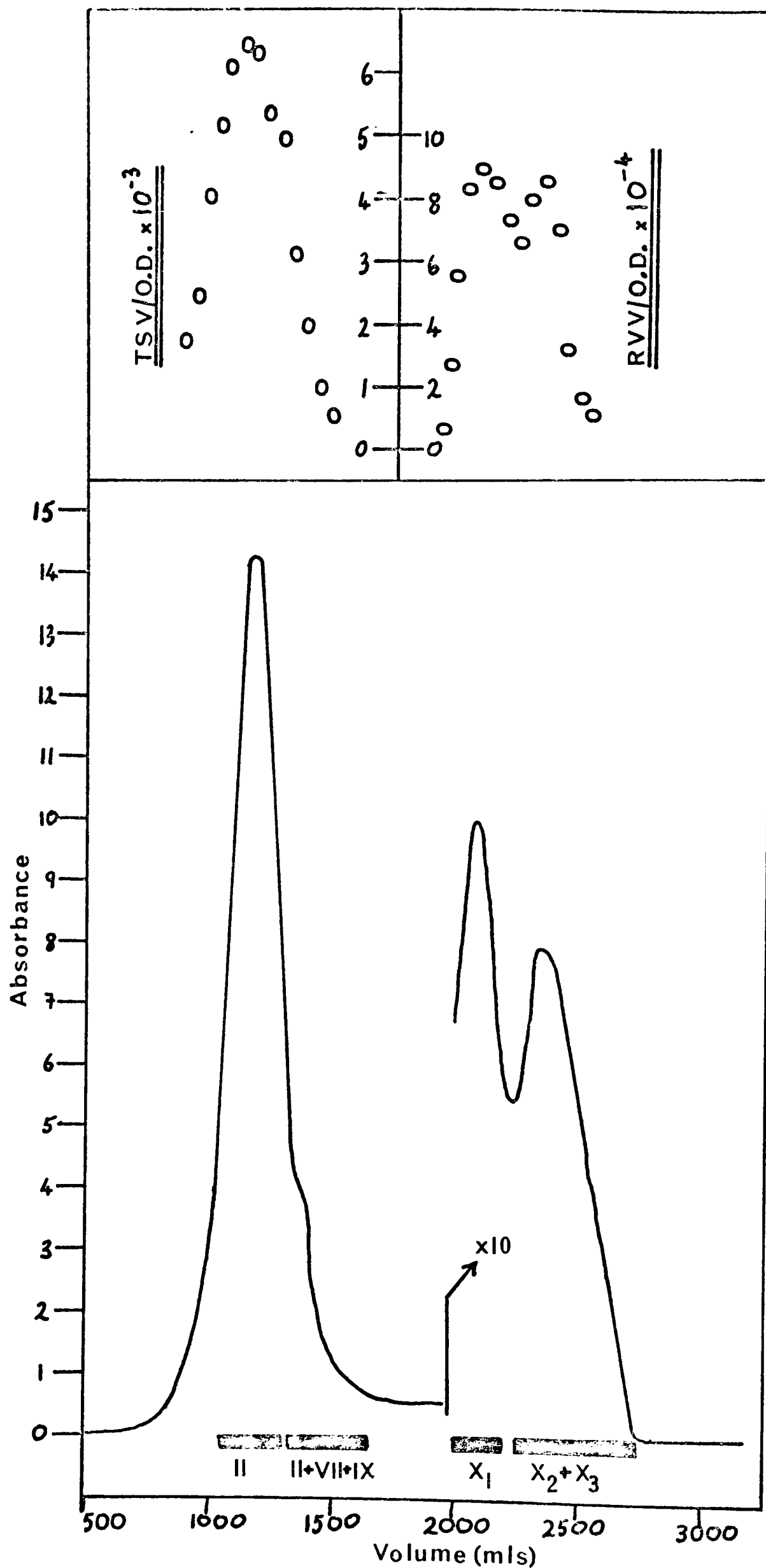


FIGURE 2•2

Preparation of Bovine Prothrombin and Factor X

The specific activity of prothrombin and Factor X are expressed in arbitrary units (as determined by the venom assays) per optical density unit. An optical density unit is defined as that quantity of protein which when dissolved in 1 ml of solvent gives rise to unit optical density.

(if present) were eluted with a (2x300ml) linear gradient from 0.1 to 0.4M NaCl in 50mM HCl/Tris pH7.5 at 20 ml/hr. 5ml fractions were collected.

If only intermediate 1 and Fragment 1 were required they were prepared by thrombin degradation of prothrombin. This was carried out as described by Owen et al (1974). Subsequent separation was carried out on DEAE-Sephadex as described above.

(c) Results and Discussion

Figure 2.2 shows the DEAE-Sephadex elution profile of the bovine preparation. The prothrombin was eluted at about 0.15M citrate and Factor X at an average of about 0.35M citrate. The slight shoulder on the trailing edge of the prothrombin peak has been shown to contain Factor VII and IX activity (Kalowsek et al 1975). The separation of Factor X into two peaks, X_1 and X_2 , has been noted by Jackson and Hanahan (1968). Jackson (1972) ascribed this separation to differences in the carbohydrate content of X_1 and X_2 , but Fujikawa et al (1972) were unable to detect this difference. This preparation also yielded Factor X_3 (Mattock and Esnouf 1973) as a contaminant of the X_2 . This protein will be discussed in more detail in Chapter 6.

In the case of the human prothrombin, which also eluted at about 0.15M citrate, no separation from Factor X

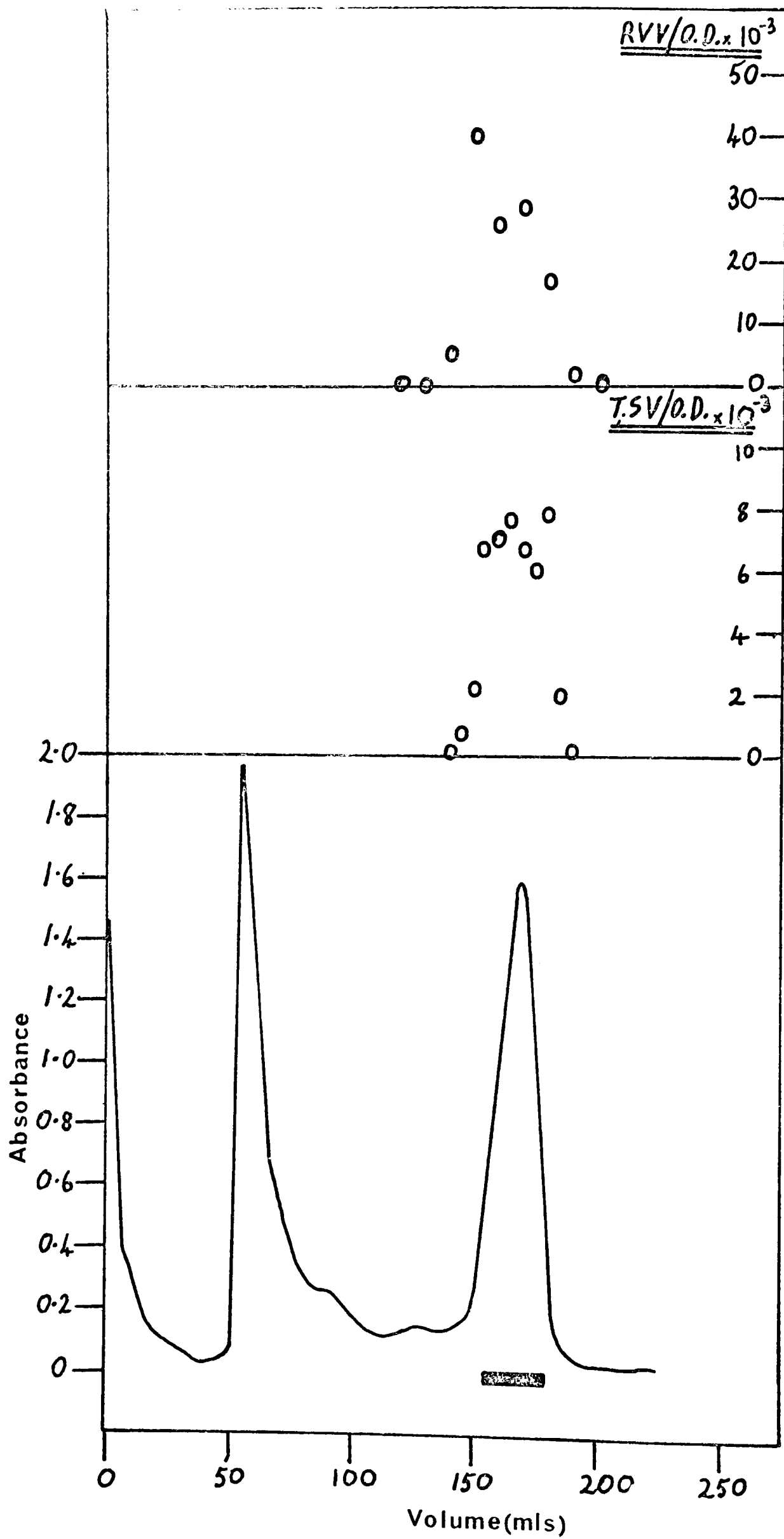


FIGURE 2•3

Preparation of human prothrombin

Specific activities of clotting factors
are expressed in the same way as for the bovine
preparation.

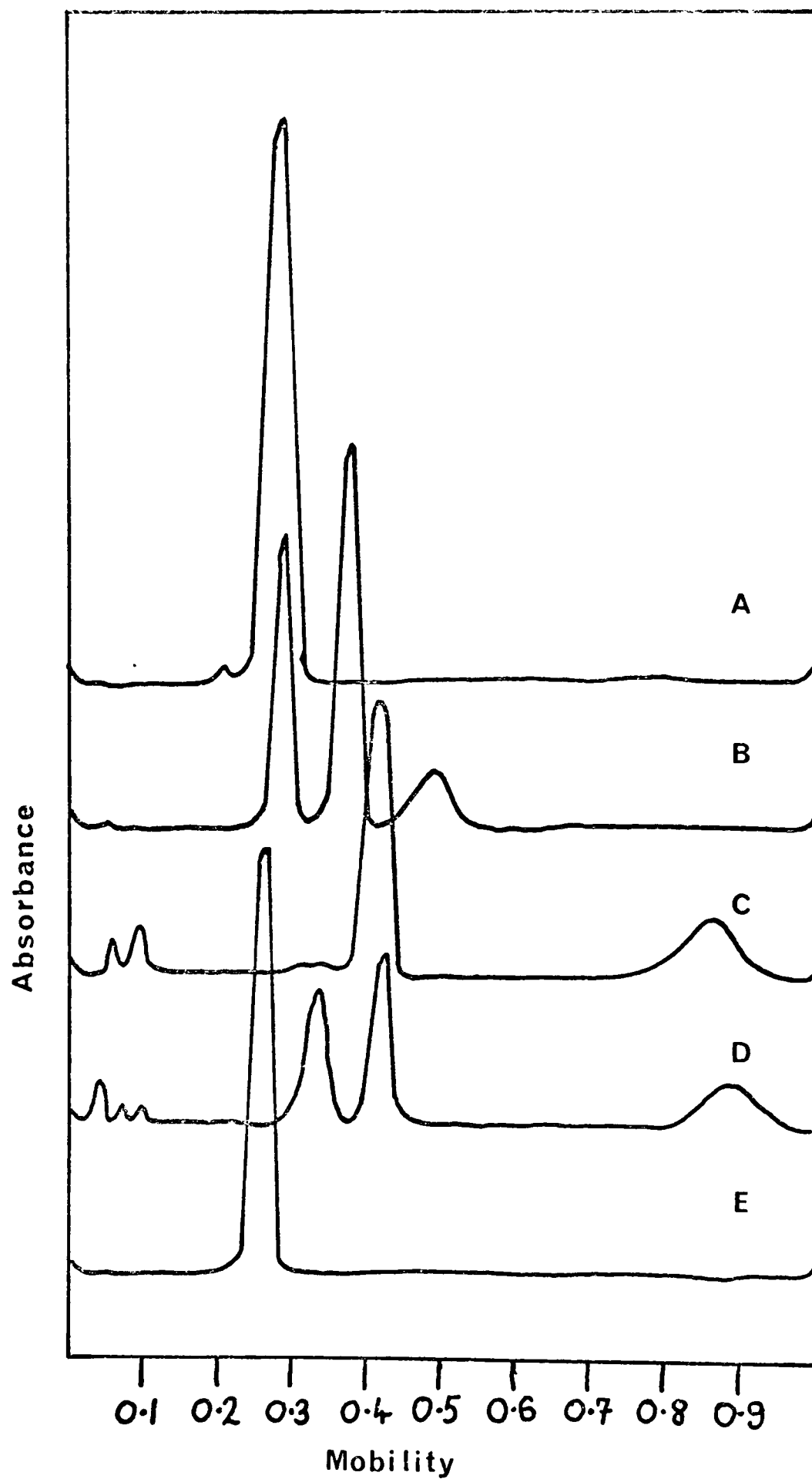


FIGURE 2•4

SDS-gel analysis of pools from
prothrombin preparations

- A: Bovine prothrombin pool.
- B: Bovine prothrombin/Factor VII/Factor IX pool.
- C: Bovine Factor X_1 pool.
- D: Bovine Factor X_2 and Factor X_3 pool.
- E: Human prothrombin pool.

was achieved (Figure 2-3). Human prothrombin prepared in a similar manner contained activity corresponding to all four Vitamin K-dependant clotting factors (Morrison and Esnouf 1973). The peak eluted at about 80mM citrate in this preparation is turbid. Many of the earlier fractions eluting of this column had a high absorption at 320nm, indicating large particle light scattering. All optical density readings were therefore corrected for Rayleigh light scattering according to Shapiro and Waugh (1966). Bovine material gave very little absorption at 320nm and the scattering correction was therefore not applied.

The proteins from either preparation were pooled as indicated, on the basis of activity measurements and SDS-gel analysis and stored at -20°C in convenient aliquots. The deep frozen proteins were quite stable for long periods in the citrate/NaOH buffer, but prothrombin was susceptible to degradation once it was dialysed out of this buffer at room temperature, or at 4°C . This may indicate the presence of trace amounts of thrombin or Factor Xa in the preparation, however SDS-gel analysis of the pooled prothrombin showed no contaminants to be present in appreciable quantities (Figure 2-4). Factor X pools did show a small amount of contamination which may be due to aggregation (Esnouf et al 1973), but this is unlikely in the presence of the denaturing solvent used to prepare samples for SDS-gel analysis. The Factor X was never

TABLE 2.3
Purification of clotting factors

Stage	Volume (mls)	Activity		Recovery (%)	Protein (OD)	Specific	Purif- cation
		units/ml	total			Activity	
						(units/OD)	
<u>Bovine prothrombin</u>							
Citrated plasma	2.5x10 ⁴	10 ³	2.5x10 ⁷	100	70	14.2	1
Barium eluate	2.5x10 ³	6.8x10 ³	1.7x10 ⁷	68	8.25	824.3	58.1
DEAE pool	235	58.8x10 ³	1.38x10 ⁷	55.3	10.5	5,600	395
<u>Bovine Factor X</u>							
Citrated plasma	2.5x10 ⁴	10 ³	2.5x10 ⁷	100	70	14.2	1
Barium eluate	2.5x10 ³	10 ⁴	2.5x10 ⁷	100	8.25	1212	85.4
DEAE pool							
X ₁	193	7.4x10 ⁴	1.43x10 ⁷	57.1	0.825	89,700	6,317
X ₂ +X ₃	303	4.0x10 ⁴	1.21x10 ⁷	48.5	0.625	64,000	4,507
				105.6			
<u>Human prothrombin</u>							
Citrated plasma	500	10 ³	5x10 ⁵	100	70	14.2	1
Barium eluate	50	8.9x10 ³	4.45x10 ⁵	89	8.1	1,113	78.4
DEAE pool	38	6.96x10 ³	2.64x10 ⁵	53	0.986	7,063	497

Protein weight has been expressed in terms of optical density units. These may be converted to mgs by division with the following extinction coefficients:

1.38 for bovine prothrombin

0.956 for bovine factor X

(Esnouf et al 1973)

1.38 for human prothrombin

(Kisiel and Hanahan 1973)

used as such; it was either converted to Factor Xa or separated into its component chains. These products were purified by column chromatography which removed the aforementioned contaminants. The degree of purification of prothrombin and Factor X, prepared by this method, are shown in Table 2-3. Comparison with recently published methods for bovine prothrombin and Factor X (Bajaj and Mann 1973) and human prothrombin (Kisiel and Hanahan 1973) showed that the procedure was comparable both in purification and yield with these methods. Both the above methods utilised a further step in the purification of the proteins after the first ion-exchange stage. Bajaj and Mann used a second ion-exchange step to achieve a final purification of 535 (at 50% yield). This was measured using a two stage assay. The final step used by Kisiel and Hanahan, preparative gel electrophoresis, increased the purification from 466 to 811 (77%), when assayed by a two-stage method, but only from 294 to 467 (45%) by a one-stage assay. These differences may be due to some alteration in the prothrombin during the purification procedure such that it is activated at a slower rate. This effect has been described by Devilee et al (1975), who assert that it is a result of the high temperature that the protein is subjected to during preparative electrophoresis. Although this procedure was not used during the present study, one-stage assays will generally give slightly lower values than two-stage, thus explaining the minor differences between the present results and those of the above authors, the

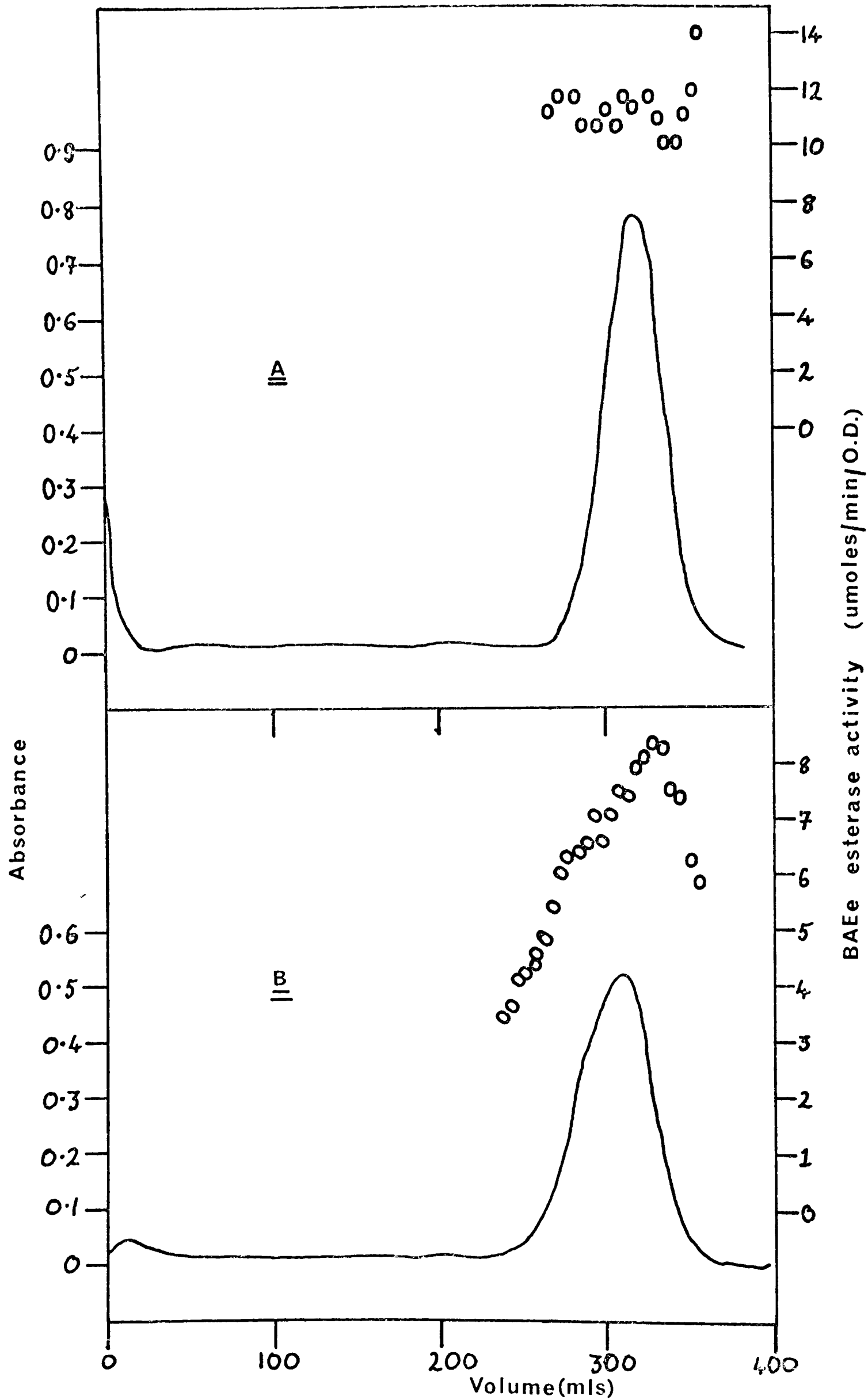


FIGURE 2.5

Chromatography of prothrombin activation
mixtures on sulphopropyl-Sephadex

A: Sample applied at a concentration of 3.0 mg/ml.

B: Sample applied at a concentration of 0.3 mg/ml.

Thrombin was assayed by the esterase method.

Isolated Intermediate 2 has no esterase activity.

prothrombin being essentially pure as judged by SDS-gel analysis. Note also that our results are expressed in terms of O.D. units rather than mg.

The purification procedure described is a quick and simple method of obtaining substantially pure prothrombin and Factor X in good yield. The prothrombin may be rerun on DEAE-Sephadex to give a marginally purer^e product; this has been done in the preparation of some material for crystallisation studies. However as no contamination was apparent on SDS-gels and in view of the fact that most of this study was concerned with the physicochemical properties of this material, it was used as described above.

Prothrombin was activated with Factor Xa and calcium, and thrombin isolated as a single species by chromatography on sulphopropyl-Sephadex. If the isolation was carried out on an activation mixture in which conversion had not gone to completion some of the Intermediate 2 also bound to the SP-Sephadex column. This was detected by SDS-gel analysis and by the gradient of esterase activity across the protein peak, which indicated that Intermediate 2 was eluted just ahead of thrombin. The extent to which Intermediate 2 was bound to the SP-column depended on the absolute concentration of the activation mixture load. At higher concentrations most of the Intermediate 2 was eluted in the dead volume of the column (Figure 2.5a). This was ascribed to competition between the two proteins

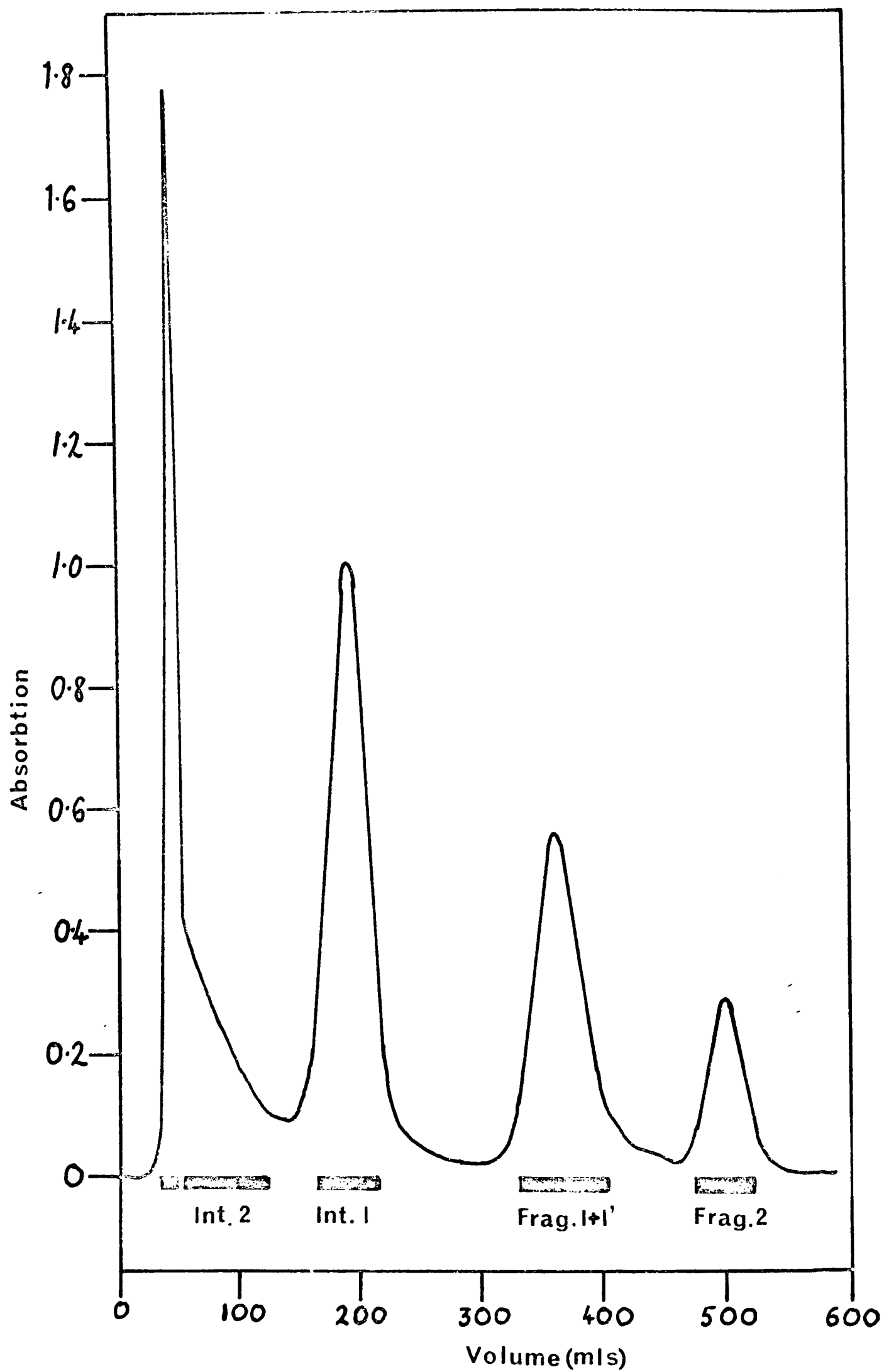


FIGURE 2•6

Chromatography of prothrombin activation
mixtures on DEAE-Sephadex

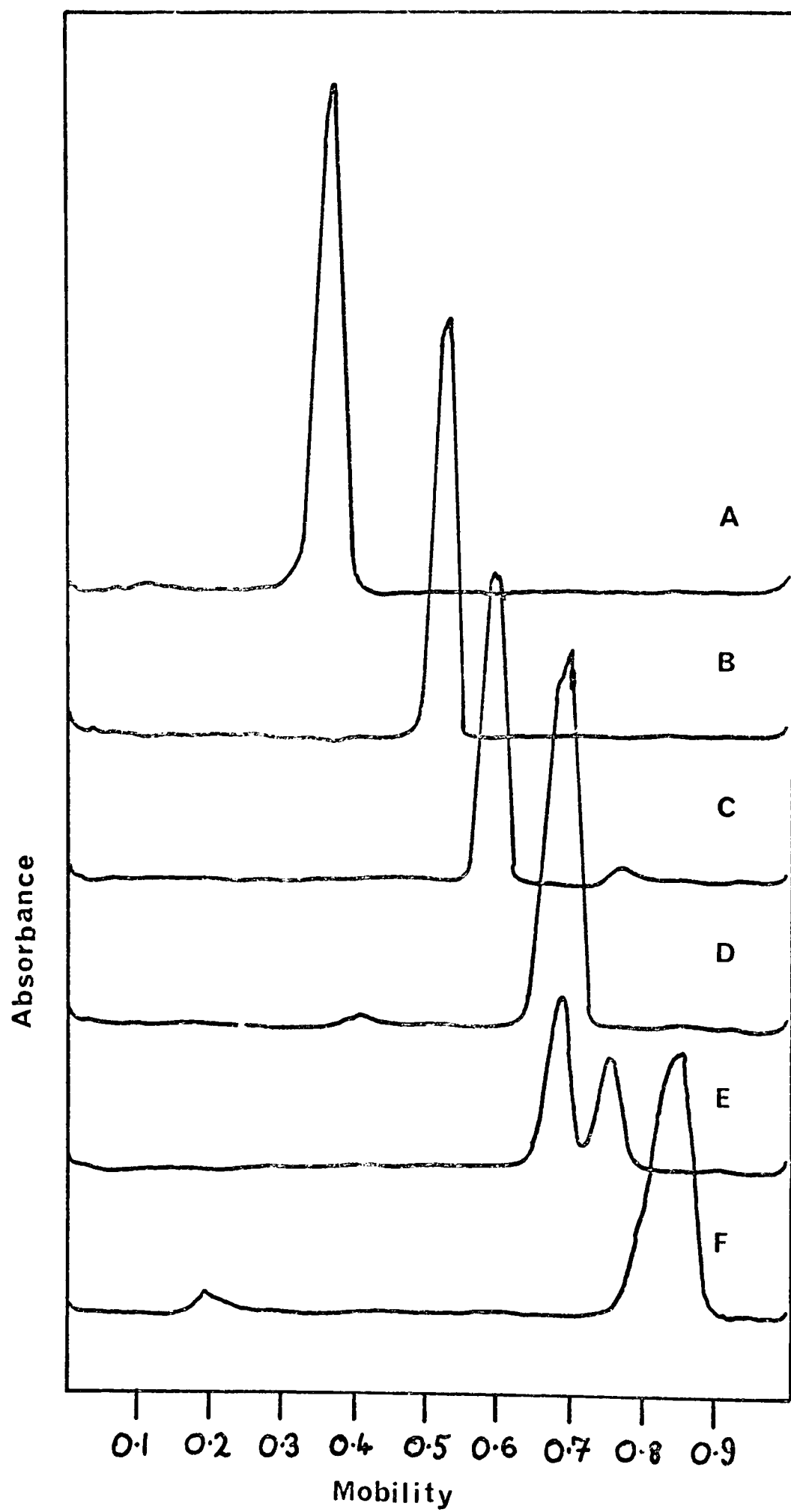


FIGURE 2•7

SDS-gel analysis of isolated prothrombin
activation products

- A: Intermediate 1. (reduced).
- B: Intermediate 2. (reduced).
- C: Thrombin (reduced).
- D: Fragment 1 + 1° (unreduced).
- E: Fragment 1 + 1° (reduced).
- F: Fragment 2. (reduced).

for available binding sites, thrombin binding due to its slightly greater affinity for these sites. At lower concentrations of column sample more of the Intermediate 2 was bound and the elution profile was as shown in Figure 2-5b. Human thrombin and Intermediate 2 differ slightly from the bovine proteins in that better resolution was achieved on the SP-Sephadex than in the bovine case.

When the SP-Sephadex column breakthrough was applied to a DEAE-Sephadex column the Intermediate 2 did not bind appreciably to the column. The breakthrough consisted of slightly contaminated Intermediate 2 whereas the Intermediate 2 that did bind to the column, albeit marginally, was eluted in pure form (Figure 2-7). Other activation products were eluted from the DEAE-Sephadex column in the order Intermediate 1, Fragment 1 and Fragment 2 (Figure 2-6). In activation mixtures containing Factor Xa (i.e. those in which the Xa-Sepharose column was not used) it was eluted after the Fragment 2.

During this work it was noted that during slow activations Fragment 1 was partially degraded to a product (Fragment 1°) of the same size as Fragment 1 on non-reduced SDS-gels but giving rise to a new component of apparent molecular weight 20,000 daltons in the presence of mercaptoethanol. This was interpreted as due to the formation of a two chain form of Fragment 1 containing 20,000 and 5,000 dalton polypeptide chains connected by

TABLE 2.4

Molecular weights and N-terminal residues of bovine prothrombin, its activation products and Factor X.

Protein	Molecular weights			MW _{sum}	MW** _{sum}	N-terminal
	SDS-gels	6M-GuHCl plates	MW*			
Prothrombin	73,000	71,000	72,500	60,200	66,100	Ala
				+	+	
				3 CHO	3 CHO	
Intermediate 1	52,500	52,000	50,100	45,300	48,100	Ser
				+	+	
				CHO	CHO	
Intermediate 2	37,000	32,000	38,000	34,500	35,400	Thr
				+	+	
				CHO	CHO	
Thrombin	35,000	30,000	35,000	34,100	35,400	Thr
				+	+	+
				CHO	CHO	Ileu
	B chain only			A and B chains		
Fragment 1	25,300	21,700	22,400	15,300	17,900	Ala
				+	+	
				2 CHO	2 CHO	
Fragment 1	26,400	20,800	-	15,300	-	Ala
+	+			+		+
Fragment 1	20,600			2 CHO		Asx
Fragment 2	16,900	12,200	12,800	12,900	12,800	Ser
Factor X	44,000	-	42,000	-	-	Tryp
	+		+			+
	15,800		22,000			Ala

CHO indicates a carbohydrate side chain.

*: figures of Owen et al (1973)

**: figures of Magnusson et al (1975)

interchain disulphide bond(s). The smaller chain is not seen on SDS-gel analysis as it will not be fixed by the method used. It is probable that this degradation was due to thrombin, rather than Factor Xa, as the conversion also took place in thrombin activations of prothrombin (used to prepare Intermediate 1 and Fragment 1 only). The various products were pooled as indicated in Figure 2-6 and stored deep frozen at -20°C . Figure 2-7 shows the results of SDS-gel analysis of the purified proteins. Gels run in parallel but stained for carbohydrate showed that all the activation products except for Fragment 2 contained carbohydrate.

To ensure that the isolated products corresponded to those described by other groups (Heldebrant et al 1972, Owen et al 1974, Magnusson et al 1975) they were characterised physicochemically. Molecular weights were determined by SDS-gel electrophoresis and by thin layer gel filtration in 6M GuHCl. The results (Table 2-4) were in agreement with those obtained by the above authors. Fragment 1 has a high apparent molecular weight by SDS-gel analysis, presumably due to its high carbohydrate content. The amino acid content and N-terminal residue of the activation products were also determined (Tables 2-4 and 2-5) and the molecular weight (MW) was calculated from the composition data (MW_{sum}) by addition of the contributions of each residue, allowing for the water lost in peptide bond formation. These values may be compared with those

TABLE 2.5
Amino acid analysis of prothrombin and
its activation products.

	<u>1.</u>	<u>2.</u>	<u>3.</u>	<u>4.</u>	<u>5.</u>	<u>6.</u>	<u>7.</u>	<u>8.</u>	<u>9.</u>	<u>10.</u>	<u>11.</u>
LYS	30.3	30.6	31.6	4.8	5.8	25.8	28.8	24.6	2.4	26.4	22.2
HIS	8.9	8.5	8.8	1.7	2.0	6.8	7.3	6.1	0	7.3	6.1
ARG	37.8	39.9	38.7	13.3	12.1	26.6	30.2	26.5	7.4	22.8	19.1
ASP	59.3	60.1	59.9	13.4	13.2	46.7	50.0	43.5	17.7	32.3	27.8
THR	29.8	28.2	29.1	8.2	9.1	20.0	19.4	18.8	5.1	14.3	13.7
SER	34.2	34.4	34.4	8.8	8.8	25.6	25.2	27.2	9.6	15.6	17.6
GLU	66.3	66.1	65.8	20.3	20.0	45.8	51.2	50.7	15.9	35.3	34.8
PRO	29.6	29.7	29.2	7.6	7.1	22.1	21.9	21.8	7.9	14.0	13.9
GLY	<u>49</u>	<u>49</u>	<u>49</u>	<u>10</u>	<u>10</u>	<u>39</u>	<u>38</u>	<u>38</u>	<u>12</u>	<u>26</u>	<u>26</u>
ALA	32.8	33.2	32.8	9.2	8.8	24.0	26.1	23.1	10.6	15.5	12.5
CYA	20.0	22.5	20.5	9.1	7.1	13.4	15.6	15.3	5.1	10.5	10.2
VAL	24.8	24.0	24.4	6.1	6.5	17.9	20.2	21.7	5.6	14.6	16.1
MET	6.0	5.6	6.0	0.8	1.2	4.8	3.8	5.2	0	3.8	5.2
ILEU	15.5	14.8	15.2	2.9	3.3	11.9	11.8	11.4	0.7	11.1	10.7
LEU	40.5	41.8	42.2	8.9	9.3	32.9	36.3	39.5	10.5	25.8	29.0
TYR	17.5	16.7	16.9	3.6	3.8	13.1	15.1	14.1	3.7	11.4	10.4
PHE	17.9	18.5	18.4	3.7	3.6	14.8	17.1	15.8	3.1	14.0	12.7
TRYP* <u>14</u>	<u>14</u>	<u>14</u>	<u>14</u>	<u>3</u>	<u>3</u>	<u>11</u>	<u>11</u>	<u>11</u>	<u>2</u>	<u>9</u>	<u>9</u>
TOTAL	534	538	535	135	135	402	429	416	119	310	298
TOTAL*	582			156		426			118	308	308

*: figures from Magnusson et al (1975).

-the results are calculated relative to glycine.

values for glycine are taken from Owen et al (1974)

- 1. Prothrombin 2. Intermediate 1 + Fragment 1
- 3. Intermediate 1 + Fragment (1 + 1')
- 4. Fragment 1 5. Fragment (1 + 1')
- 6. Intermediate 1 7. Intermediate 2 + Fragment 2
- 8. Thrombin + Fragment 2
- 9. Fragment 2 10. Intermediate 2
- 11. Thrombin.

reported by Magnusson et al (1975) derived from sequence studies. The figures obtained are lower than Magnusson's, but it is likely that this reflects a low recovery of the hydrophobic residues after hydrolysis for only 48 hours. This effect is most noticeable in the prothrombin (MW_{sum} 60,000 as compared to 66,000). The values obtained for MW_{sum} are lower than those obtained by the other methods as no allowance was made for the carbohydrate contribution. It was thought that in view of the results obtained it was not worth carrying out estimations of tryptophan as the methods are protein destructive; however the most convenient method would appear to be that of Liu (1972) in which the protein is hydrolysed with p-toluene sulphonic acid in the presence of tryptamine, rather than the earlier direct spectro-photometric methods (e.g. Edelhoch 1967).

The N-terminal residues of the products were identical with those reported by other investigators. The new N-terminal residue exposed by degradation of Fragment 1 to Fragment 1° is aspartic acid (or asparagine). Inspection of the amino acid sequence of prothrombin (Magnusson et al 1975) shows that, in view of the known arginine specificity of the clotting serine proteases, a possible site for this cleavage is the Arg-52 - Asn-53 bond in the sequence Glu-Ser-Ala-Arg-Asn-Pro-. Activation products were also obtained from human prothrombin by the above methods. Their molecular weights by SDS-gel analysis were the same as those

of the corresponding bovine proteins, except for Intermediate 1 which was apparently larger ($MW_{app} = 55,000$ daltons).

Characterisation of the size and composition of the activation products of prothrombin thus shows them to be substantially the same as those described by other authors. In view of their origin and the method of activation it was thought reasonable to conclude that they are the same products.

CHAPTER 3

COUMARIN THERAPY AND THE PURIFICATION OF ABNORMAL PROTHROMBINS

(a) Introduction

Soon after the introduction of specific antisera for the study of the effects of coumarin drugs, it was demonstrated that an abnormal form of prothrombin appears in the plasma of both human patients (Josso et al 1968, Nilehn and Ganrot 1968) and cattle (Stenflo and Ganrot 1972) during treatment with coumarin drugs. This abnormal prothrombin is immunologically identical with normal prothrombin (Josso et al 1968, Stenflo 1972), but does not bind calcium (Nelsestuen and Suttie 1972c, Stenflo and Ganrot 1973) or to insoluble barium salts (Nelsestuen and Suttie 1972b, Stenflo and Ganrot 1972) to the same extent and is not activated by the normal physiological activators (Nelsestuen and Suttie 1972b, Stenflo and Ganrot 1972, Ganrot and Nilehn 1968). On isolation of the abnormal prothrombin it was found to have a very similar amino acid and carbohydrate composition and to have the same molecular weight and N-terminal residue as the normal protein (Nelsestuen and Suttie 1972c, Stenflo 1970, Stenflo 1972).

Abnormal prothrombin that does bind to barium citrate has been demonstrated in bovine (Malhotra and Carter 1971) and in human (Morrison and Esnouf 1973) coumarin plasma. These proteins also have low physiological but normal immunological activity. During treatment with therapeutic levels of coumarin drugs there is a preponderance of the above barium-absorbable prothrombin over the non-absorbable

protein, as opposed to the contrary situation when higher dose levels are used as in the rather drastic regimes of Stenflo (1972) or Nelsestuen and Suttie (1972b). The barium-absorbable form of the protein might therefore be of more relevance in the clinical situation, in which coumarin drugs may be prescribed for long periods.

The two abnormal forms of bovine prothrombin, barium-absorbable (prothrombin-Ba) and non-absorbable (prothrombin-super), have therefore been isolated from cattle on relatively long-term courses of Warfarin treatment (up to 3 months) in the hope of establishing the difference between them and normal prothrombin. In a parallel study barium-absorbable prothrombin was isolated from the plasma of patients undergoing long-term coumarin therapy.

(b) Methods

1. Preparation of Antisera.

Rabbits were immunised against purified proteins, prepared as described in Chapter 2, by multiple site intradermal injections of a total of 1mg of antigen in 1 ml of Freund's complete adjuvant. A booster dose was given after 2 weeks and blood collected from an ear vein about 10 days later. The antisera were stored as 1 ml aliquots at -20°C and used without further purification.

2. Quantitative immunoelectrophoresis.

This was performed by the one-dimensional technique of Laurell (1966). 17.5 mls of a 1% (w/v) agarose solution in 60mM sodium barbitone/HCl pH8.65, containing 50 to 200 ul of antiserum, was poured onto a (10x20cm) grease-free glass plate. When set 19 holes were cut along one edge and 5 ul of sample, containing up to 0.1ug of antigen, was added to each well. The antigen was electrophoresed into the gel at 5V/cm overnight, or until precipitation was complete. The 'rocket' heights were measured after staining with Coomassie Brilliant Blue. Normal plasma was used as a standard and was defined as containing 1000 arbitrary units/ml.

3. Administration of Warfarin.

a. Cattle.

Cattle were provided and housed by Dr. J.M. Payne, Director of the ARC Institute for Research on Animal Diseases, Compton, Newbury. Cows were given 100mg of sodium Warfarin daily, by Mr. A. Russell, as a drench in 1% NaHCO₃ in milk. This increases the chance of the Warfarin entering the third and fourth stomach, thus reducing the required dose.

b. Human patients.

Blood from coumarin treated patients was obtained by voluntary donation from outpatients attending the

Anticoagulation Clinic at the Radcliffe Infirmary.

The majority of these patients had been undergoing treatment with Marevan (sodium Warfarin) for periods of more than six months. The reason for these prophylactic courses was usually a history of pulmonary embolism or deep vein thrombosis, or followed a major operation such as heart valve replacement. Patients received a loading dose of 15, 10 and 5mg of Warfarin on successive days and subsequent dosage was adjusted so as to provide a level of between 10 and 20% of normal by the Owren Thrombotest (Owren 1959). This test is analogous to the Quick PT and measures a combination of prothrombin, Factor VII and Factor X activity, a useful measure of the overall clotting ability of the patient. When assayed by the one-stage TSV assay for prothrombin activity coumarin plasma contained about 50% of the normal level (Morrison and Esnouf 1973).

4. Preparation of barium-absorbable prothrombin (prothrombin-Ba).

Blood (usually 2 l) was collected from the jugular vein of the Warfarin treated cows (sedated with 2% Rompun) by gentle suction into 0.1 volume of 4% trisodium citrate containing 1g BZ per litre of blood. The container was packed into ice until the cells could be removed by centrifugation at 2,000g for 45 minutes. The preparation was then carried out as for normal prothrombin but on a smaller scale, except that the barium citrate eluate was dialysed

into 20mM citrate/NaOH pH6-9, 1mM BZ. The DEAE-Sephadex column was also pre-equilibrated in this buffer and elution of the (1.5x20)cm column was accomplished with a (2x200ml) linear gradient from 20 to 400mM citrate/NaOH pH6-9, 1mM BZ at 20 ml/hr. 5 ml fractions were collected.

b. Human.

Human prothrombin-Ba was purified exactly as described for the normal protein, utilising pooled deep-frozen coumarin plasma in 100 to 200 ml batches.

5. Preparation of prothrombin not absorbed to barium citrate (prothrombin-super).

a. Bovine

Initially prothrombin-super was isolated according to the procedure given in Prowse et al (1976). This method gave very low yields, mainly due to the inclusion of an isotachopheresis stage. This procedure may also affect the activity of the prothrombin due to the heat lability of the calcium binding site (Devilee et al 1975) if the temperature during the run becomes too great. A second method was devised whereby the supernatant plasma, after barium citrate absorption, was made to 40% saturation with ammonium sulphate to precipitate the prothrombin-super and also any barium remaining in solution as barium sulphate. This had the advantage of removing any last traces of barium absorbable prothrombin from the solution. The

Purification of Prothrombin-super.						
Stage	Volume (mls)	Activity (units/ml)	Total activity (units)	Yield (%)	Protein (O.D.)	Specific Activity (units/O.D.)
Barium supernatant	17.9x10 ³	270	4.8x10 ⁶	100	70	3.86
(from Cow 2)						1
Ammonium sulphate	6.27x10 ³	400	2.5x10 ⁶	52	>6	-
O-LC ₂₀ cut						
DEAE-citrate pH6.0	1.84x10 ³	999	1.84x10 ⁶	38	>6	-
DEAE-Cl pH7.5	130	9264	1.74x10 ⁶	35	>6	-
Sephadex G-200	142	9000	1.28x10 ⁶	26	6.7	1350
QAE-citrate pH6.9	35	4708	1.65x10 ⁵	3.4	1.05	4484
						1161

-activity is expressed in Laurell antigen units, defined at 1000/ml normal plasma

-protein is expressed in O.D. units as defined earlier.

poor yield in this step may be indicative of a fair proportion of the remaining prothrombin being absorbed by the barium sulphate (Table 3-1). Cow 2 (see results section) supplied the plasma for this preparation and was given Warfarin at a dosage such that the level of prothrombin-Ba was relatively high compared to prothrombin-super. Subsequent steps in the preparation are summarised in Table 3-1 and consisted of chromatography on DEAE-Sephadex, firstly in citrate at pH6.0 and then in 50mM HCl/Tris pH7.5. This was followed by gel filtration on Sephadex G-200 and a final QAE-Sephadex ion exchange step in citrate/NaOH buffer pH6.9. The final product, although over 90% pure by SDS-gel analysis (Figure 3-4), was only obtained in low yield.

b. Human

An attempt was made to purify the small amount of prothrombin-super present in human coumarin plasma. This was abandoned when it was realised that the total yield from 1 l of absorbed plasma (which took about 8 months to collect) would be of the order of 1 mg at the most.

(c) Results

The level of bovine plasma prothrombin during Warfarin therapy was measured both by the one-dimensional immunoelectrophoretic method of Laurell (1966) and by the TSV assay of Jobin and Esnouf (1966). Rather than

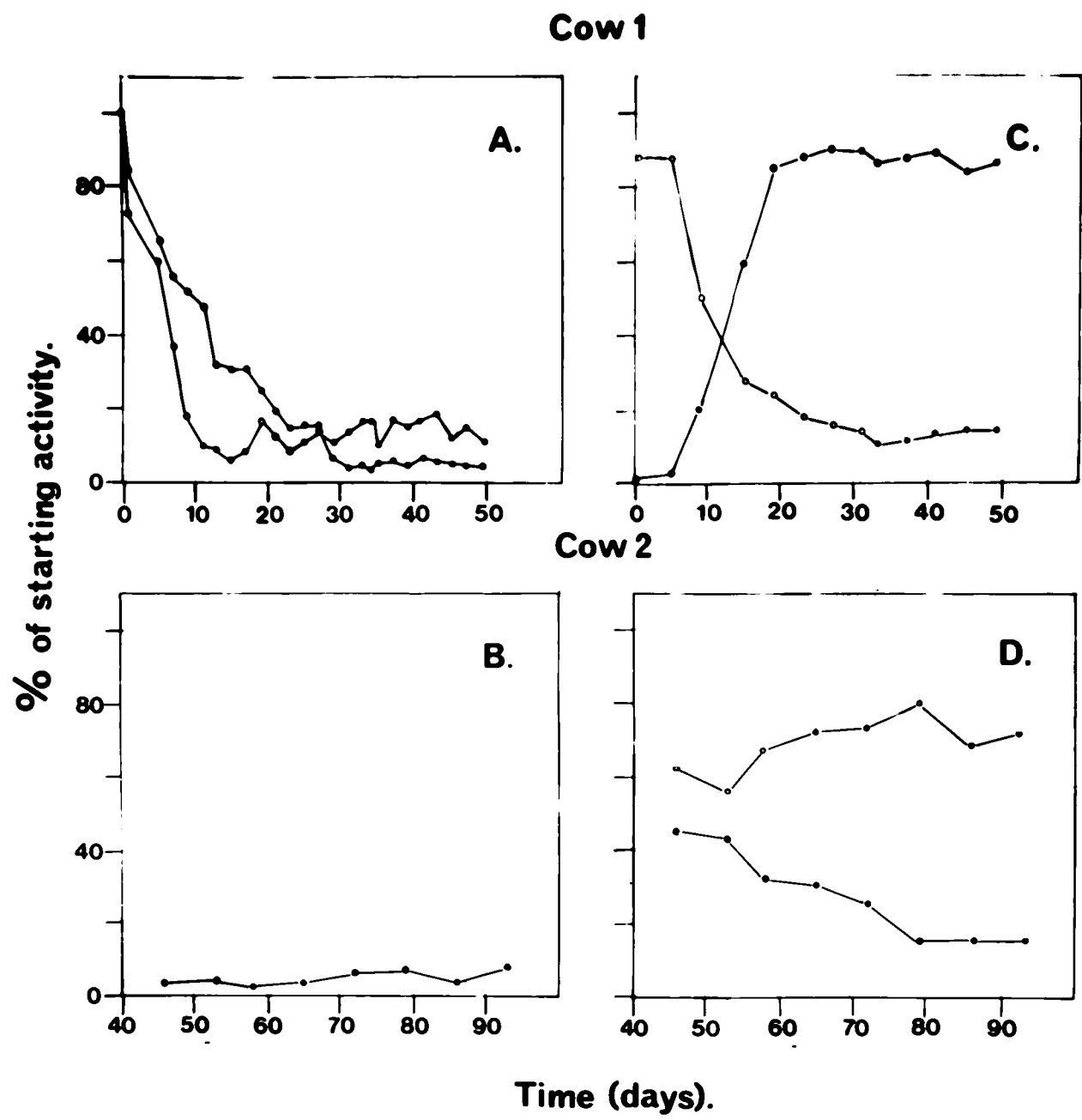


FIGURE 3-1

The effect of administration of Warfarin on the
biological activity of plasma prothrombin and the
level of barium-absorbable and non-absorbable
prothrombin.

A and B: bioassay ● — ● : prothrombin.

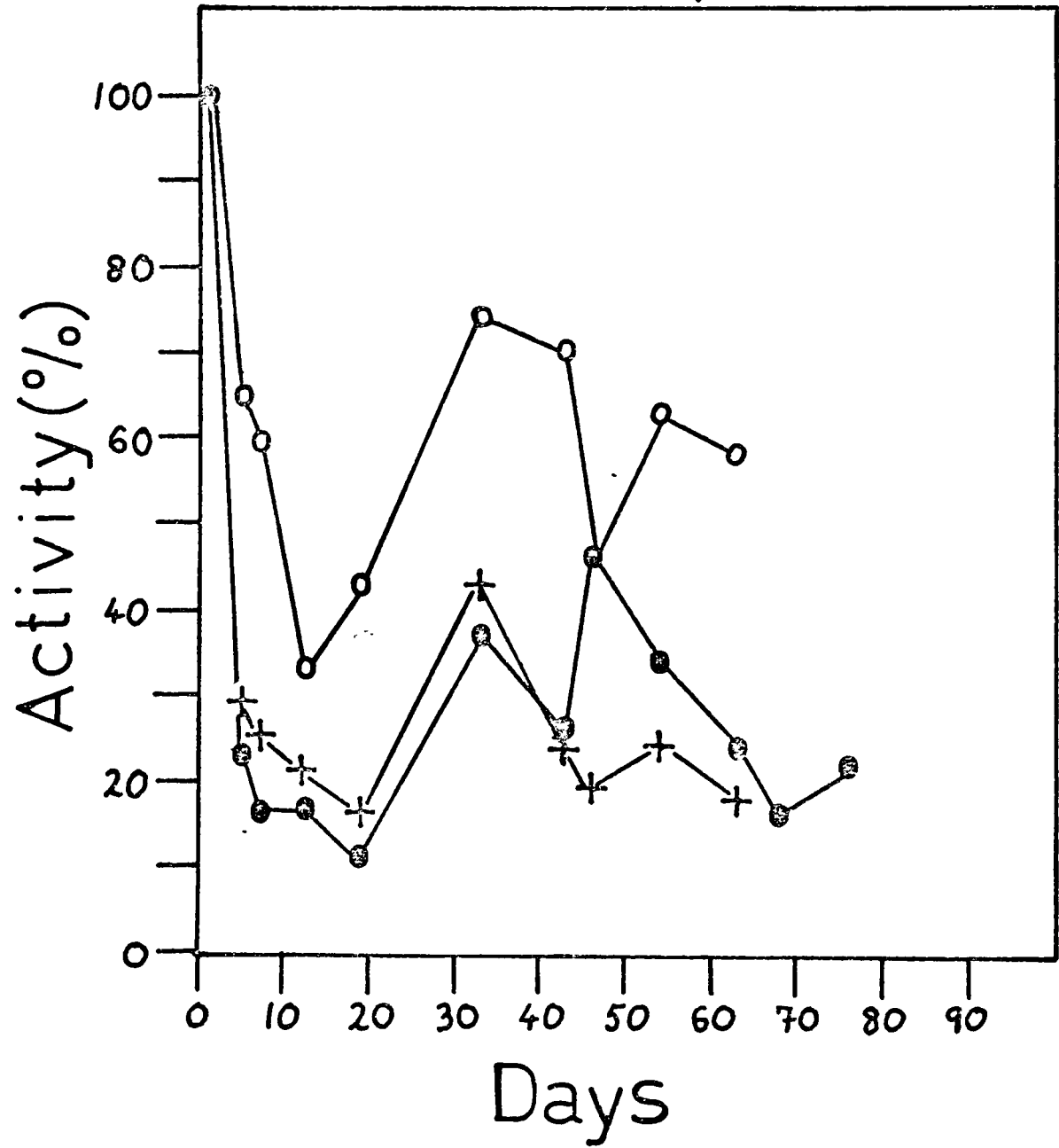
○ — ○ : Factor X.

C and D: immunoassay ○ — ○ : barium-absorbable.

● — ● : barium non-absorbable.

measuring the total prothrombin antigen, which did not change appreciably during the course of treatment, barium absorbable and non-absorbable prothrombin antigen were measured separately. Figure 3-1 shows that in Cow 1 (daily dose 1mg/4.75kg) the activity of prothrombin (measured by the TSV assay) dropped to a steady state level of about 5%, levelling off at around day 30. Factor X activity was also followed and this fell to a similar level although the response was quicker. This would be expected as plasma Factor X has a shorter half life than prothrombin (Hemker et al 1967). The amount of barium-absorbable prothrombin antigen fell in parallel with the decrease in activity, this was accompanied by a corresponding rise in non-absorbable antigen (Figure 3.1). These results confirm those of Stenflo and Ganrot (1972); they, however, then concentrated on the non-absorbable prothrombin (prothrombin-super). On isolation of the barium-absorbable prothrombin (prothrombin-Ba) it was found to have a lower specific activity than normal prothrombin. As only small amounts of prothrombin-Ba were obtained from the first cow, a second cow was treated. The same total daily dose of Warfarin was used but as this cow was larger this corresponded to a dosage of 1 mg/6.5kg. The first cow had shown that steady-state activity was not reached until around day 30 at the earliest. Collection of the blood samples from Cow 2 was therefore started on day 43. Although the lower dosage resulted in approximately the same steady-state level of prothrombin activity, the

Figure 3.2
Coumarin therapy
of a human patient



o—o Prothrombin (TSV)
+—+ Factor X (RVV)
●—● Thrombotest

TABLE 3.2

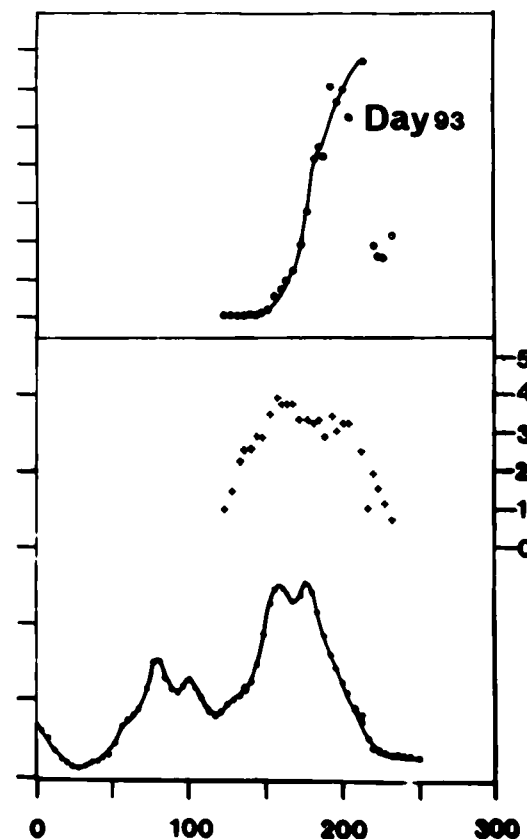
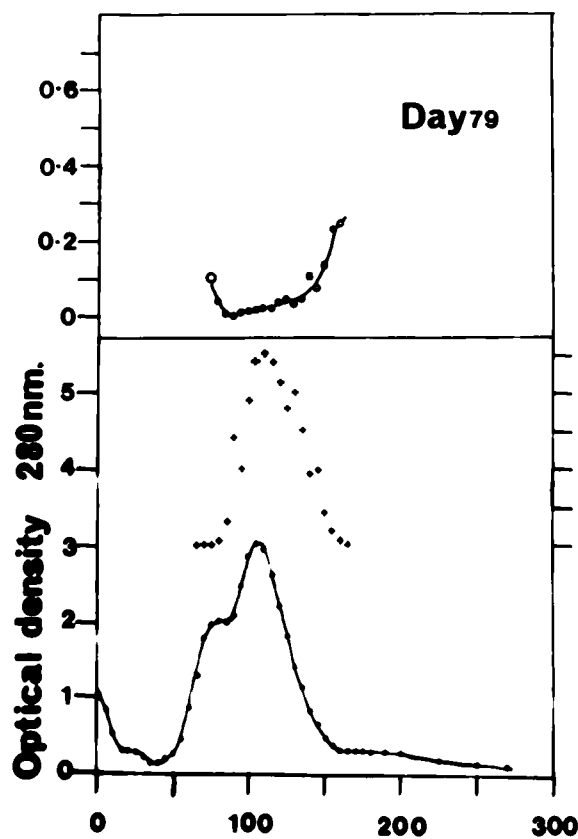
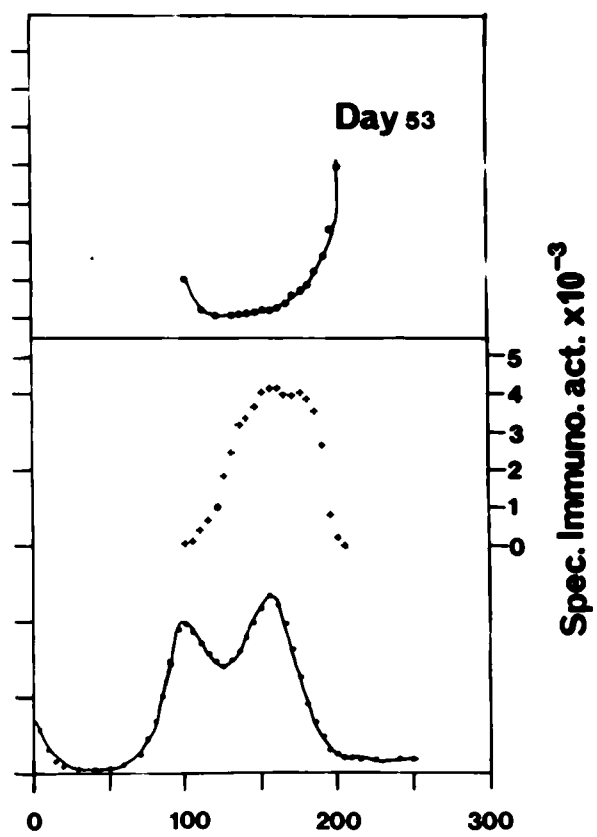
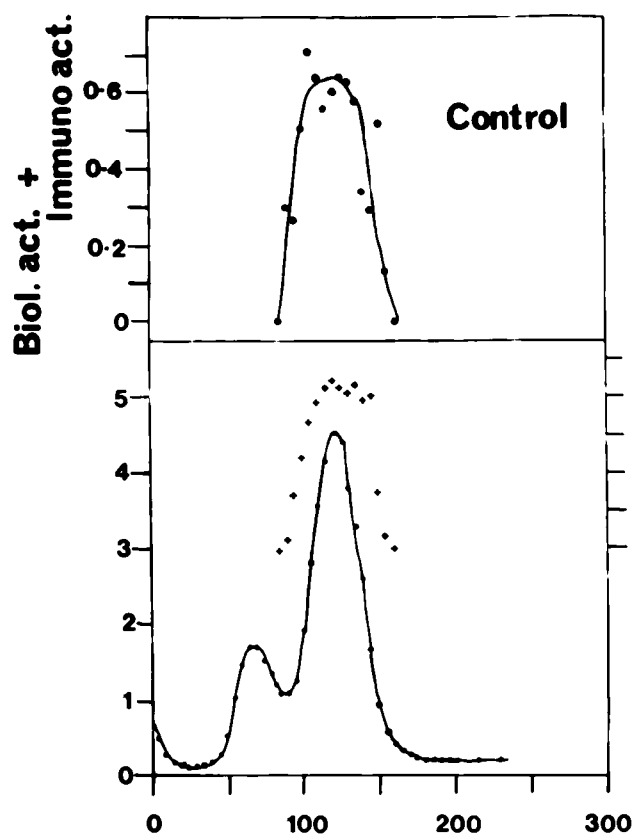
Average prothrombin levels, during coumarin therapy.

	<u>Biological Activity</u> (TSV units)	<u>Immunological Activity</u> (Laurell units)
<u>Cow 2</u>		
Plasma	50 units/ml	970 units/ml (800-1040)
Barium-adsorbed plasma	10 units/ml	270 units/ml (200-400)
Prothrombin-Ba	228 units/OD	3910 units/CD
Normal bovine prothrombin	5200 units/OD	4044 units/OD
<u>Human</u>		
Plasma	440 units/ml (200-600)	720 units/ml (700-900)
Barium-adsorbed plasma	10 units/ml	190 units/ml (100-200)
Prothrombin-Ba	2054 units/CD	5161 units/OD
Normal human prothrombin	6986 units/OD	5816 units/CD

- an OD unit is defined as that quantity of protein required in 1 ml of solution to yield unit optical density.
- both TSV and Laurell units are defined on the basis of 1000 units/ml in a standard of normal plasma.

level of prothrombin-Ba was much higher, confirming the low biological specific activity of this material. This would not have been noticed in the earlier studies of Stenflo and Ganrot (1972) or Nelsestuen and Suttie (1972b) as they used a higher dose of Warfarin than used here and treatment was only continued for 7 or 8 days. In this situation prothrombin-super becomes the major species present and the cow is liable to haemorrhage. This was not seen in the present study, even at the site from which the blood was taken.

Strict control of therapy in the human patient is obviously much more difficult to achieve. Figure 3·2 shows the commencement of therapy in a male patient suffering from recurrent pulmonary embolism. The extent of therapy was fairly well controlled by the Thrombotest procedure apart from a lapse around day 30, coinciding with the Christmas holiday period. Prothrombin activity fluctuated rather widely whereas the Factor X activity corresponded more closely to the Thrombotest results, suggesting that the limiting factor in this test may be Factor X. Table 3·2 shows average figures for prothrombin levels in the human (and bovine) coumarin plasma samples utilised to prepare prothrombin. These again show that prothrombin-Ba has a lower specific activity than normal prothrombin and that in human patients prothrombin-Ba represents the major species of prothrombin present in the plasma.



Volume (ml.)

FIGURE 3•3

Elution profiles from DEAE-Sephadex
columns of bovine prothrombin from
control and Warfarin-treated cows.

In the cow Warfarin had little effect on the total level of prothrombin antigen, whereas in man the level was slightly reduced (Table 3-2). Warfarin-treated rats have very little prothrombin present in their plasma but prothrombin antigen, with no physiological activity, accumulates in the liver (Carlisle et al 1975). This protein has been isolated from the liver of Warfarin-treated rats (Esmon et al 1975a) and has similar characteristics to prothrombin-super. It is also converted to prothrombin in microsomal system containing Vitamin K and certain other factors (Esmon et al 1975c). In cows very little precursor of prothrombin accumulates in the liver during Warfarin treatment (Carlisle et al 1975) presumably due to its release to form prothrombin-super. On this basis it seems reasonable to assume that the level of human liver prothrombin precursor rises slightly during Warfarin treatment, the majority being released into the plasma as prothrombin-super and prothrombin-Ba.

The elution profiles from DEAE-Sephadex of prothrombin-Ba isolated from Cow 2 on days 53, 79 and 93 are shown in Figure 3-3, together with a control preparation from normal plasma. The abnormality of prothrombin-Ba has been represented by the ratio of the biological to the immunological activity. Prothrombin-Ba was eluted, after an initial peak of fibrin(ogen), with a gradient of activity across the protein peak, the lower activity material eluting first. The ratio of biological to immunological activity

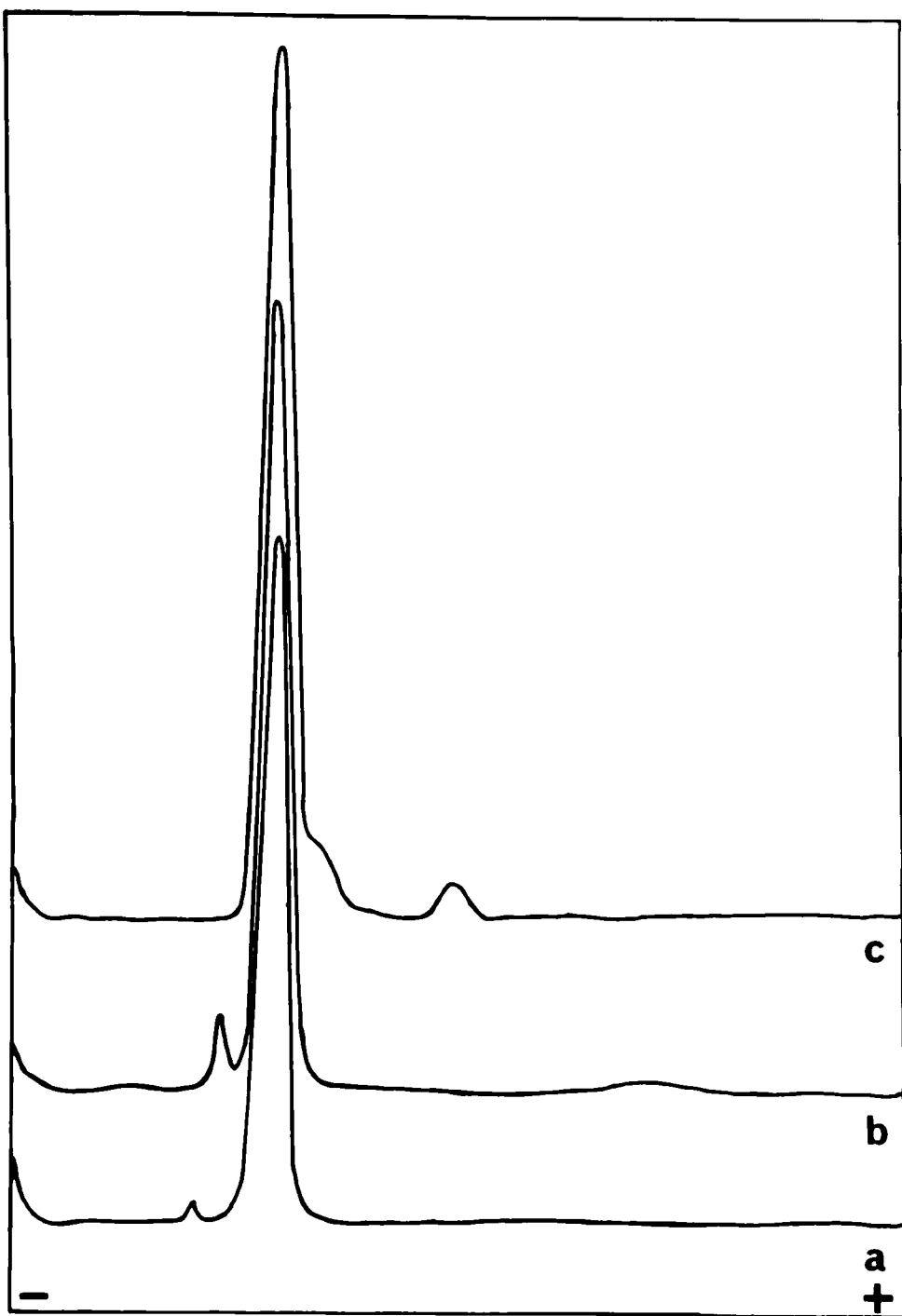


FIGURE 3•4

SDS-gels of various bovine prothrombin

- a. normal prothrombin
- b. prothrombin-Ba
- c. prothrombin-super

varied from 0 to 0.6 across the peak. The biological activity of the normal prothrombin prepared by this method was apparently lower than the material prepared by the large scale method described in Chapter 2. This could be explained in terms of contamination; however SDS-gel analysis (Figure 3.4) and immunological activity measurements (Table 3.2) showed that the prothrombin prepared by this method was substantially pure. Also the presence of contaminants will not affect the biological: immunological activity ratio. The lowered activity can be explained as due to citrate inhibition of the clotting assay due to the formation of calcium citrate. This was not seen in the large scale preparation due to the larger amounts of protein involved and hence the higher dilutions required to bring the activity into the normal plasma dilution range. The small normal preparation provides a control for this effect in the prothrombin-Ba preparations.

Stenflo and Ganrot (1972) and Nelsestuen and Suttie (1972b) tacitly assume the existence of only two forms of prothrombin (normal and abnormal). The present results might be argued to be due to mixtures of these two forms. To ensure that this is not the case, various fractions of the DEAE-Sephadex eluate of prothrombin-Ba were reabsorbed to barium citrate. In each case more than 90% of the immunological activity was reabsorbed. Thus prothrombin-Ba appears to consist of a range of species with differing biological activity, the least active of which is eluted

first from DEAE-Sephadex columns, as seen from the ratio of biological to immunological activity (Figure 3-3). Despite the low biological activity of this material it is nonetheless appreciably greater than that of the prothrombin not absorbed to barium citrate (Table 3-2).

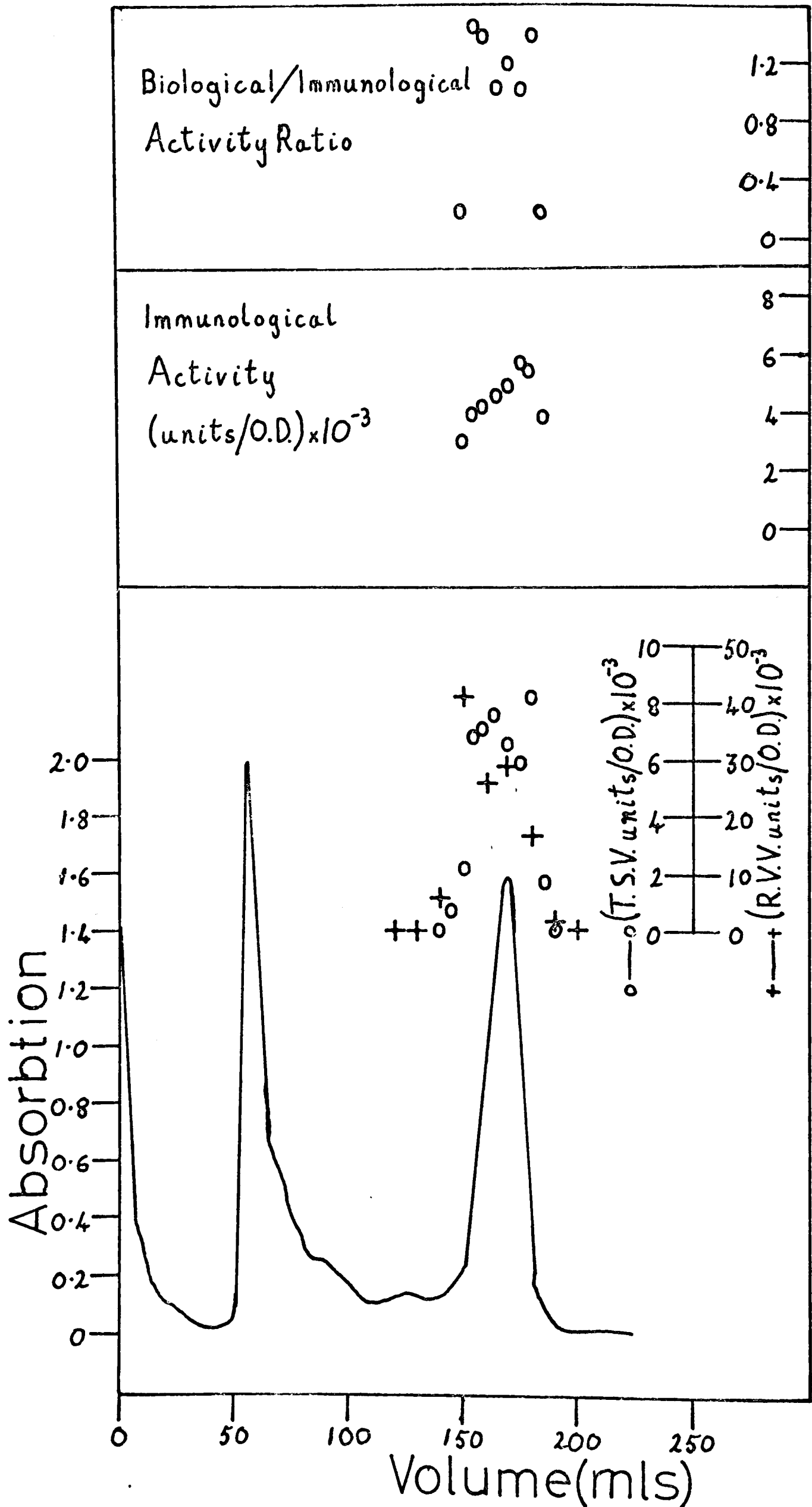
Prothrombin-super was only obtained in low yield. This was partly due to attempts to introduce different methods during the purification, e.g. chromatography on heparin-agarose, which were subsequently rejected. The eventual method was similar to that of Stenflo and Ganrot (1972) who obtained a similar purification at a higher yield (19%). They had the advantage that their treatment resulted in high levels of prothrombin-super in the bovine plasma, whereas the amount of Warfarin given in the present study resulted in high levels of prothrombin-Ba (in the case of Cow 2). The method of Nelsestuen and Suttie (1972b) gives a product quoted as only 80% pure. Recently Wallin and Prydz (1975b) have described a method of purifying prothrombin-super from bovine coumarin plasma using a column of goat antiprothrombin IgG-Sepharose. Subsequent gel filtration of the eluate gave a pure product in good yield. The method may also be applied to normal prothrombin (Wallin and Prydz 1975a) but is rather expensive in goat antisera. Prothrombin-super prepared by any of these methods has very little, if any, physiological activity but possesses normal immunological activity. SDS-gel traces of the normal prothrombin, prothrombin-Ba and

prothrombin-super obtained in the present study are shown in Figure 3.4. All three pools were substantially pure and had the same apparent molecular weight of about 72,000 daltons.

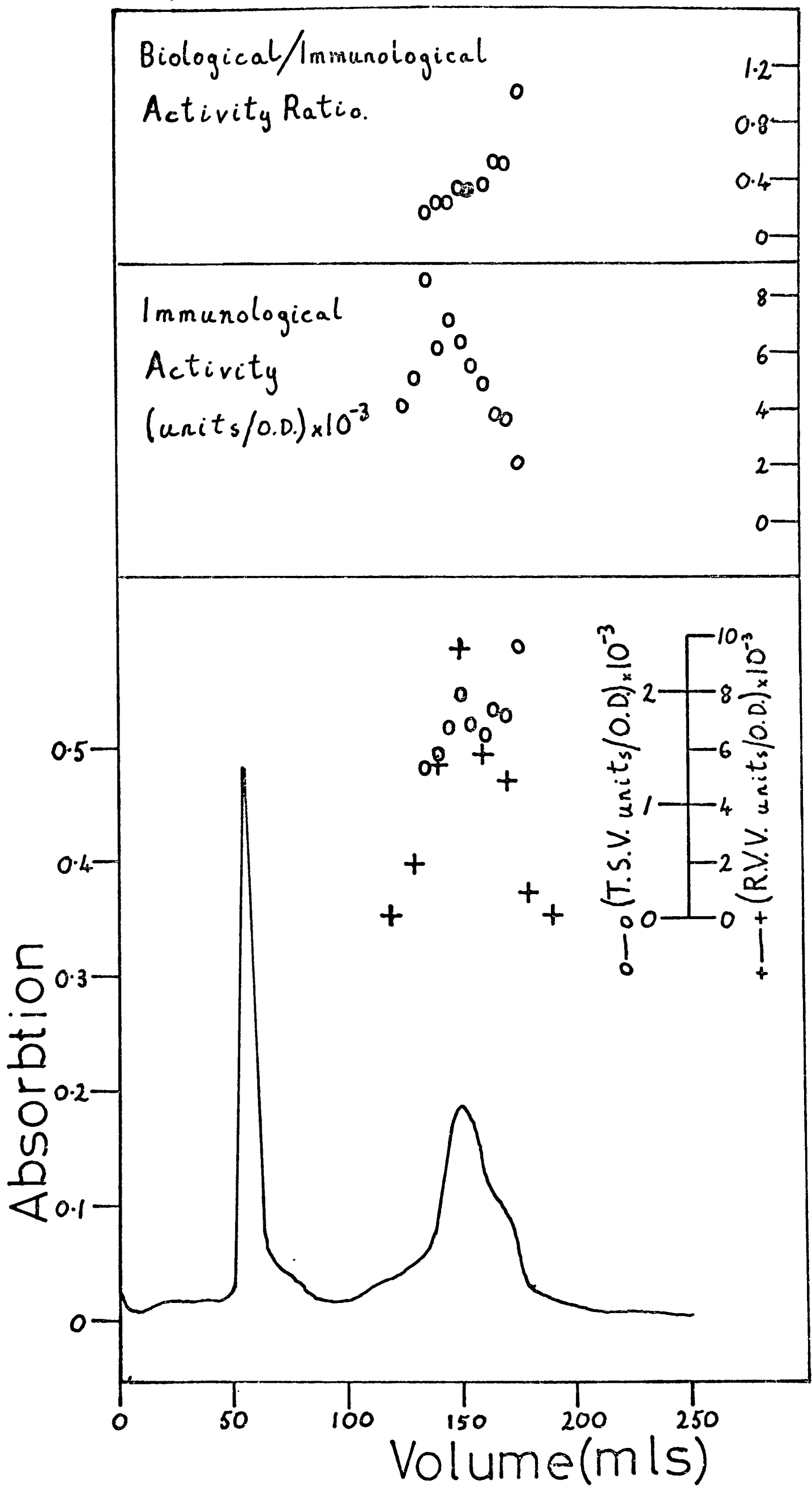
During the preparation of prothrombin-Ba attempts were made to isolate the corresponding form of Factor X, i.e. a form that binds to barium citrate but which has reduced activity. The amount of Factor X binding to barium was definitely reduced by Warfarin therapy, although a residual amount of activity could be detected eluting from the DEAE-Sephadex columns. By pooling of this material and rechromatography of it preliminary evidence of a Factor X-Ba was obtained, in that a protein peak was found with Factor X activity and with a higher immunological than biological activity. This ratio was graded across the peak in a similar manner to prothrombin. However, this material was heavily contaminated (up to 40%) and very little of it was present. Characterisation of such a species of Factor X would require a suitable coumarin regime and the processing of a good deal more plasma than has been attempted here. Abnormal forms of Factor X corresponding to prothrombin-super have been demonstrated in both cattle (Reekers et al 1973) and in human patients (Prydz and Gladhaug 1971, Larrieu and Meyer 1970).

Human prothrombin-Ba prepared according to Morrison and Esnouf (1973) shows a gradient of activity across the

Figure 3.5 (a) Normal Human Prothrombin



(b) Coumarin Human Prothrombin.



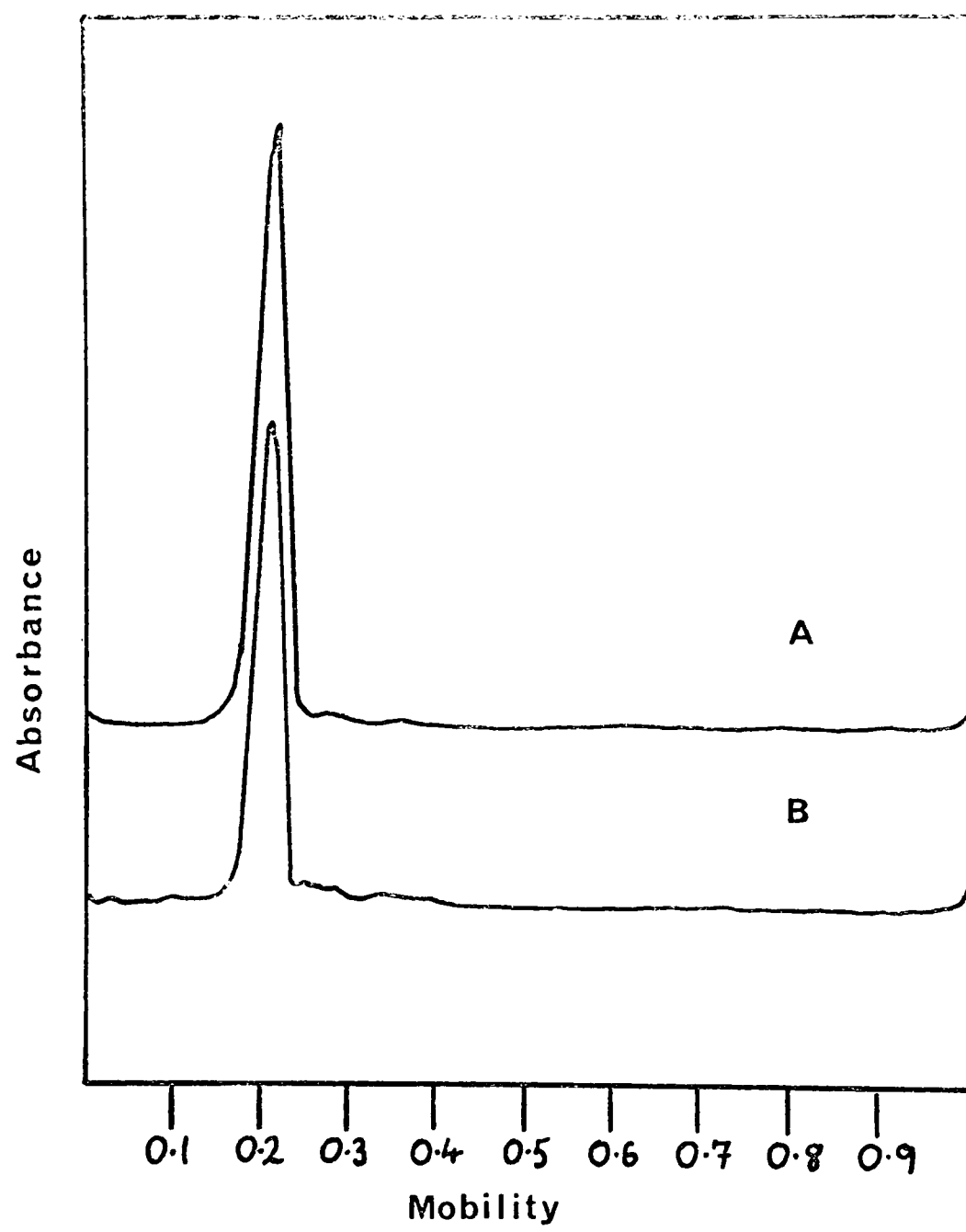


FIGURE 3-6

SDS-gel analysis of human prothrombin

- A. Normal prothrombin
- B. Prothrombin-Ba

DEAE-Sephadex column elution peak, in a similar manner to the bovine material described above. When the human material was prepared by the present method this effect was less obvious, but still present. The biological activity of the prothrombin was reduced to about a third of the normal level there being no significant change in the immunological activity (Figure 3-5, Table 3-2). The Factor X activity included in the prothrombin peak was also reduced by Warfarin therapy, but without a monospecific antiserum towards human Factor X it was not possible to assess the significance of these results in terms of the present hypothesis. SDS-gel analysis of both normal and barium-absorbable human prothrombins show no significant level of Factor X (human Factor X has a similar molecular weight to the bovine material - Rosenberg et al 1975) and that both forms of prothrombin were at least 95% pure with an apparent molecular weight of 72,000 (Figure 3-6). Human coumarin prothrombin has recently been purified by Cesbron et al (1973) by a method in which 'normal' prothrombin was absorbed to relatively small amounts of barium sulphate and 'abnormal' prothrombin then absorbed to larger amounts of the same salt. This abnormal prothrombin had low biological activity and was eluted from a DEAE-cellulose column earlier than the normal material. It should be noted that coumarin therapy in this case resulted in a plasma prothrombin activity of only 30% of normal. Viewed in terms of the present study it would seem that this abnormal prothrombin corresponds to the less active forms

of prothrombin-Ba. The procedure of using barium sulphate to absorb some of the abnormal prothrombin after a preliminary barium absorption to remove the normal material has been proposed already by Malhotra (1972b) in his study of bovine coumarin prothrombin.

(d) Discussion

During coumarin therapy a range of prothrombin species are produced with varying biological activities, but with similar immunological activities, in both cattle and human patients. This range of prothrombin species may be fractionated on the basis of their ability to bind barium citrate. The latter property may be expected to correspond to the ability of the molecules to bind calcium, required for physiological activation and for activation by TSV, although the calcium requirement for activation may not be altered in the same manner as the barium citrate binding ability. The prothrombin-Ba components may be partially resolved by chromatography on DEAE-Sephadex, when the less active material is eluted first, implying that the normal prothrombin is more negatively charged at pH6.9 than the abnormal species. Thus the abnormality in coumarin prothrombin may be explained in terms of the lack of some negatively charged prosthetic group present in the normal molecule.

When human patients are treated with coumarin drugs the dose is usually such that it produces a level of

prothrombin activity in the plasma of about 50% of the normal level. Under this regime the major species (greater than 70%) of prothrombin present in the plasma has low activity but will still absorb to barium citrate. At a suitable dose cattle treated with Warfarin for long periods give a similar result. More severe treatment results in more abnormal forms of prothrombin which will not bind to barium citrate. These differences are reflected in the ability of these proteins to be activated in the TSV assay, corresponding to the normal physiological activators. Preliminary results would indicate a similar effect in the case of Factor X.

It is interesting to note that prothrombin-super has no inhibitory effect on the assay of normal prothrombin. This makes the finding of Hemker et al (1963) that coumarin plasma contains an inhibitor of prothrombin activation, PIVKA-II, very difficult to explain in terms of the model they originally proposed.

CHAPTER 4

ACTIVATION OF THE VARIOUS PROTHROMBINS

(a) Introduction

(The terminology proposed by Gitel et al (1970) has been used throughout this thesis).

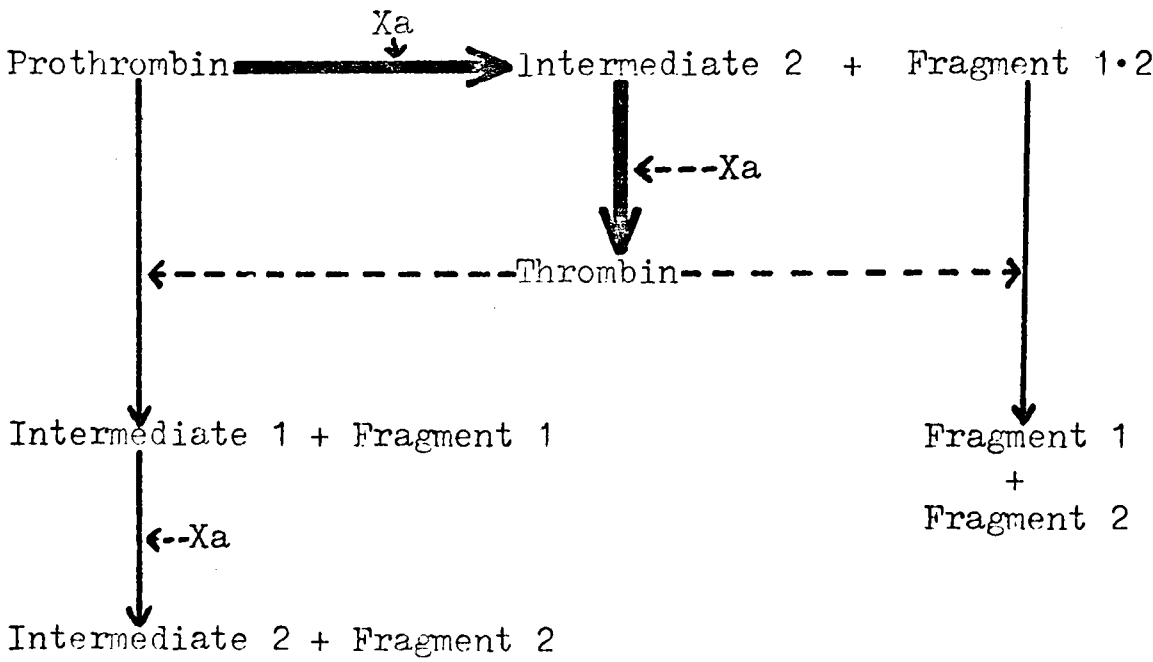
The activation of bovine prothrombin results in the formation of three end products: thrombin, Fragment 1 and Fragment 2 (Stenn and Blout 1972, Heldebrant and Mann 1973). The origin and size of these activation products is shown in Figure 2.3. During the activation two intermediates arise which contain the potential thrombin (Heldebrant and Mann 1973), Heldebrant et al 1973a and b, Owen et al 1974, Stenn and Blout 1972). Isolation of these products and study of the partial reactions of activation has shown that activation proceeds as shown in Figure 4.1, the preferential route occurring via Intermediate 2 in two steps, other products being formed subsequent to preliminary thrombin formation (Stenn and Blout 1972), Esmon and Jackson 1974a).

Prothrombin is activated by the 'prothrombinase complex' consisting of Factor Xa, Factor V, calcium ions and phospholipid. Of these components only Factor Xa is enzymically active, the other components acting as accelerators of the activation (Jobin and Esnouf 1967). Papahadjopoulos and Hanahan (1964) have shown that each of these components binds the others, in the presence of calcium ions, by column chromatography on Sephadex G-200. In the absence of calcium only the binding between Factor V and phospholipid occurs. At -1°C prothrombin is bound to the complex; if the temperature is then raised to 37°C activation proceeds and thrombin is released into the aqueous phase (Barton and Hanahan 1969).

Careful study of the partial reactions of the activation path of prothrombin by Jackson and his colleagues has demonstrated

FIGURE 4.1.

Prothrombin Activation Pathways.



(adapted from Esmon et al 1974c)

that the Factor V acceleration is dependent on the Fragment 2 region of the prothrombin (Esmon and Jackson 1974b), whereas the accelerating effect of phospholipid on prothrombin activation is dependent on its binding to the Fragment 1 portion of the molecule (Bull et al 1972, Gitel et al 1973, Esmon et al 1974b). The latter effect depends on the calcium bound to prothrombin. Calcium binding sites have been located in both Fragment 1 and Fragment 2 regions of the molecule, however the 5 or 6 high affinity sites are all associated with the Fragment 1 portion (Benson et al 1973, Bajaj et al 1975). Thus activation accelerators all act by means of their binding to the pro-portion of prothrombin. This introduces the great anomaly of prothrombin activation. The accepted model of the 'prothrombinase complex' is that all the components, accelerators, enzyme and substrate, are grouped on the phospholipid surface. If this is so the first cleavage shown in Figure 4.1, resulting in the formation of Intermediate 2, separates this potentially thrombinogenic portion of the molecule from the binding sites for the enzyme and accelerators that can cleave it to thrombin. One possible solution to this problem is the demonstration that Intermediate 2 forms a non-covalent complex with Fragment 1.2 (and Intermediate 1 with Fragment 1) which could prolong the time of association of Intermediate 2 with the pro part of prothrombin (and hence the prothrombinase) sufficiently long for the second thrombin-forming cleavage to occur (Esmon et al 1974b). This explanation is supported by the finding that, although the path of activation is unaffected by the accelerators Factor V and phospholipid, the presence of Fragment 1.2 is required for the kinetic pathway of activation to be as shown in Figure 4.1 during activation with the complete prothrombinase. This was shown by comparison of the rate of thrombin formation from prothrombin with that from Intermediate 2 with or without Fragment 1.2. (Esmon and Jackson 1974a and b, Esmon et al 1974b and c).

In the human case the situation is further complicated by the existence of a third intermediate, necessitating a third cleavage before thrombin is formed (Downing et al 1976 - see Results).

Prothrombin-super, as isolated by Stenflo and Ganrot (1972) and by Nelsestuen and Suttie (1972b), is not activated by the prothrombinase complex. This may be explained in terms of the inability of this species of prothrombin to bind calcium (Stenflo and Ganrot 1973, Nelsestuen and Suttie 1972b) and the resultant lack of phospholipid or calcium participation in the Factor Xa catalysed activation. Factor Xa alone will only activate prothrombin at about 1/50,000 the rate of the full prothrombinase complex. (Jobin and Esnouf 1967).

With this in mind the differences between normal prothrombin, prothrombin-Ba and prothrombin-super (see Chapter 3), as seen in the TSV assay situation, may be further specified by carrying out activations with only some of the components of the complete prothrombinase complex. Activations were carried out with purified bovine Factor Xa, with or without calcium and phospholipid.

(b) Methods

1. Esterolytic assay of thrombin

Thrombin hydrolyses peptide-like esters of lysine and arginine (Sherry and Troll 1954) ; however, although proteolytic and esterolytic activity reside at the same site in thrombin (Gladner and Laki 1956), the clotting activity may be differentially lost as compared to the esterase activity (Landbaru and Seegers 1959, Mann et al 1971a). Thus esterolytic activity is a valid measure of activity but may not be directly comparable to coagulant activity.

The activity of the thrombin formed in activation mixtures was measured by the method of Schwert and Takenaka (1955). 1ml of 3mM N-alpha-benzoyl arginine ethyl ester in 50 mM Tris/HCl pH8.0 was mixed with 0.05 to 0.2ml of the activation mixture and the volume made to 3mls with 50mM Tris/HCl pH8.0, in a 1cm cuvette. The decrease in absorbance at 253nm was measured in a recording spectrophotometer (a Beckmann DU spectrophotometer fitted with a Gilford automatic cuvette positioner) at 25°C. The rate of hydrolysis of the ester by the activation mixture was compared with a control incubation mixture containing Factor Xa alone, as Xa also hydrolyses this ester at a slow rate. Experiment showed that the esterolytic activity of Factor Xa was unaffected by calcium, phospholipid or thrombin.

2. Activation of prothrombin

Prothrombin was dialysed into 50mM Tris/HCl pH8.0 prior to activation. The period of dialysis was kept to a minimum as the prothrombin was susceptible to degradation on prolonged dialysis against the above buffer. This effect would appear to be due to traces of thrombin as the products were Intermediate 1 and Fragment 1 (Esmon and Jackson 1974a). Activations were carried out in plastic tubes as thrombin absorbs to glass surfaces; for this reason all cuvettes were washed in concentrated nitric acid prior to their use in assays.

Incubation mixtures contained 0.2mg/ml prothrombin and 60ug/ml Factor Xa, isolated by the method of Jesty and Esnouf (1973), dissolved in 50mM Tris/HCl pH8.0. In

experiments in which calcium was included the concentrations of the components were 0.2mg/ml prothrombin, 3ug/ml Factor Xa and 5mM CaCl_2 . Activations were also carried out with calcium and phospholipid, in which case the mixture contained 0.2mg/ml prothrombin, 5mM CaCl_2 , 0.01ug/ml Factor Xa and 0.3mg/ml Inosithin (diluted from the TSV assay stock solution of 10mg/ml). The calcium and calcium/phospholipid acceleration of human prothrombin activation was not nearly as great as in the bovine case; the concentration of Factor Xa was therefore maintained at 20ug/ml for both these activations.

In all cases the mixtures were incubated at 37°C in plastic tubes; aliquots were withdrawn at intervals for esterolytic assay and for SDS-gel electrophoretic analysis (performed as described in Chapter 2).

(c) Results

1. Bovine

Incubation of normal bovine prothrombin with Factor Xa, in the absence of calcium, resulted in a slow but progressive conversion of prothrombin to thrombin (Figure 4.2A). SDS-gel analysis (Figure 4.3A) showed that this conversion was virtually complete by 380 minutes. In those activations involving relatively high concentrations of Factor Xa, this protein was visible on all gels during the activation. Smaller peptides on the gels have not been

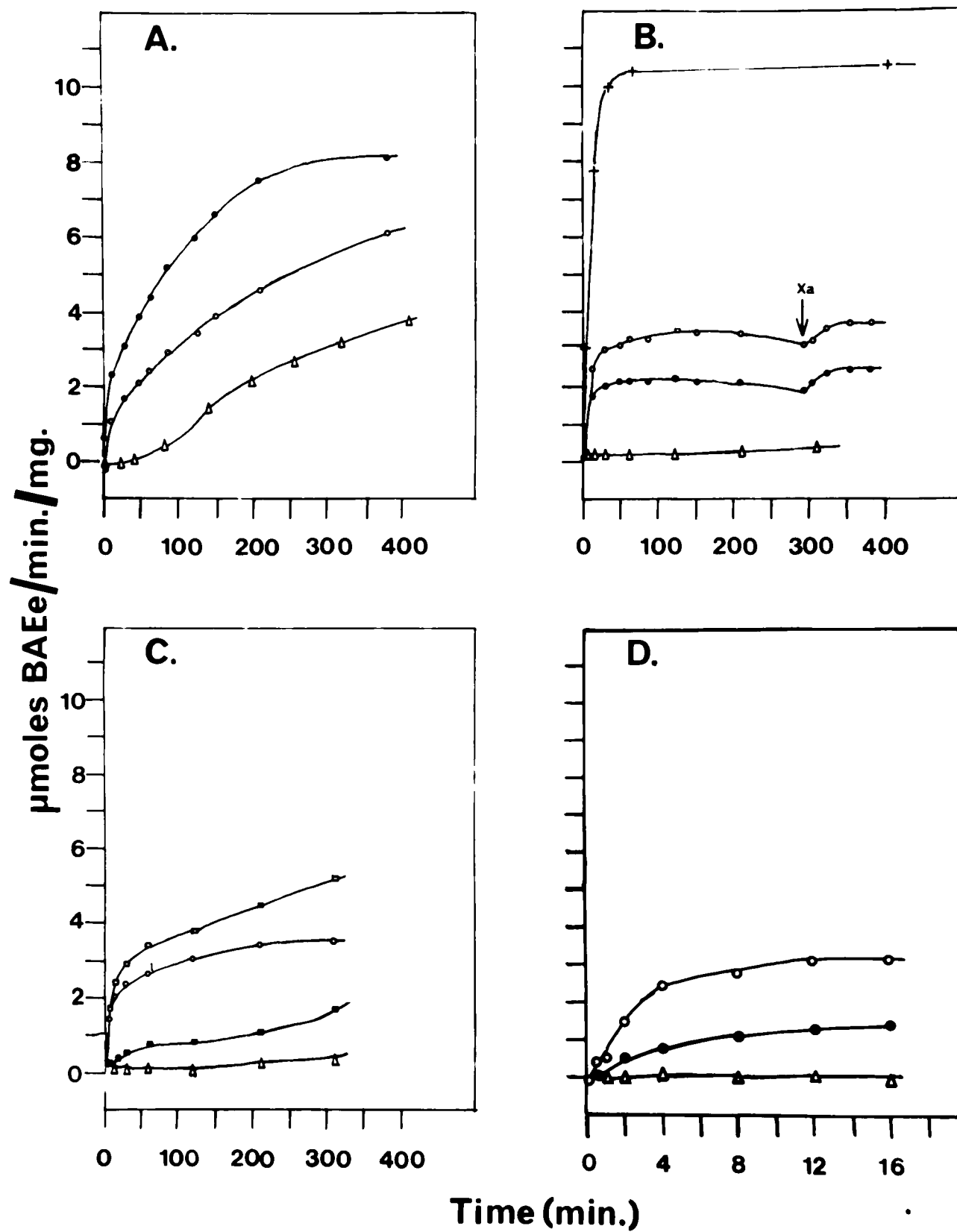


FIGURE 4-2

Activation of various bovine prothrombins

- conditions as described in the text.

A: activation by Factor Xa alone.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

△ — △ : prothrombin-super

B: activation by Factor Xa and calcium.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

△ — △ : prothrombin-super

+ — + : activation at equimolar Factor Xa

C: conditions as for B.

○ — ○ : normal prothrombin

△ — △ : prothrombin-super

□ — □ : normal prothrombin + prothrombin-super

■ — ■ : (normal + super) - normal (calculated)

D: activation by Factor Xa, calcium and phospholipid.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

△ — △ : prothrombin-super

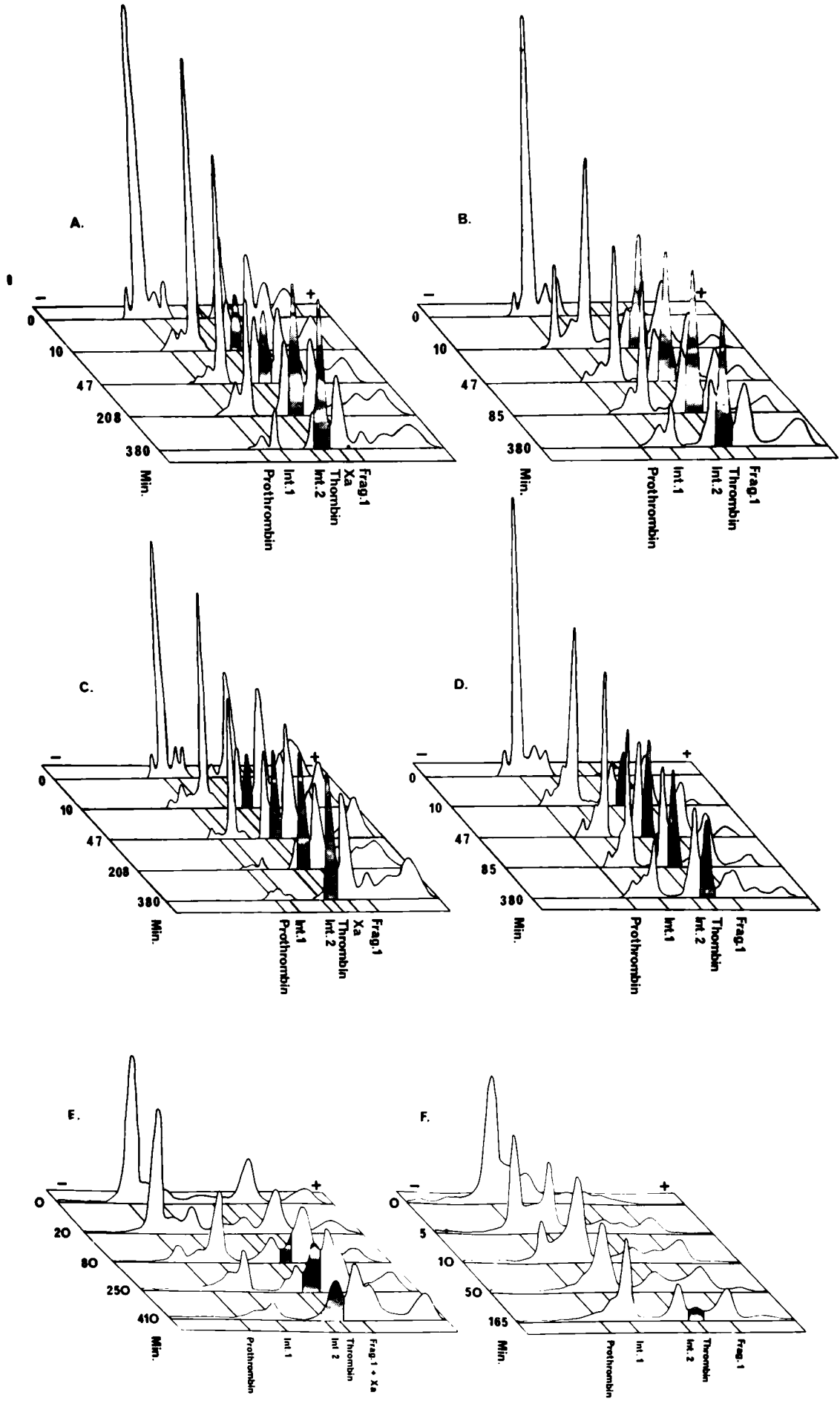


FIGURE 4-3

SDS-gel analysis of bovine
prothrombin activations

- conditions as described for Figure 4-2.

Factor Xa alone on normal prothrombin (A)

prothrombin-Ba (C) and prothrombin-super (E).

Factor Xa and calcium on normal prothrombin (B),

prothrombin-Ba (D) and prothrombin-super (F).

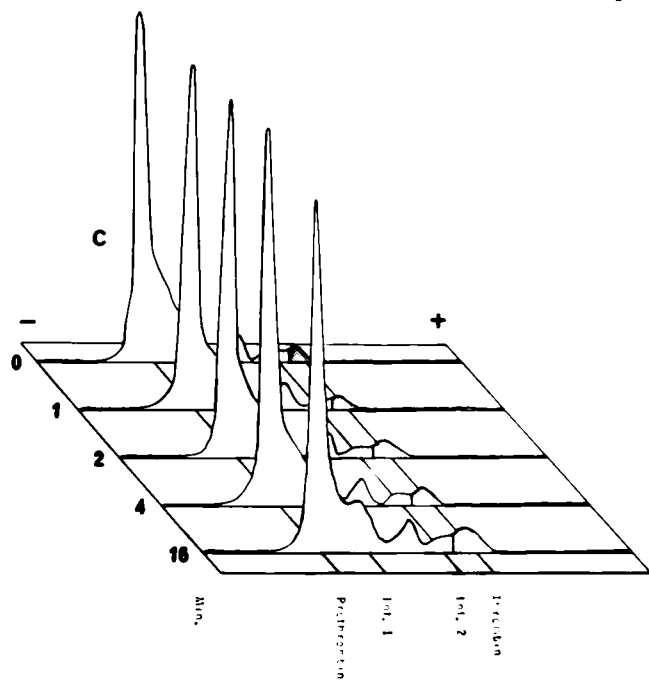
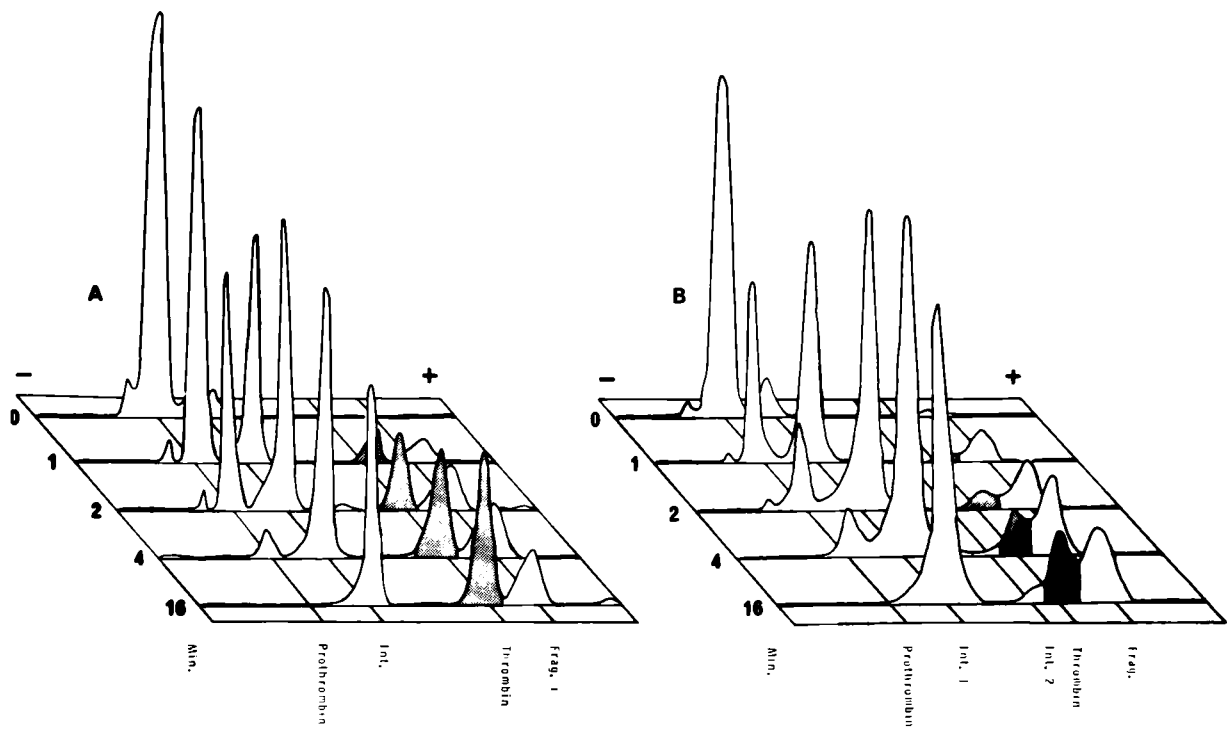


FIGURE 4-4

SDS-gel analysis of bovine
prothrombin activations

Factor Xa, calcium and phospholipid on
normal prothrombin (A), prothrombin-Ba (B) and
prothrombin-super (C).

identified but include Factor Xa light chain, thrombin A chain, Fragment 1 degradation peptides and Fragment 2. The activation of prothrombin-Ba by Factor Xa alone was essentially similar to that of normal prothrombin (Figures 4•2A and 4•3C), but apparently proceeded at a slightly faster rate; prothrombin-super activation, on the other hand, occurred much more slowly and had a sigmoid characteristic (Figure 4•2A). This may have been due to the presence of minute amounts of normal prothrombin in the prothrombin-super pool as it was found that addition of normal prothrombin to the prothrombin-super aided its activation (Figure 4•2C). SDS-gel analysis of the activation of prothrombin-super (Figure 4•3E) showed that the increase in esterase activity occurred after the prothrombin had been degraded to Intermediate 1. Thus Intermediate 1 would appear to be more readily activated, in this system, than prothrombin-super and the ability of normal prothrombin to aid this activation is presumably due to the formation of thrombin, which then degrades prothrombin-super to Intermediate 1.

In activations including calcium the Factor Xa concentration was reduced to 1/20 the above amount. In this system normal prothrombin was rapidly activated until about a third of the potential thrombin was formed (Figure 4•2B), after which thrombin production virtually ceased, the remainder of the prothrombin being cleaved to Intermediates 1 and 2 only, with a build up occurring at the Intermediate 2 stage (Figure 4•3B). When the Factor Xa concentration was doubled after reaching

this plateau only a very small ~~amount of~~ extra amount of thrombin was formed; however if an equimolar amount of Factor Xa was used for the activation, from the start, the theoretical yield of thrombin could be achieved (Figure 4.2B). This apparent product inhibition could be due to the formation of Intermediate 1 by thrombin degradation of prothrombin and this Intermediate 1 then being a poor substrate for Factor Xa. This was shown to be the case (Figure 4.5D); however the effect was only seen in the presence of calcium. Since the Fragment 1 region of prothrombin contains the calcium binding site the most likely mode for a calcium-dependent inhibition of prothrombin activation would appear to be a direct competition between Fragment 1 and prothrombin for Factor Xa, in the presence of calcium. Other authors have shown that Fragment 1 inhibits the activation of prothrombin (Jesty and Esnouf 1973, Benson et al 1973, Jackson et al 1974, Mann 1974) and this effect was also seen in the present study (Figure 4.5D), albeit only to a small degree. More significantly it was shown that addition of EDTA, as a calcium chelating agent, would release the inhibition occurring during Factor Xa/calcium activation of normal prothrombin. Further activation obviously only occurred slowly as the EDTA also removes the acceleration due to the calcium (Figure 4.5D). The binding of I^{125} -labelled Fragment 1 to Factor Xa-Sepharose (see Appendix 1), in the presence and absence of calcium, has also been studied in this laboratory. It has been shown that a definite calcium-dependent binding of Fragment 1 to

Factor Xa occurs with a dissociation constant of about 1×10^{-5} (Merson 1976). This is of course to insolubilised Factor Xa and may not correspond completely to the situation in solution.

The activation of prothrombin-Ba by Factor Xa was also considerably enhanced by the addition of calcium and again the reaction did not go to completion but stopped, after an initial burst, at a level slightly lower than that found for normal prothrombin (Figure 4•2B). SDS-gel analysis showed that there was a build-up of Intermediate 2 during the plateau phase, as in the normal activation (Figure 4•3D). Prothrombin-super was unaffected by the addition of calcium to the reaction mixture (Figures 4•2B and 4•3F).

Activation of the three forms of prothrombin by Factor Xa/calcium/phospholipid (Figure 4•2D) clearly shows the gradation of activity already seen in the TSV assay situation. The acceleration due to phospholipid depends on the calcium-binding ability of the Fragment 1 region of prothrombin (Bull et al 1972, Gitel et al 1973). Activations in the presence of phospholipid differed qualitatively from those carried out with Factor Xa and calcium only in that no Intermediate accumulated during the plateau phase of the activation (Figure 4•4). Prothrombin-super was not appreciably activated at the very low Factor Xa concentration used in these activations, during the short times for which this reaction was observed, although cleavage would presumably occur slowly in a manner corresponding

to that with Factor Xa alone. The other two forms of prothrombin were rapidly cleaved to thrombin, although the yield of thrombin from prothrombin-Ba was less than from the normal prothrombin. The remainder of the prothrombin (not activated to thrombin) was degraded to Intermediate 1. This pattern presumably arises from the differing abilities of normal prothrombin and prothrombin-Ba to bind calcium (see Chapter 5) and hence to be activated by Factor Xa/calcium/phospholipid and from competition between the resultant thrombin and the Factor Xa/calcium/phospholipid for the remaining unactivated prothrombin.

2. Human

The activation of human prothrombin by bovine Factor Xa resulted in similar products to those found for bovine prothrombin, i.e. products of MW_{app} (by SDS-gels) 72 (prothrombin), 54 (Intermediate 1), 37 (Intermediate 2), 34 (thrombin-B chain), 24 (Fragment 1), 22 (Fragment 1') and 15 (Fragment 2) thousand daltons, by any of the methods of activation used. This is in agreement with the results of Kisiel and Hanahan (1973) who have also shown that the reaction pathway is the same if human Factor Xa is used, although this material has a much lower activity than the bovine protein. If activation is carried out in the presence of hirudin (Kisiel and Hanahan 1974) or

μmoles benzoyl arginine ethyl ester/min/mg,

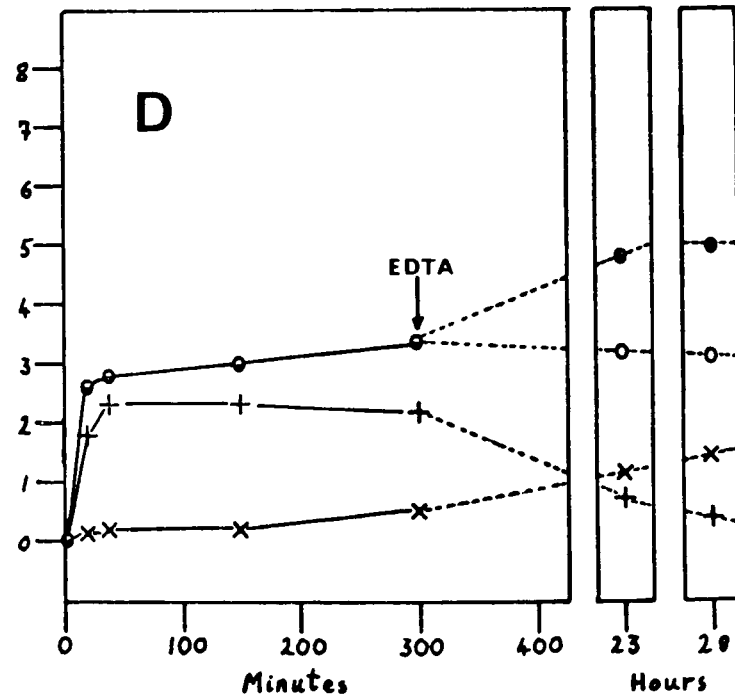
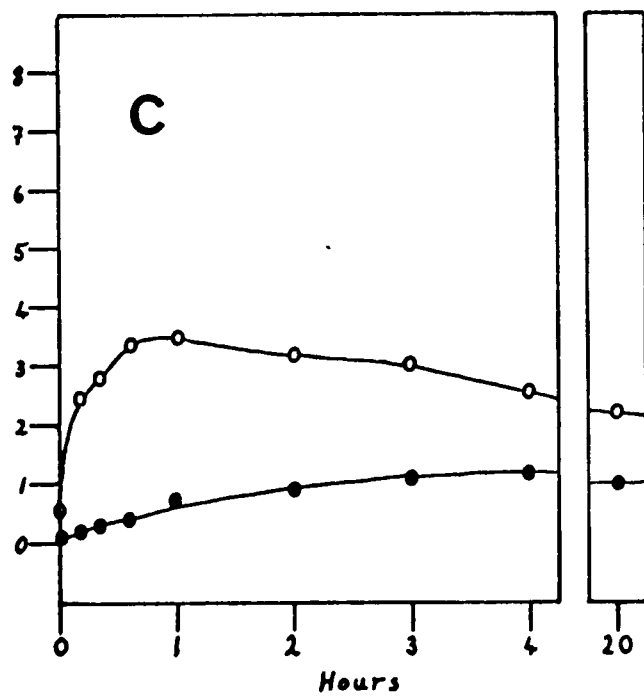
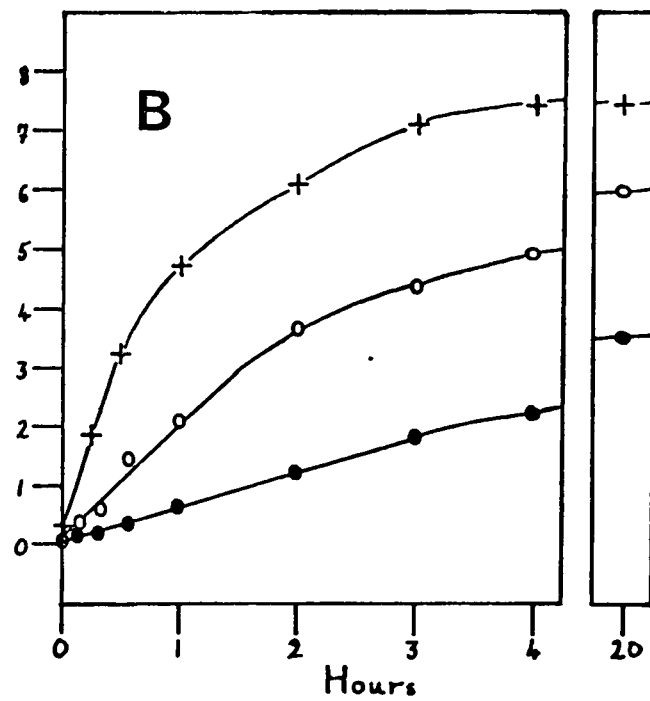
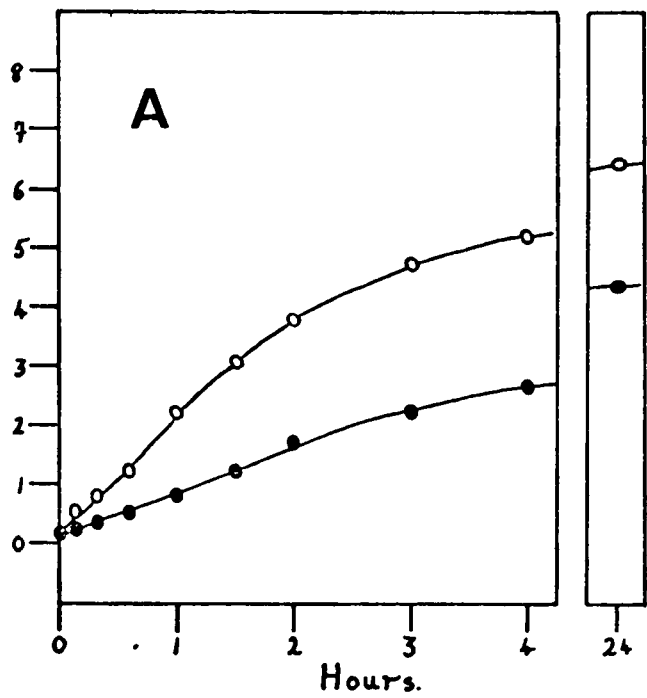


Figure 4.5.

Activations of human prothrombins

- conditions as described in the text.

A: Factor Xa.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

B: Factor Xa and calcium.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

+ — + : normal prothrombin and equimolar Xa

C: Factor Xa, calcium and phospholipid.

○ — ○ : normal prothrombin

● — ● : prothrombin-Ba

D: Factor Xa and calcium and BOVINE proteins.

○ — ○ : normal prothrombin

● — ● : normal prothrombin with EDTA added
at 300 minutes to 10mM

+ — + : normal prothrombin (0.2mg/ml) with
Fragment 1 included at 0.1mg/ml

X — X : Intermediate 1 at 0.15mg/ml

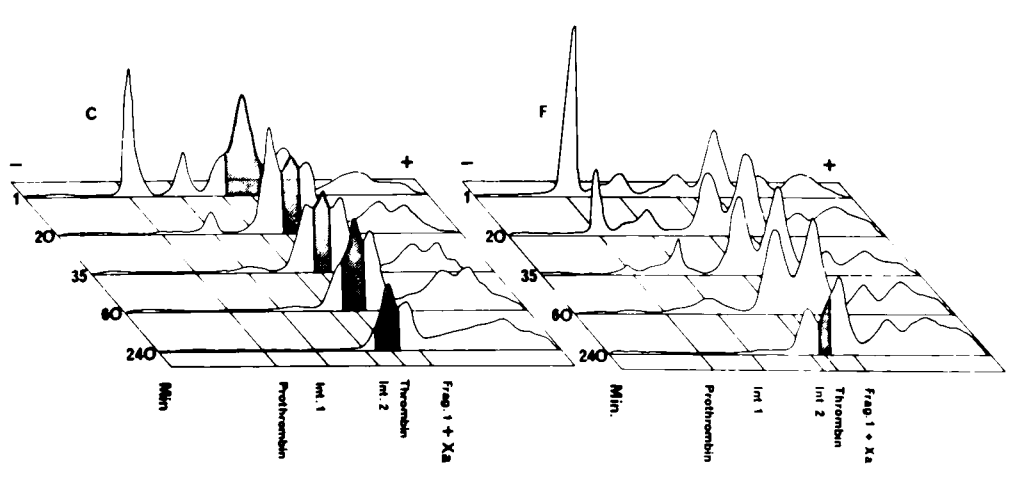
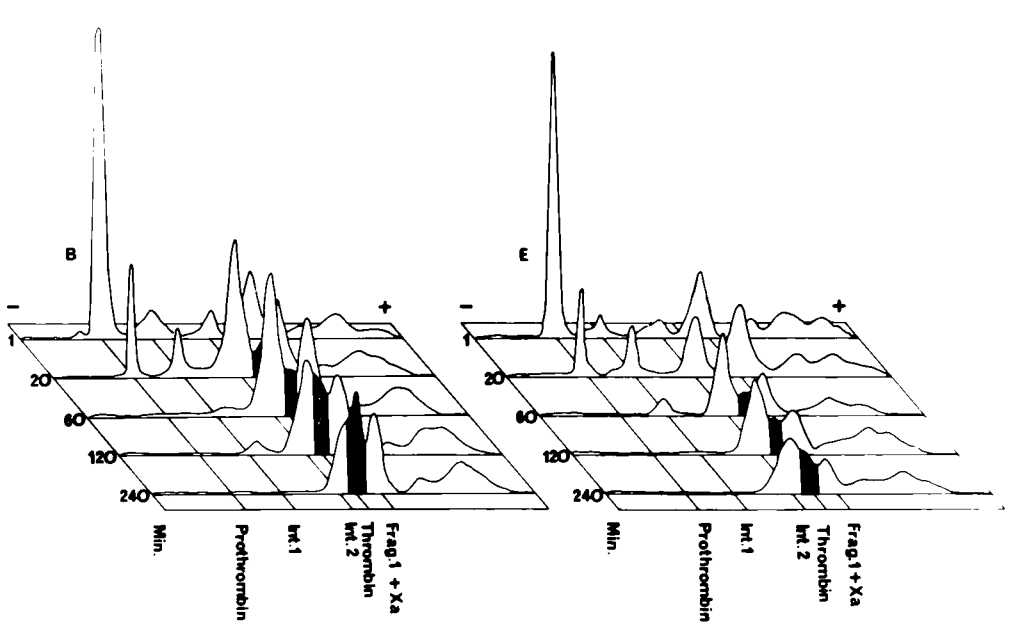
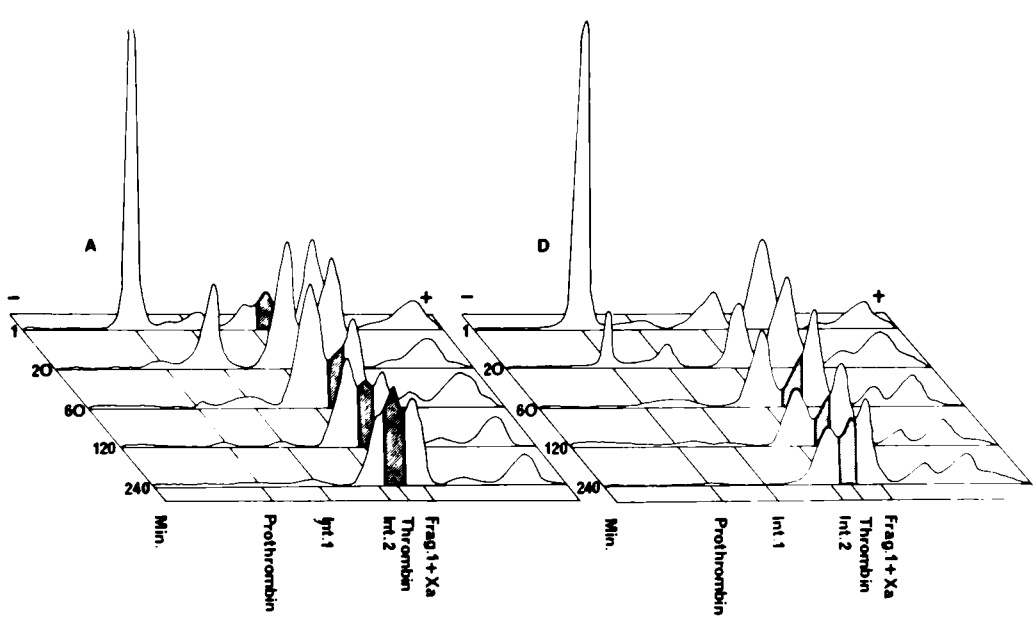


FIGURE 4-6

SDS-gel analysis of human
prothrombin activations

Factor Xa alone (A and D), Factor Xa and calcium (B and ^E~~B~~), and Factor Xa, calcium and phospholipid (C and F) on normal prothrombin (A, B and C) and prothrombin-Ba (D, E and F).

antithrombin III (Rosenburg et al 1975), to trap thrombin as it is formed, Fragment 1.2 is seen, as in the case of thrombin-inhibited bovine prothrombin activations (Esmon et al 1974a). Recent investigation of the human prothrombin activation products by Downing et al (1976) has demonstrated the existence of an additional reaction intermediate (Intermediate 2') 13 residues shorter than Intermediate 2 and formed by thrombin cleavage of the Intermediate 2. Thus human thrombin has an A chain 13 residues shorter than the bovine protein, although the N-terminal residue is also threonine.

All three modes of activation: Factor Xa, Xa/calcium and Xa/calcium/phospholipid, resulted in a lower rate of activation of human prothrombin-Ba than of normal human prothrombin (Figures 4.5 and 4.6). Thus, unlike the bovine protein, human abnormal prothrombin is not as good a substrate for bovine Xa as the normal human prothrombin, when activated by Factor Xa alone. All else being equal this is presumably due to differences in the conformation of the two forms of the molecule. The acceleration of activation by calcium and phospholipid was not nearly as great as in the bovine case. Thus a Factor Xa concentration of 20ug/ml was required for suitable activation rates. Also activation of normal human prothrombin with equimolar Factor Xa, in the presence of calcium, resulted in a relatively slow formation of thrombin. This may reflect the species specificity of the bovine Factor Xa. SDS-gel analysis of these human activations (Figure 4.6) showed that in all cases there

was a build up of Intermediate 2 during the plateau phase of the activation, even those including phospholipid, the inclusion of which abrogated this effect in the activation of bovine prothrombin. The activation of human prothrombin-Ba also showed the persistence of uncleaved prothrombin for longer times in the reaction mixture than in the corresponding normal prothrombin activations. The latter effect must be due to the poorer association between the enzyme and the abnormal prothrombin. The build up of Intermediate 2 may be associated with the extra Intermediate (Intermediate 2') in the human activation mechanism although this intermediate was not resolved in the analytical system used here. Alternatively it could be argued that Intermediate 2 builds up due to the inability of this intermediate to be cleaved in the absence of Fragment 1•2 (this having been cleaved by thrombin to Fragment 1 and Fragment 2) which could bind Intermediate 2 to the activating enzyme. This mechanism would also explain the build up of Intermediate 2 in the Factor Xa/calcium activations of bovine prothrombin but does not explain why the addition of phospholipid releases this build up in the bovine case.

(d) Discussion

The reason for the lower specific activity of the abnormal forms of prothrombin shown by the TSV assay was studied in greater detail by activating them with only some of the components of the prothrombinase complex. The lower activity of bovine prothrombin-Ba

was not noticeable unless calcium was involved in the activation complex; phospholipid enhanced the differences resulting from the inclusion of calcium. Bovine prothrombin-super, on the other hand, was only activated slowly, if at all, in all of the systems used here. This effect may be associated with the Fragment 1 region of the molecule as Intermediate 1 was apparently a better substrate for Factor Xa than the complete prothrombin-super. This is shown by the steeper portion of the sigmoid activation curve for bovine prothrombin-super not occurring until most of the prothrombin has been cleaved to Intermediate 1. Similarly the defect in prothrombin-Ba can be associated with the Fragment 1 region as differences between this molecule and normal prothrombin depend on the presence of calcium and the calcium binding is due to sites in this part of the molecule. Parallel studies on human prothrombins showed that the differences found in the TSV assay system corresponded to those found in Factor Xa/calcium and Factor Xa/calcium/phospholipid systems. However in this case a similar gradation was also seen in activation with Factor Xa alone (corresponding to the case of bovine prothrombin-super). The poor ability of calcium and phospholipid to enhance the activation of human prothrombin by bovine Factor Xa may be due to the species specificity of the latter enzyme.

CHAPTER 5

LOCALISATION AND IDENTIFICATION OF THE DEFECT IN ABNORMAL PROTHROMBIN

a. Introduction

In 1973 Morrison and Esnouf described the isolation of a barium-absorbable abnormal prothrombin from human coumarin plasma. Characterisation of this protein apparently showed that it had a lower carbohydrate content than normal prothrombin, in agreement with the work of Pereira and Couri (1971) on the effect of Vitamin K on rat prothrombin, although the amino acid composition was normal. Morrison (1973) also suggested that this structural defect might result in the formation of a low specific activity thrombin, on the basis of activation experiments with the prothrombinase complex. The latter results, however, could also be explained in terms of a slow activation of the abnormal prothrombin.

In contrast to these results no carbohydrate difference was found between normal bovine prothrombin and the abnormal proteins isolated by Stenflo and Suttie, which do not absorb to insoluble barium salts (Stenflo and Ganrot 1972b, Nelsestuen and Suttie 1972b). In fact the only defect found by these two groups was a reduced ability to bind calcium in the abnormal protein (Stenflo and Ganrot 1973, Nelsestuen and Suttie 1972b), associated with low activity in the standard prothrombin assays. In non-physiological activation systems, such as trypsin, Echis carinatus venom, Dispholidus typus venom and staphylocoagulase activation of both normal and abnormal proteins gave a

similar yield of thrombin (Nelsestuen and Suttie 1972a, Hemker et al 1975). However the yield of thrombin in these activations was low.

Digestion of normal prothrombin has yielded peptides that bind barium and calcium and which have subsequently been located in the N-terminal portion of the molecule (Nelsestuen and Suttie 1973, Stenflo 1974, Magnusson et al 1974). These peptides have anomalous charge by electrophoresis at pH6.5 (Magnusson et al 1974), whereas a corresponding peptide from the abnormal protein has a charge consistent with its composition (Stenflo 1974).

The abnormal protein in this study has been analysed for carbohydrate, amino acid and free carboxyl group content. The differences observed in the activation experiments described in the previous chapter were further investigated by isolating the thrombin and N-terminal Fragment 1 region of each species of prothrombin. The abnormality was found to lie in the N-terminal region and peptides from enzymic digests of the abnormal barium-absorbable prothrombin have been isolated and characterised.

b. Methods

1. Carbohydrate analysis

Carbohydrate analysis of proteins was performed by the method of Chambers and Clamp (1971). Protein solutions

(1mg/ml) were dialysed against 0.1M NH_4HCO_3 before addition of mannitol as an internal standard, and the solutions, containing 1 to 2mg of protein, dried at 60°C for 1 to 2 days and then under vacuum for 1 day. 1ml of methanolic HCl (1M) was added to each sample and methanolysis was allowed to proceed in screw capped vials (Soveril) under nitrogen for 18 hours at 85°C for 18 hours. Excess acid was neutralised with silver carbonate and re-N-acetylation was performed for 8 hours by the addition of 10mg of silver acetate followed by 50ul of acetic anhydride. The supernatant was decanted and rotary evaporated at 40°C . After drying under vacuum overnight, trimethylsilyl derivatives were prepared by the addition of 100ul of a mixture of trimethylchlorosilane, hexamethyldisilazane and pyridine in the ratio of 1:1:5. Typically 2 to 4ul of each sample was analysed using an F & M Hewlett Packard or an F11 Perkin Elmer chromatograph fitted with a 2m x 4mm glass column packed with 3% OV-1 on Diatomite CQ 85-100 mesh. The carrier gas was oxygen-free nitrogen.

The oven temperature was programmed to rise at 1°C / minute from 130 to 220°C , after an initial delay of 10 minutes. Individual carbohydrate residues were estimated from the chromatographic traces by measuring the areas under the appropriate peaks and comparing them with standards. All estimates were carried out at least in duplicate. Protein weights were estimated by optical absorbance corrected for light scattering, by dry weight determinations and by the

Lowry colorimetric technique (1951), standardised with bovine serum albumin.

2. Estimation of free carboxyl groups

The method of Hoare and Koshland (1967) was used to estimate the number of free carboxyl groups in proteins. 0.5mls 2M GEE (glycine ethyl ester) was mixed with 0.5g GuHCl. Approximately 10nmoles of protein was dissolved in this solution and the pH adjusted to 4.9. Reaction was commenced by the addition of 40mg EDC (1-ethyl 3-dimethyl aminopropyl carbodiimide) and the pH maintained between 4.8 and 5.0. An additional 20mg of EDC was added after 2 hours and reaction continued overnight. Excess reagents were removed by extensive dialysis and the extra glycine incorporated into the protein estimated on the amino analyser, using the other amino acids as internal standards.

3. Zone electrophoresis

Prothrombin and Fragment 1 were electrophoresed in 60mM sodium barbitone/HCl pH8.65, containing 1% agarose, essentially as described by Johansson (1972), except that fixing was carried out for 15 minutes in 20% trichloroacetic acid, followed by two 5 minute washes in ethanol. After drying the plate was stained in 0.1% Coomassie blue in 25% isopropanol, 10% acetic acid and then destained in 45% methanol, 10% acetic acid. This procedure was carried out

either in the presence of 2mM calcium lactate or 2mM EDTA.

4. Estimation of gamma-carboxy glutamic acid

This was carried out as described by Fernlund et al (1975). About 1mg of dry protein was taken up in 0.2ml of 15% NaOH and transferred to a small capped polypropylene tube. Hydrolysis was carried out at 110°C for 16 hours. After dilution, the samples were desalted on a (1 x 3)cm column of Dowex 50-X8 (NH₄⁺ form), elution being carried out with 1M-NH₃. The sample was rotary evaporated to dryness at 50°C, taken up in water and applied to the long column of the amino acid analyser. Gla appeared at 25 minutes (aspartate at 45 minutes) on this column, as shown by co-chromatography with a sample of the amino acid prepared as described in Appendix 2. Experiment showed that the yield of aspartic acid and proline from acid and alkaline hydrolysis was the same and this result was used in calculating the Gla content of proteins. The ninhydrin colour value of Gla was taken to be the same as that for glutamic acid as the values obtained for synthetic Gla quickly decreased on storage, presumably due to the formation of the pyrocarboxy amino acid.

5. Preparation of peptides

Preliminary small scale chymotryptic digests were performed, at the same molar ratio as described below, on

3mg of Fragment 1, and characterised by a calcium diagonal technique. This was carried out in the normal pH6.5 paper electrophoretic system except that 10mM calcium acetate was included in the second dimension buffer.

The main digest was carried out on 100mg of Fragment 1. 100mg of prothrombin-Ba Fragment 1 in 0.15M NH_4HCO_3 was digested for 2 hours with chymotrypsin at an enzyme to substrate ratio of 1:50. Chymotrypsin was pretreated with SBTI (1mg of soy bean trypsin inhibitor/mg enzyme) for 30 minutes at room temperature, to inhibit tryptic activity. The digest was stopped by the addition of DPCC (0.5ml of a stock solution of 19.6mg diphenyl carbamyl chloride in 10mls of methanol, per mg of enzyme), and then freeze dried to remove ammonia. The residue was taken up in 5mls 0.1M Tris 2mM EDTA and the pH checked to be 8.6. Some insoluble material remained. This was dissolved by the addition of 5g GuHCl . Peptides were reduced by the addition of 9.6mg DTT (3-fold excess if dithiothreitol), flushing with nitrogen and incubating for 1.5 hours at room temperature; and then carboxymethylated by incubation with 75mg ^{14}C -labelled sodium iodoacetate (3-fold molar excess with respect to DTT) for 30 minutes. The pH was adjusted to that of 10% acetic acid and the peptides desalted on a (2x120)cm Sephadex G-10 column in 10% acetic acid at 20 ml/hr. 5ml fractions were collected.

All fractions containing peptide material were pooled,

lyophilised, taken up in a minimum volume of 20mM $\text{NH}_4\text{HCO}_3/\text{NH}_3$ pH8.3 and applied to a (1X60)cm DE-52 cellulose (Whatman) column at 10ml/hr. This column was eluted with a (2x150ml) linear gradient from 20 to 500mM $\text{NH}_4\text{HCO}_3/\text{NH}_3$ pH8.3 at 10ml/hr, collecting 20 minute fractions. Elution was completed with a (2x75ml) gradient from 0.5 to 1M buffer. Individual peptides were further purified by preparative paper electrophoresis at pH 6.5 and 2.1, after preliminary analytical runs.

Pool II peptide from the above run was further digested (see results) with pepsin as follows: 400nmoles of pool II peptide in 0.1M HCl was incubated with pepsin at a molar ratio of 1:30 for 5 hours and then lyophilised. The peptides were taken up in 2mls 20mM NH_3 , applied as a 15cm streak to 3MM paper and subjected to high voltage electrophoresis at pH6.5 for 40 minutes at 3kV. Side strips were stained for arginine and with ninhydrin-cadmium stain. A 1cm side strip was also taken for a second dimensional run at pH2.1. On the basis of these results individual peptides were isolated by elution from the paper before or after further preparative paper electrophoretic purification.

High voltage paper electrophoresis was carried out by standard methods at 3kV in buffers of pH 6.5 and 2.1 as described by Ambler (1963) and stained as described by Offord (1969), except for peptides that contained arginine

which were stained for this amino acid with phenanthrenequinone and visualised under ultraviolet light, according to Yamada and Itano (1966). Mobilities were calculated as described by Offord (1966). Approximate amino acid analyses of hydrolysed peptides were carried out quickly by high voltage electrophoretic separation of amino acids at pH 2.1 (5kV, 17 minutes) followed by staining with ninhydrin-collidine. N-terminal analysis of peptides was carried out as described by Gray (1972).

6. Heat treatment of Gla-containing peptides

Gla-containing peptides were taken up in the standard pH 2.1 buffer and then dried down again. They were then sealed in small glass vials, under vacuum, and heated at 140°C for 20 minutes. The vials were opened and the contents dissolved in pH 6.5 buffer before electrophoretic analysis.

7. Mass spectrometry of peptides

Peptides containing Gla were characterised by Dr. Howard Morris of Imperial College, London by mass spectrometry according to procedures described by him (Morris et al 1976). Positions identified as containing Gla in the acetylated, permethylated peptides were confirmed as such by the methyl iodide isotopic labelling method described in the above paper.

c. Results

At the time that the present study was started (September 1973) there was general agreement that the various abnormal prothrombins produced during coumarin therapy did not differ significantly in amino acid composition from normal prothrombin. These results were based on the normal procedure of hydrolysis in 6M HCl. There was, however, conflicting evidence as to whether a difference existed in carbohydrate composition between normal and abnormal prothrombin. Earlier work in this laboratory had suggested that abnormal human prothrombin had a reduced carbohydrate content and that this might be associated with a low activity 'coumarin' thrombin (Morrison and Esnouf 1973, Morrison 1973). Initial work was therefore carried out on the composition and activity of abnormal human, and subsequently bovine, prothrombin relative to the normal protein.

Compositions of the three bovine and two human prothrombins are presented in Table 5-1. The amino acid compositions are in good agreement with published values (Stenflo 1972b, Nelsestuen and Suttie 1972a, Downing et al 1975) for abnormal and normal prothrombin, except for the methionine value for human material. Other authors have found a methionine content closer to the value of 7 found for bovine material. The results show that the amino acid compositions, obtained by acid hydrolysis, of the abnormal

TABLE 5.1.

Analyses of various prothrombins.

<u>Residue</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>
LYS	32.2	33.7	33.3	31.1	33.0
HIS	10.6	11.2	13.5	10.6	11.1
ARG	39.3	38.8	39.9	26.4	28.4
ASP	63.9	61.5	65.0	65.8	65.3
THR	30.6	29.8	34.4	32.1	30.5
SER	36.6	39.1	40.9	38.4	37.8
GLU	68.5	67.1	68.0	73.9	68.1
PRO	33.9	34.6	38.0	33.7	34.9
GLY	49.0	49.0	42.0	49.0	49.0
ALA	36.0	34.3	38.5	37.3	36.0
CYS	20.0	20.1	18.8	19.9	20.0
VAL	28.3	28.8	24.0	36.6	33.7
MET	7.6	6.9	6.5	3.0	3.2
ILEU	18.5	18.5	18.0	20.0	19.7
LEU	42.6	45.2	41.5	41.9	39.6
TYR	19.8	19.9	21.5	18.0	17.5
PHE	19.5	20.1	21.8	20.3	18.4
Mannose	8.3	8.2	8.5	8.9	8.6
Galactose	6.8	6.8	7.3	7.8	7.2
Glucose*	0.9	1.2	0.6	1.8	2.1
Glucosamine	14.1	13.8	12.1	11.3	10.6
Sialic Acid	13.9	15.3	16.4	9.3	7.6

* Glucose is probably a contaminant derived from chromatography media.

A; normal bovine prothrombin

B: bovine prothrombin-Ba

C: bovine prothrombin-super

D: normal human

E: human prothrombin-Ba.

prothrombin.

prothrombins do not differ significantly from those for the normal proteins. Further, human prothrombin has a composition very similar to the bovine material, except for the methionine content about which some doubt exists, as described above. Bovine prothrombin-super shows minor variation in composition from the normal prothrombin. This may be ascribed to the minor contaminant shown by SDS-gel analysis to be present in this material (Figure 3-4).

The carbohydrate content was estimated by gas liquid chromatography (glc) of trimethylsilyl derivatives of the methanolysed glycosyl residues, as opposed to the more widely used procedure of estimating hexosamine on the amino acid analyser and hexose and sialic acid by colorimetric methods. Glc allows the estimation of the individual sugar residues, however the method gives somewhat variable results for sialic acid (present as N-acetyl neuraminic acid in all the proteins studied in this work) partly due to the cracking of this residue at the higher temperatures used in the terminal portion of the glc programme. It was found possible to avoid this to some extent by carrying the methanolysis stage out in 0.5M methanolic HCl for only 1.5 hours. The low level of glucose found in these analyses is probably a contaminant derived from the dextran matrix of the Sephadex used during the protein purification, as experiment has shown that careful washing of the protein prior to methanolysis reduced the glucose content to minimal levels.

As one of the initial propositions of the present study was that coumarins might affect the carbohydrate component of prothrombin, carbohydrate analyses were repeated a number of times under a variety of different control conditions. The major source of error in these analyses was estimation of the amount of protein initially present, at the stage of addition of the mannitol internal standard (in some runs perseitol was used as an internal standard, but was found to be inferior to the more commonly used mannitol, despite its better resolution from other peaks). Protein was therefore estimated by three methods:

1. by its optical absorbance at 280nm, corrected for light scattering according to Shapiro and Waugh (1966);
2. on the basis of dry weight measurements (obtained by drying in vacuo over P_2O_5 at $110^{\circ}C$ to constant weight, correction being made for ^{any} salt content by drying of buffer alone);
3. by the use of the colour reaction described by Lowry et al (1951) using bovine serum albumin as a standard.

Method 2 had the incidental advantage of demonstrating that no difference existed between abnormal and normal prothrombin in respect of the molar extinction coefficient at 280nm.

The results presented in Table 5.1 are based on the absorbance measurements and show no differences between

normal and abnormal prothrombin, in contrast with the results of Morrison and Esnouf (1973). Similarly, no difference was observed when analyses were carried out using the other two methods, although the absolute values varied slightly as the methods measure different physical parameters. Also the dry weight measurements involved prolonged heating at 110°C , which had a slight degradative effect.

As in the case of the amino acid composition, the carbohydrate composition of human and bovine prothrombin are very similar, except in the case of sialic acid. Whether the latter difference is significant or not is doubtful.

It was shown in Chapter 3 that abnormal prothrombin is eluted ahead of normal prothrombin during chromatography on DEAE-Sephadex. This suggests that the abnormal protein is lacking in some negative prosthetic group(s) present in the normal prothrombin. The most obvious candidate for such a negative group would be some form of carboxyl moiety. In order to investigate this attempts were made to quantitate the number of free carboxyl groups in the various forms of prothrombin. The method of estimation used was that of Hoare and Koshland (1967) whereby glycine ethyl ester is conjugated to the free carboxyl groups through the agency of water soluble carbodiimide. The number of extra glycines in the derivatised molecule, corresponding to the free

TABLE 5.2

Free-carboxyl content of prothrombins.

(single experiment)

	<u>Calculated*</u>	<u>Amino acid analysis</u>	<u>¹⁴C-incorporation</u>
<u>Bovine</u>			
Normal prothrombin	88	93	101
Prothrombin-Ba	88	89	115
Prothrombin-super	88	107	124
Fragment 1 from prothrombin-Ba	36	35	-
<u>Human</u>			
Normal prothrombin	88**	90	-
Prothrombin-Ba	88**	95	-

* calculated from the composition given by
Magnusson et al (1975) for amide and free
acid residues, assuming no carboxylation.

** figures for bovine material.

carboxyl content, may be quantitated by amino acid analysis or by the use of labelled glycine ethyl ester. The results of this experiment are shown in Table 5-2 and would seem to indicate that there are an excess of carboxyl groups in prothrombin over that indicated by amino acid analysis after acid hydrolysis. However no clear gradation of this effect was observed, corresponding to the activity differences, in this experiment, which was only carried out once as subsequent developments indicated a more direct procedure. The differences in carboxyl content were difficult to quantitate accurately as they involve differences of less than 5% in glycine values in the amino acid analyses. The labelling method, although potentially more accurate, gave unreliable results in this experiment as only very small amounts of label were introduced into the molecule by the technique used. For the required accuracy a much higher activity label or alternatively a much greater amount of protein (about 100-fold greater) would be required. This assay method would be of greater use if the prosthetic group could be obtained attached to a smaller portion of the molecule, thereby reducing the background level of glycine incorporation due to glutamic and aspartic residues.

The reduced activity of abnormal prothrombin could in theory be due to either a 'coumarin' thrombin of reduced activity or to the poor ability of this prothrombin to act as a substrate for the 'prothrombinase' complex. The results presented in Chapter 4, together with those of

TABLE 5.3

Activity of various thrombins.

	<u>Bovine</u>		
	<u>Prothrombin</u> <u>normal</u>	<u>Prothrombin</u> <u>Ba</u>	<u>Prothrombin</u> <u>super</u>
Clotting Activity			
NIH units/mg.	2750±260	3244±1170	2597±380
Esterase Activity			
umoles/min/mg.	21.7±1.3	18.0±1.4	21.3±2.9

	<u>Human</u>	
	<u>Normal prothrombin</u>	<u>Prothrombin-Ba</u>
Clotting Activity		
NIH units/mg.	3933±683	3695±549
Esterase Activity		
umoles/min/mg.	26.5±1.8	25.6±5.0

- calculated using $E_{280}^{1\%} = 19.5$ for bovine thrombin

(Winzor and Scheraga 1964)

= 19.2 for human thrombin

(Thompson 1976)

Nelsestuen and Suttie (1972a), would indicate that the thrombin has normal activity; this conclusion is based on thrombin present in the activation mixtures and not as the pure protein. Components of the activation mixture may affect the yield of thrombin, as described in Chapter 4. The conclusions of Nelsestuen and Suttie are based on the use of non-physiological activators that do not give as high a yield of thrombin as the normal activators. Thrombin was therefore isolated from the various prothrombins by the method described in Chapter 2 and assayed for clotting and esterolytic activity. The results of these experiments (Table 5-3) show that 'coumarin' thrombin is fully active in both human and bovine cases.

The abnormality would therefore appear to affect the ability of coumarin prothrombin to act as a substrate and to be associated with the 'pro' portion of the molecule. As bovine coumarin prothrombin, that does not bind to barium, has a reduced ability to bind calcium (Nelsestuen and Suttie 1972b, Stenflo and Ganrot 1973) the defect may be associated with the calcium-binding Fragment 1 region of the prothrombin molecule.

In the absence of the fairly large amounts of protein required for calcium binding studies by equilibrium methods, the calcium binding ability of prothrombin and Fragment 1 was qualitatively assessed by electrophoresis in the presence and absence of calcium. The results of such an experiment for bovine

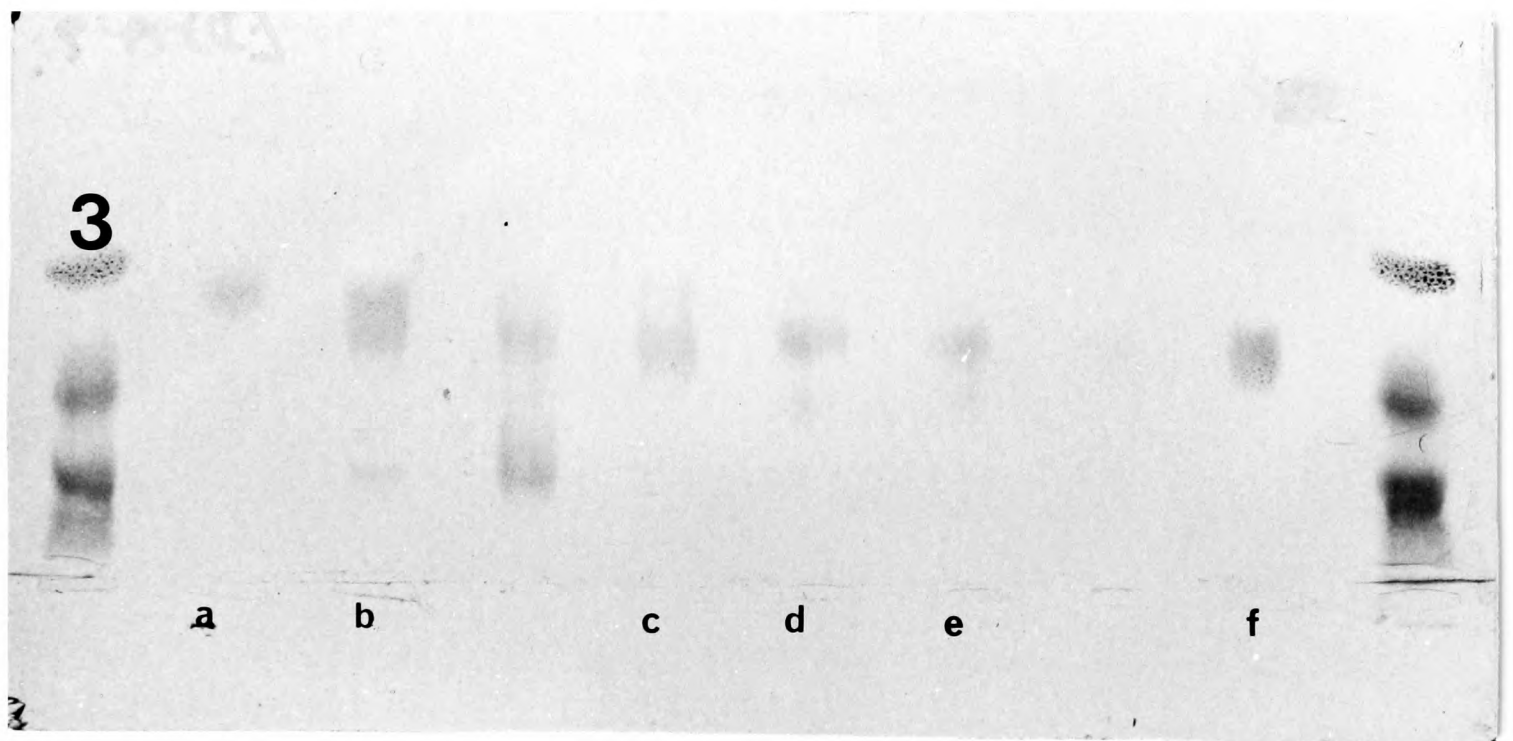
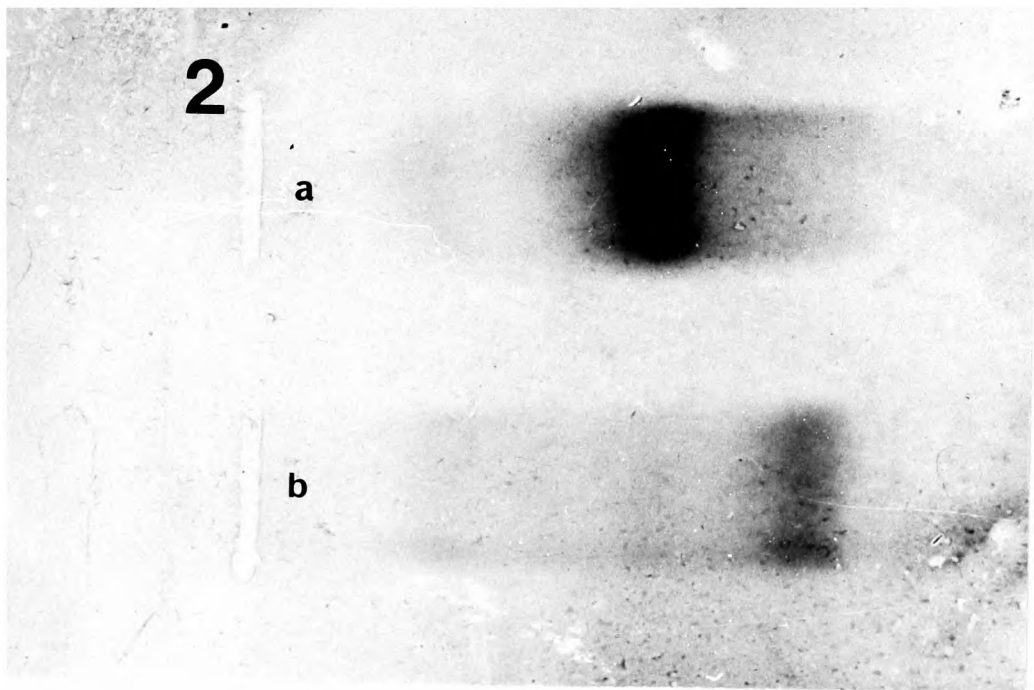
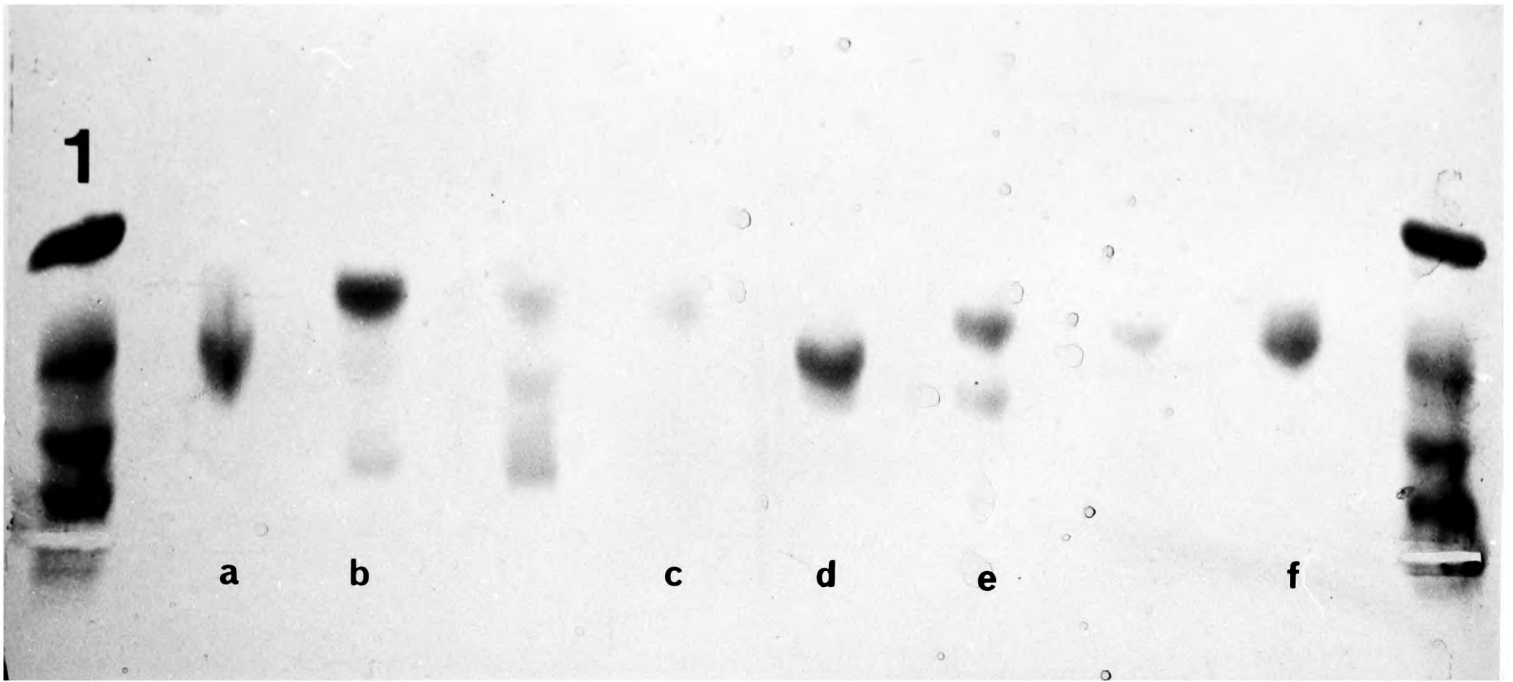


FIGURE 5•1

Zone electrophoresis of prothrombin and Fragment 1

- 1 & 2: in 60mM barbitone pH 8•65, 2mM calcium lactate
3: in 60mM barbitone pH 8•65, 1mM EDTA.
a: normal Fragment 1.
b: Fragment 1 from prothrombin-Ba.
c: Fragment 1 from prothrombin-super.
d: normal prothrombin.
e: prothrombin-Ba.
f: prothrombin-super.

prothrombins and Fragment 1's are shown in Figure 5-1. This shows that the mobility of normal prothrombin and Fragment 1 are reduced by the inclusion of calcium in the electrophoresis buffer as compared to the abnormal proteins, whereas this difference was not seen when EDTA replaced the calcium. Thus normal prothrombin binds sufficient calcium at pH 6.85 to affect its mobility while both forms of abnormal prothrombin bind calcium to a lesser extent. Parallel experiments on human prothrombin and Fragment 1 did not distinguish between the normal and abnormal proteins.

In 1974 Girardot et al showed that a microsomal rat liver system would synthesise prothrombin in the presence of bicarbonate. If the bicarbonate was labelled with ^{14}C , the label was introduced into the prothrombin to form an acid residue. Under the acid hydrolysis conditions normally used for amino acid analysis this residue gave rise to glutamic acid. This lead was quickly taken up by other groups working on the structure of prothrombin.

All three groups working in this area demonstrated the existence of the amino acid gamma-carboxy glutamic acid (Gla) in peptides from the N-terminal of prothrombin (Magnusson et al 1974b, Nelsestuen et al 1974, Stenflo et al 1974). Magnusson et al showed that 10 of these carboxylated residues exist in bovine prothrombin (Magnusson et al 1974, Morris et al 1976), all in the first 35 residues from the N-terminal (Figure 5-2). These results have been

FIGURE 5.2

N-terminal sequence of bovine prothrombin.

1.	10.
Ala-Asn-Lys-Gly-Phe-Leu-Gla-Gla-Val-Arg-	
11.	20.
Lys-Gly-Asn-Leu-Gla-Arg-Gla-Cys-Ieu-Gla-	
21.	30.
Gla-Pro-Cys-Ser-Arg-Gla-Gla-Ala-Phe-Gla-	
31.	40.
Ala-Leu-Gla-Ser-Leu-Ser-Ala-Thr-Asp-Ala-	
41.	50.
Phe-Trp-Ala-Lys-Tyr-Thr-Ala-Cys-Glu-Ser-	
51.	60.
Ala-Arg-Asn-Pro-Arg-Glu-Lys-Leu-Asn-Glu-	

(from Magnusson et al 1974).

confirmed by the other two groups (Fernlund et al 1975, Howard et al 1975). A similar peptide containing up to 14 Gla has been isolated from a tryptic digest of normal Factor X by barium citrate absorption (Howard and Nelsestuen 1975).

The identification of Gla in the above studies depended on its heat lability under acid conditions, resulting in a decreased mobility after heating on electrophoresis at pH6.5; on mass spectrometry of a peptide and on comparative proton magnetic resonance spectroscopy between small peptides from normal prothrombin and the corresponding synthetic peptides containing glutamic acid. However the only direct demonstration of uncarboxylated Gla (i.e. glutamic acid) in the abnormal prothrombin has been the sequencing of the peptide:

Gly-Phe-Leu-Glu-Glu-Val-Arg-

representing residues 4-10 in prothrombin-super (Stenflo and Ganrot 1974), although indirect evidence for lack of carboxylation in the abnormal prothrombin has been presented. Tryptic digestion of normal prothrombin yields the peptide, residues 12-34, whereas the abnormal protein yields smaller peptides from this portion of the molecule; in this connection it has been proposed that carboxylation affords some protection against tryptic attack (Stenflo ~~and Ganrot~~ 1974). Nelsestuen and Suttie (1973) have isolated the peptide 12-42 from a tryptic digest of normal prothrombin by barium citrate absorption. Abnormal prothrombin gave

TABLE 5.4

Average values from Gla estimations of prothrombins.

	<u>Gla released by</u> <u>alkaline hydrolysis*</u>	<u>Gla content</u> <u>(corrected**)</u>
<u>Bovine</u>		
Normal prothrombin.	8.7	10.0
Prothrombin-Ba.	5.7	6.6
Fragment 1 from		
prothrombin-Ba.	5.1	5.9
Prothrombin-super	3.5	4.0
<u>Human</u>		
Normal prothrombin	8.7	10.0
Prothrombin-Ba	6.0	6.8

*- assuming a ninhydrin colour value equal to that of
glutamic acid and using Asp and Pro as internal
standards

** - assuming 10 Gla in normal prothrombin

(Magnusson et al 1974b).

only very low yields of this peptide (less than 5%) from the barium absorption stage. Also the ability of the normal peptide to bind barium citrate can be blocked by reaction of the carboxyl groups with glycine ethyl^{ester}, (all the carboxyl groups in this peptide are derived from Gla).

All the above methods require the use of fairly small peptides and are not suitable for the identification of Gla in larger polypeptides such as prothrombin. To solve this problem two methods have been developed, the difficulty being the heat lability of Gla under acid conditions. In the first method these conditions are avoided by the use of alkaline hydrolysis of the protein. This gives a good yield of Gla which can then be identified by the conventional amino acid analysis procedures (Fernelund et al 1975). Alternatively acid residues in the protein may be converted to the corresponding alcohols by treatment with diborane, thus Gla yields dihydroxyleucine. These residues are acid stable and may be estimated conventionally (Zytcovicz and Nelsetuen 1975). The method only gives poor yields of Gla (approx. 30%) but may be used as a qualitative test for the presence of Gla.

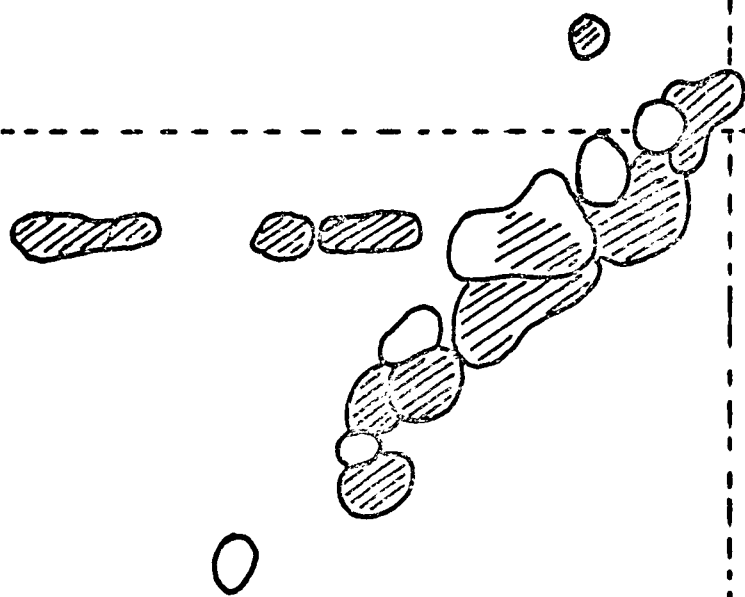
The results of assay, by the alkaline hydrolysis procedure, of the Gla content of the various forms of prothrombin are presented in Table 5.4 and demonstrate that prothrombin-Ba, derived from both bovine and human coumarin plasma, has a reduced content of Gla. In the bovine case

assay of the Fragment 1 shows that all the Gla is located in this region of the molecule, as expected. It was also shown that prothrombin-super contains some Gla. In order to confirm this result peptides were isolated from the N-terminal region of prothrombin-Ba, through the co-operation of Dr. Staffan Magnusson of Aarhus University. The sequence of the Gla containing region of normal prothrombin is shown in Figure 5°2 for reference.

A useful technique in the preliminary identification of Gla-containing peptides is the heat diagonal technique described by Magnusson et al (1974). This has the advantage of allowing identification of Gla-containing peptides but suffers in that larger peptides tend to become fixed to the paper during the heating process. This can be overcome by running heated and unheated peptides in one dimension only. This method was tried out on a 42 residue peptide isolated from normal prothrombin by thermolytic digestion and barium citrate absorption. The amino acid composition and N-terminal residue of this peptide were consistent with its being residues 1-42. Heat treatment of this peptide resulted in a decrease of its mobility (relative to aspartic acid) from 0.78 to 0.54 during high voltage electrophoresis at pH6.5. However a fair amount of insoluble material remained at the origin in the run on the heated sample.

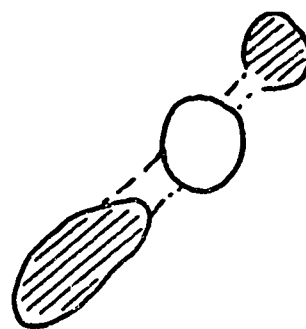
To overcome the problems caused by heating, a calcium diagonal method was devised whereby the mobility of calcium-

(a)

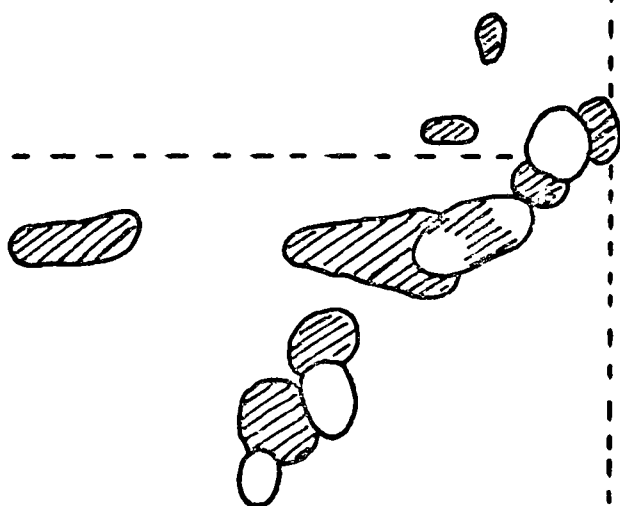


○ ? DNS-OH

+



(b)



○ ? DNS-OH

+

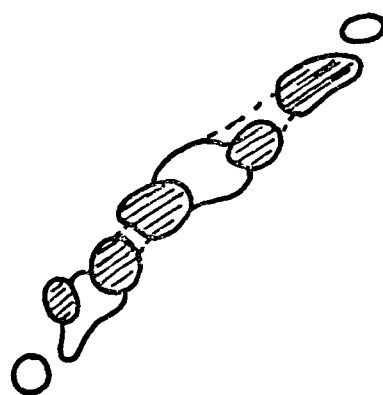


FIGURE 5•3

Calcium diagonals on chymotryptic digests of Fragments

1 derived from :

(a) normal prothrombin

(b) prothrombin-Ba

- high voltage electrophoresis was carried out in the normal pH6•5 buffer except that 10mM calcium acetate was included in the buffer for the second dimension (vertical in this diagram).
- the aspartate marker ran as far as the edge of the map as drawn.
- dotted lines indicate the position of the neutral marker (DNS-arginine).
- cross-hatching indicates peptides containing arginine.

binding peptides is retarded in one dimension by the inclusion of 10mM calcium acetate in the standard pyridine/acetic acid pH6.5 buffer. This has the advantage of being less harsh than the heat treatment. The results, however, indicate that precipitation of some calcium-binding peptides may occur in this method. In the purely qualitative sense in which this method was used in the present study this is of no consequence.

The studies of Stenflo et al (1974) have shown that the Gla residues of normal prothrombin may afford some protection against enzymic degradation. Chymotryptic digests of Fragment 1 derived from normal prothrombin and prothrombin-Ba were therefore characterised by the calcium diagonal technique, which showed that there was indeed a difference in the mode of degradation of the two forms of the protein (Figure 5.3). As a result of this preliminary test it was felt that the normal procedure of absorbing the N-terminal Gla-peptides from the chymotryptic digest with barium citrate (in normal prothrombin this yields residues 1-35, Morris et al 1976) would not be justified as some of the peptides from prothrombin-Ba will contain glutamic acid rather than Gla and might not bind to the barium citrate. Less specific peptide isolation techniques were therefore adopted.

Following a large scale chymotryptic digest of prothrombin-Ba Fragment 1 and ^{14}C -carboxymethylation, the

Figure 5.4.

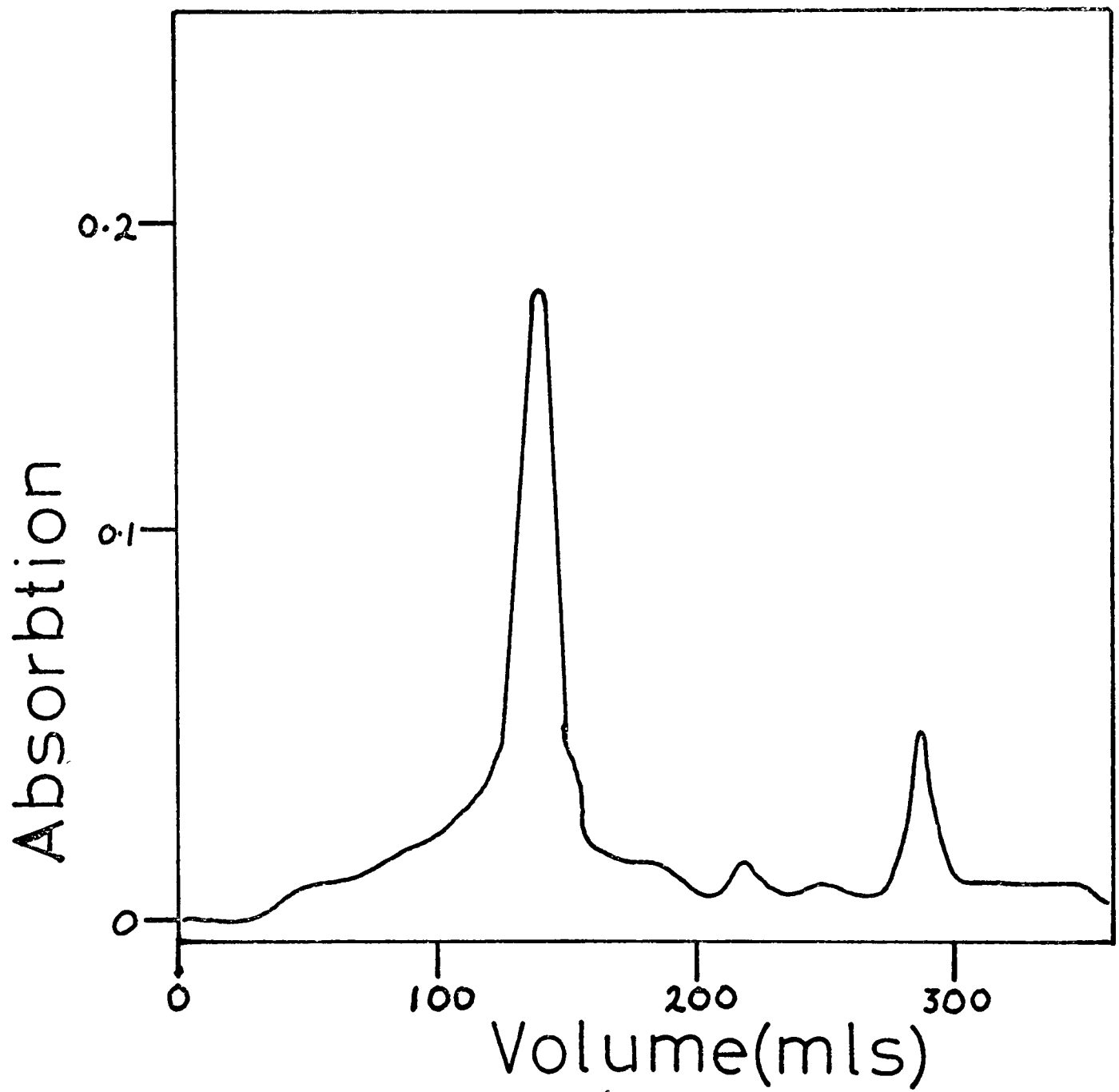


FIGURE 5•4

Chromatography of chymotryptic digest of Fragment 1
from bovine prothrombin-Ba on DEAE-cellulose.

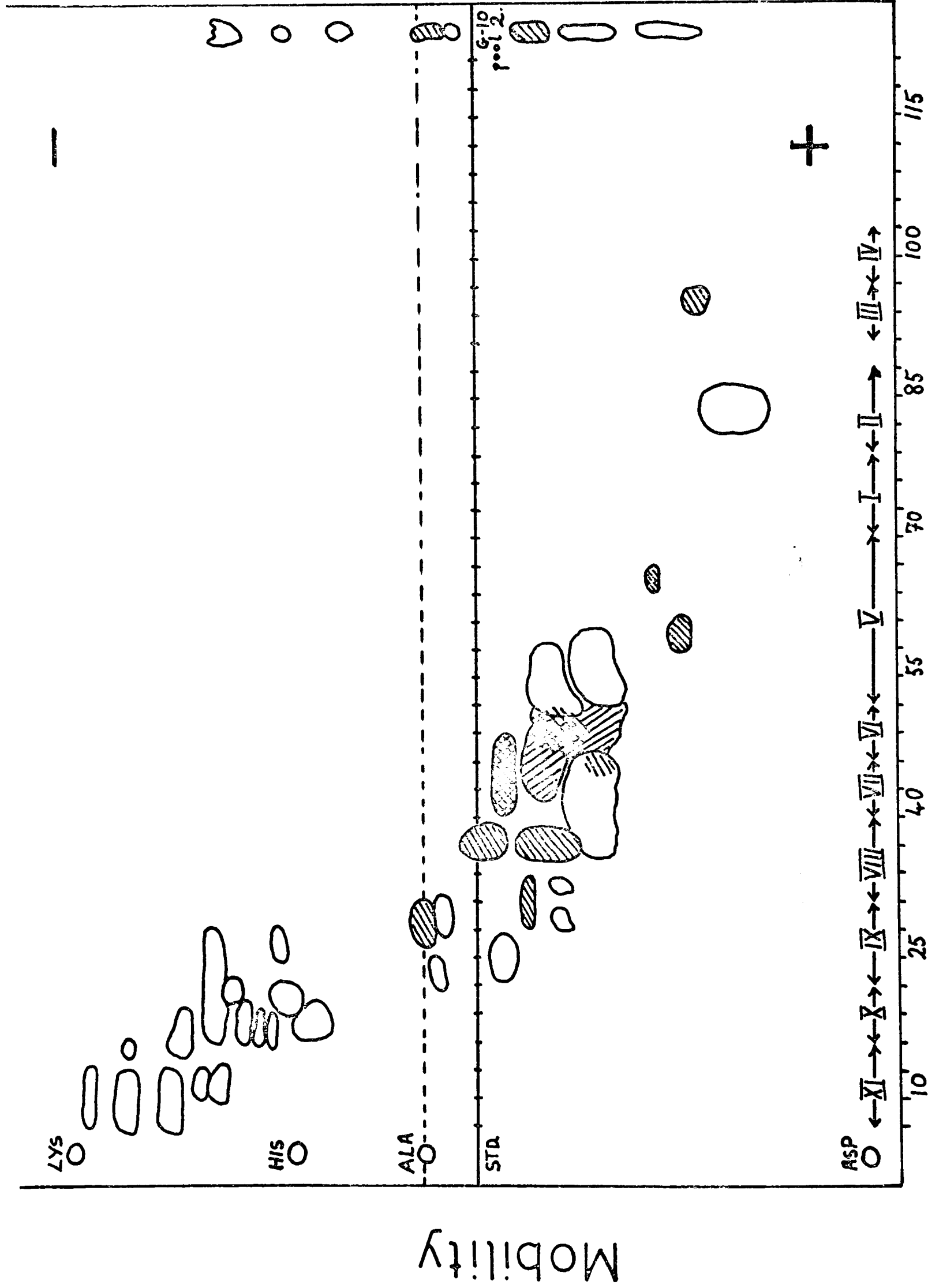


FIGURE 5.5

pH 6.5 electrophoretic analysis of
chymotryptic digest DEAE-cellulose
run.



: cysteine peptides (as shown by autoradiography).



: peptides that stain for tryptophan.

-peptides were pooled as indicated by the roman numerals.

-samples for analysis were taken from every third fraction.

DNS-Arg-Arg

DNS-Arg

SER.

A-1 A-2 A-3 A-4
A-5 A-6 A-7
A-8 A-9 A-10
A-11 A-12 A-13
A-14 A-15 A-16
A-17 A-18 A-19
A-20 A-21 A-22
A-23 A-24 A-25
A-26 A-27 A-28
A-29 A-30 A-31
A-32 A-33 A-34
A-35 A-36 A-37
A-38 A-39 A-40
A-41 A-42 A-43
A-44 A-45 A-46
A-47 A-48 A-49
A-50 A-51 A-52
A-53 A-54 A-55
A-56 A-57 A-58
A-59 A-60 A-61
A-62 A-63 A-64
A-65 A-66 A-67
A-68 A-69 A-70
A-71 A-72 A-73
A-74 A-75 A-76
A-77 A-78 A-79
A-80 A-81 A-82
A-83 A-84 A-85
A-86 A-87 A-88
A-89 A-90 A-91
A-92 A-93 A-94
A-95 A-96 A-97
A-98 A-99 A-100

A-1 A-2 A-3 A-4 A-5 A-6 A-7
A-8 A-9 A-10 A-11 A-12 A-13 A-14
A-15 A-16 A-17 A-18 A-19 A-20 A-21
A-22 A-23 A-24 A-25 A-26 A-27 A-28
A-29 A-30 A-31 A-32 A-33 A-34 A-35
A-36 A-37 A-38 A-39 A-40 A-41 A-42
A-43 A-44 A-45 A-46 A-47 A-48 A-49
A-50 A-51 A-52 A-53 A-54 A-55 A-56
A-57 A-58 A-59 A-60 A-61 A-62 A-63
A-64 A-65 A-66 A-67 A-68 A-69 A-70
A-71 A-72 A-73 A-74 A-75 A-76 A-77
A-78 A-79 A-80 A-81 A-82 A-83 A-84
A-85 A-86 A-87 A-88 A-89 A-90 A-91
A-92 A-93 A-94 A-95 A-96 A-97 A-98
A-99 A-100

1ST DIMENSION

NINHYDRIN-CD STAIN
ARGININE STAIN

FIGURE 5•6

Electrophoretic separation of peptides from peptic

digest of pool II at pH 2•1 and pH 6•5

- cross-hatching indicates peptides that stained
†
after treatment wick chlorine.

TABLE 5.5.

Electrophoretic Properties of Gla-containing peptides.

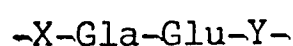
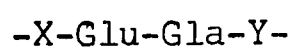
<u>Peptide.</u>	<u>Position in</u> <u>Sequence. *</u>	<u>Mobility at</u> <u>pH6.5.</u>	<u>Charge.</u>	<u>Expected charge</u> <u>if all Gla.</u>	<u>Number of</u> <u>Gla's.</u>	<u>Mobility</u> <u>at pH2.1.</u>
II	1-35	0.75				
II-P-A-1	15-21	1.11	-5	-8	1 (4)**	0.42
II-P-A-2	15-21	1.00	-4	-8	0 (4)	0.68
II-P-A-3-1	30-33	0.93	-3	-4	1 (2)	0.46
II-P-A-3-2	mixture.	0.93	-	-	-	0.39
II-P-A-4	22-32	0.85	-4	-6	1 (3)	0.60
II-P-A-5-1	mixture	0.69	-	-	-	0.79
II-P-A-5-2	31-35	0.69	-2	-2	1 (1)	0.51
II-P-A-6-1	32-34	0.56	-1	-2	0 (1)	0.80
II-P-A-7-1	mixture	0.51	-	-	-	0.82
II-P-A-7-2	1-8	0.51	-2	-3	1 (2)	0.74
II-P-B-1	9-14	-0.56	+2	+2	0 (0)	1.52
II-P-B-2	9-14	-0.51	+2	+2	0 (0)	1.57

* - as indicated by amino acid composition and N-terminal analysis.

** - the figures in brackets are those expected for corresponding peptides from normal prothrombin. Calculated from the data of Magnusson et al (1974b).

resultant peptides were desalted on a column of Sephadex G-10 in 10% acetic acid. Most of the peptides were eluted as a main peak, but a small proportion eluted between this peak and the salt peak. This was pooled separately, while the main peak was resolved into its component peptides by chromatography on DEAE-cellulose. The absorbance scan of this run is shown in Figure 5.4. A much more useful analysis of the run was given by pH6.5 electrophoresis of the fractions, staining with ninhydrin- Cd^{2+} and for tryptophan and arginine; and by autoradiography to detect carboxymethylcysteine containing peptides (Figure 5.5). The fractions were pooled as indicated in Figure 5.5. Amino acid analysis of the pools indicated that pool II consisted of the N-terminal peptide, residues 1-35. This peptide was only obtained in low yield (about 10% or 400nmol) and on isolation was further digested with pepsin. The resultant peptides were purified by preparative paper electrophoresis at pH6.5 and 2.1 (Figure 5.6). These peptides were located in the prothrombin sequence by amino acid and N-terminal analysis (Figure 5.7); this together with electrophoretic data allowed an estimate of the number of Gla residues within each peptide (Table 5.5).

Attempts were made to confirm the distribution of Gla residues by mass spectrometry, a technique which allows direct sequencing of Gla-containing peptides. However the method is by its nature only qualitative and if a mixture of peptides such as:



which cannot be separated by electrophoresis, is analysed on the mass spectrometer the results will only demonstrate the

Figure 5.7

Localisation of isolated peptides from prothrombin-Ba
Fragment 1 in the N-terminal sequence.

-peptides contain Glu or Gla as indicated.

1
Ala- Asn- Gly- Phe- Leu- Glu* -Glu* - Val- Arg- Lys- Gly- Asn- Leu
1 Glu, 1 Glu

15
Glu* Arg- Glu* Cys Leu- Glu/Glu¹²-Glu¹³

(1) no Glu or (2) 1 Glu, 3 Glu

Pro- Cys- Ser Arg- Glu^{*} Glu^w- Ala- Phe- Glu^w-Ala- Leu- Glu/Glu^w-Ser- Leu-

1 Gla, 2 Glu

denotes a Glu residue in normal prothrombin.

Possible sequence of peptide 1-35 from prothrombin- Ba

presence of Glu at both positions. As some of the Glu decarboxylates during analysis it is not practically possible, although perhaps theoretically feasible, to quantitate the Glu content of a particular position in the starting mixed peptides.

In preliminary mass spectrometry on the peptides isolated from prothrombin-Ba Glu was shown to be present at positions 7 and 8. However, as described above, this does not necessarily exclude the presence of Glu at these positions, indeed the electrophoretic data would indicate that in any one peptide only one of these positions is carboxylated. Spectra were also attempted on some of the other peptides but the results were inadequate to assign sequences unambiguously due to the small amounts of peptide available and due to the prolonged storage of these peptides having resulting in some degradation.

(d) Discussion

Physicochemical studies on the various forms of prothrombin show that they are indistinguishable in terms of carbohydrate content, molecular weight by SDS-gel analysis, immunological activity or amino acid content following acid hydrolysis. As all prothrombins could be activated to thrombin with similar clotting and esterase activity to normal thrombin it is obvious that the functional defect in the abnormal proteins lies in the pro portion of the proteins. This is confirmed by the reduced electrophoretic mobility of normal, but not abnormal bovine Fragment 1 in the presence of calcium. Thus abnormal prothrombin has a reduced calcium binding ability due to a defect in the Fragment 1 portion of the molecule.

By using an alkaline hydrolysis procedure it was shown that

the gradation of activity in the various prothrombins corresponded to a gradation in the extent of protein carboxylation. The use of a glycine ethyl ester coupling assay to monitor the extent of gamma-glutamyl carboxylation in proteins was found to be inadequate for this purpose. Alkaline hydrolysis unexpectedly showed that prothrombin-super does contain some Gla, despite the assumption that the abnormal prothrombin isolated by Suttie and Stenflo is completely uncarboxylated; this having only been directly demonstrated for residues 7 and 8, and indirectly for residue 15. It would thus appear that 'coumarin prothrombin' is heterogenous in respect of the degree of carboxylation, although the results could also be interpreted in terms of a mixture of fully carboxylated and uncarboxylated forms of prothrombin. This possibility was excluded by demonstration of full reabsorption of individual prothrombin-Ba fractions to barium citrate and by the isolation of partially carboxylated peptides from prothrombin-Ba. Unfortunately the yields of peptides in the latter experiments were too low to permit deductions to be made as to whether particular glutamic acid residues are preferentially carboxylated or not.

The above studies also showed that all of the Gla of prothrombin-Ba resides in the Fragment 1 region of this protein. The results do not necessarily preclude the possibility of additional defects in the Fragment 2 region of abnormal prothrombin. However this is deemed unlikely in view of Magnusson's report (1974a) of the complete primary structure of normal bovine prothrombin, the only unusual residues in the molecule being the Gla's in the Fragment 1 region. Also as the only demonstrable defects in abnormal prothrombin are due to low calcium binding ability, the abnormality is completely explicable in terms of the Fragment 1 region.

Chapter 6

OTHER VITAMIN K-DEPENDENT PROTEINS

A. FACTOR X_3

a) Introduction

Factor X_3 was first described by Mattock and Esnouf (1973) who showed that during the preparation of Factor X a single chain protein was eluted from DEAE-Sephadex at a similar ionic strength to Factor X_2 . On the basis of molecular weight measurements by SDS-gel analysis and an apparent immunological cross-reactivity with Factor X it was proposed that this species represented a single chain precursor of the two chain Factor X molecule. These studies were carried out on material taken from the trailing edge of the combined Factor X_2/X_3 peak during DEAE-Sephadex chromatography, a process that only partially resolves these two proteins. The proportion of $X_2:X_3$ in this peak varies from preparation to preparation but was usually of the order of 25 to 30% by SDS-gel analysis, initially the only method of recognising Factor X_3 .

As described in Chapter 3, the Factor X DEAE-Sephadex peak disappears during coumarin therapy of cattle, although a small amount of X activity can still be detected in the appropriate fractions. As Factor X_3 elutes at the same position it appeared that this protein was also affected by coumarin therapy and that as a result it was worthy of further characterisation.

b) Methods

Purification of Factor X_3

Preliminary purification of Factor X_3 was carried out using the same procedure as described for normal bovine prothrombin and Factor X (Chapter 3), on 50 l lots of bovine blood. This yields a pool containing about 25% X_3 and 75% X_2 . Attempts at further purification are detailed in the results section.

Preparation of antiserum to Factor X_3

The above pool was rechromatographed in the DEAE-Sephadex pH6:9 citrate buffer system three times, pooling for X_3 each time.

The resultant Factor X_3 was shown to be free of Factor X by SDS-gel analysis and by its lack of activity in the RVV assay. Factor X_3 used as immunogen was taken from the trailing edge of the third rechromatography and antiserum to it produced by multisite intradermal injections of rabbits as described in Chapter 3, except that much smaller amounts of immunogen were used (about 0.1mg of X_3 both for loading and booster doses); this proved to be quite adequate. The antiserum was used to assay for Factor X_3 by the one-dimensional "rocket" technique of Laurell (1966) using 150-250ul of antiserum for each (10 x 20)cm plate; and for the immunological characterisation of the protein by the Ouchterlony method (1958).

Rabbit antiserum to Factor X_1 was produced in a similar manner, using X_1 from the first DEAE-Sephadex run in the above purification as immunogen.

Specific absorption coefficient

Protein, at a concentration of about 5mg/ml was dialysed extensively against 0.1M ammonium bicarbonate (pH8.0) and the optical density of the solution at 280nm determined using a Unicam SP500 ultraviolet spectrophotometer. 0.3ml samples of the dialysed solution were added to small vial of accurately known weight. The solution was dried down at 80°C overnight and then in vacuo over P_2O_5 at 120°C for 24 hours. Experiment showed that this treatment was sufficient to give a constant weight. The vials were then allowed to equilibrate to room temperature for 2 hours in a vacuum dessicator and then weighed on a Mettler M5 microbalance.

These dry weights, corrected for solvent controls, allow the calculation of the original protein concentrations of the dialysed solution and hence the specific absorption coefficient.

Differential refractometry

The interference optics of the analytical ultracentrifuge show concentration in terms of interference fringes. It is therefore convenient to measure the initial concentration of the solution

under study in fringes also. The difference in refractive index between a solution and its solvent is given by:

$$\Delta n = \gamma c$$

where γ , the specific refractive increment, is the proportionality constant between the concentration of the solute, c , in g/ml and Δn the refractive index increment between a solution and its solvent. In the ultracentrifuge cell the difference in refractive index results in a displacement of J fringes according to the equation:

$$J = \frac{a \Delta n}{\lambda}$$

a being the path length of the cell, λ the wavelength of the light used in the interference optics. At 546 nm (the wavelength of maximum transmission for the light filter used in both the ultracentrifuge and the differential refractometer),

$$J = 2.2 \times 10^4 \times \Delta n.$$

Differences in the refractive index between the solution and solvent were measured at 546 nm on a Brice-Phoenix differential refractometer (Phoenix Precision Instrument Co. Ltd., Philadelphia, USA) at room temperature, the instrument being calibrated with standard solutions of KCl (Kruis 1936).

Molecular weight determination by sedimentation equilibrium

Sedimentation equilibrium runs were performed in a Beckmann Model E analytical ultracentrifuge. Initial runs were performed by the method of Chervenka (1970) in which the concentration of solute at the meniscus is set at zero by layering solvent over the protein solution at the start of the run, by use of a synthetic boundary double sector cell. Later runs were performed by the more accurate conventional method of van Holde and Baldwin (1958) using low speed runs with 3mm columns. In either case the weight average molecular weight was calculated from a plot of $\ln c$ versus r^2 , and by use of the equation (van Holde and Baldwin, 1958):

$$M_{app} = \frac{2RT}{(1-\bar{v}_p)w^2} \times \frac{d \ln c}{d r^2}$$

where M_{app} is the weight average molecular weight under the stated conditions, R the gas constant, T the temperature, $\bar{v}$ the partial specific volume of the solute, ρ the density of the solution, ω the angular velocity of the rotor and c the concentration of solute at a radial distance r from the axis of rotation.

Sequence analysis

The N-terminal sequence of Factor X_3 was determined on a Beckmann sequenator (model 890C) on a 100nmole sample of the protein, using a 0.1M Quadrol programme, through the cooperation of Dr. L. Mole of the MRC Immunochemistry Unit, Oxford.

Synthetic PTH-Gla was synthesised from Gla (see Appendix 2) by dissolving the Gla in 0.5ml 1M N-methyl morpholine, followed by the addition of the phenyl isothiocyanate in heptane. After removing the heptane in a stream of nitrogen coupling was carried out at 50°C for 30 minutes, followed by drying the sample under nitrogen flush. The dried sample was washed three times in 0.5mls of benzene, drying under nitrogen each time. Cyclisation was carried out in 1M HCl at 50°C for 10 minutes and the sample dried down as before. For chromatography the PTH-Gla was taken up in 1M NH_3 .

Identification of the PTH-amino acids delivered by the sequenator was carried out by glc on a Hewlett Packard 5830A system, by tlc on polyamide sheets by the method of Summers et al (1973), using the BBOT fluorescent indicator, and by back hydrolysis and amino acid analysis.

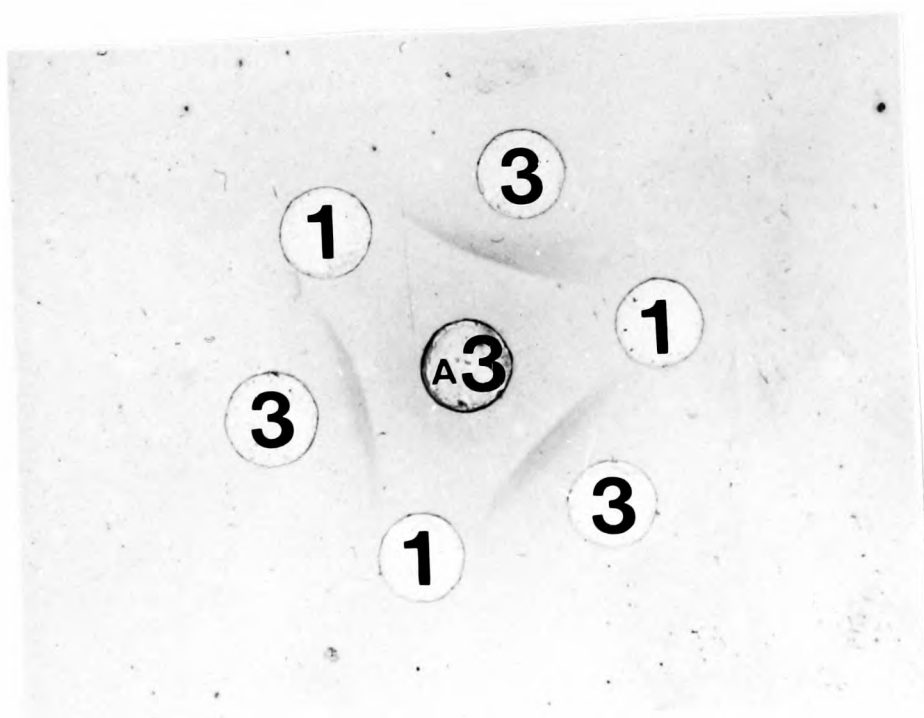
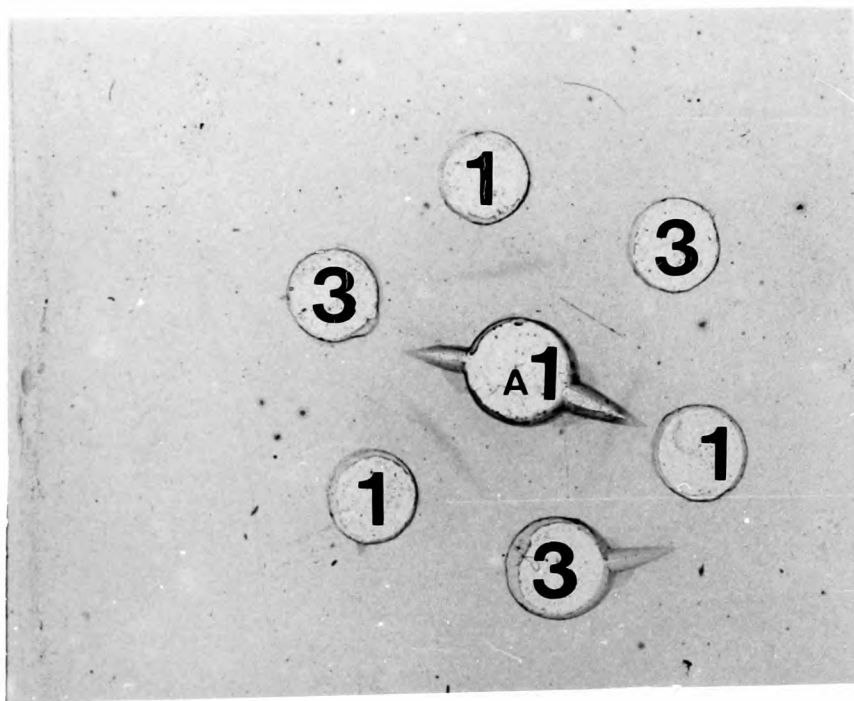
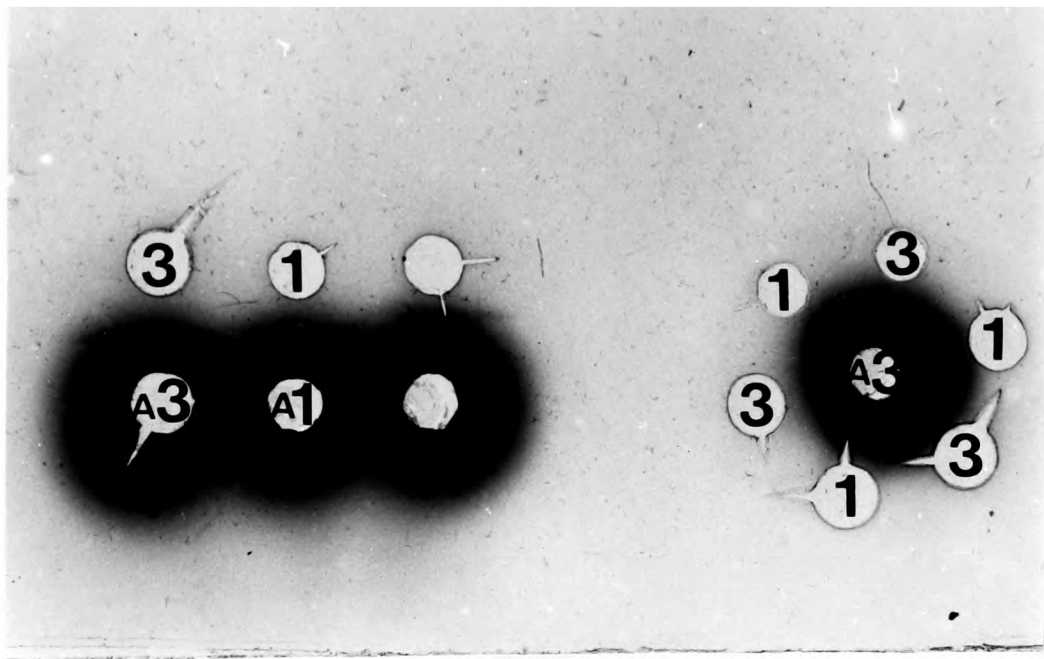


FIGURE 6•1

Immunological non-identity of

Factors X_1 and X_3

1 : wells contain Factor X_1

3 : wells contain Factor X_3

A1 : wells contain antiserum to Factor X_1

A3 : wells contain antiserum to Factor X_3

c) Results

The availability of specific antisera against Factor X_1 and Factor X_3 allowed the immunological characterisation of Factor X_3 in terms of its possible role as a single chain precursor of Factor X. By the use of the Ouchterlony technique it was shown that no cross reaction occurs between X_1 and X_3 (Figure 6.1). This is in contrast with the results of Mattock and Esnouf (1973), the difference presumably being due to the presence of a small amount of Factor X in the X_3 used as immunogen by these authors. Thus Factor X_3 represents a vitamin K dependent protein of unknown function. During this work a similar protein was reported by Stenflo (1976). This protein 'C' is also of unknown function and its calcium binding ability and plasma concentration are affected by the administration of Warfarin. Due to the generosity of Dr. Stenflo we obtained some antiserum to protein C. This antiserum did not precipitate Factor X_3 and in fact no C antigen was demonstrable in any of the fractions obtained from the DEAE-stage of our preparative procedure. These fractions contained Factors II, VII, IX, X and X_3 activity and would be expected to contain the C activity as the preparative method described by Stenflo does not differ greatly from that used here.

Assay by the Laurell technique was utilised in attempts to achieve a better purification method for Factor X_3 as the yield from the recycling ion exchange chromatography, described above, ^{was} gave very low yields. These attempts are summarised in list form:

1. Barium elution

The normal barium elution method gave a poor yield of Factor X_3 (Table 6.1). Increasing the ionic strength of the citrate elution buffer to 0.2M increased this yield to about 80% but this resulted in the formation of a barium precipitate during the dialysis step resulting in clogging of the subsequent DEAE-Sephadex column. This resulted in a very poor yield of prothrombin and made the method unreliable although when it worked the X_3 yield was good.

2. con A-Sepharose

It has been demonstrated in this laboratory that prothrombin will bind to Sepharose bound conconavalin A (Morrison 1973) despite the absence of exposed mannose in this molecule (Nelsestuen and Suttie 1972). On the other hand Factor X will not bind to this affinity matrix and could thus yield a method of separating X from X_3 if the latter protein does bind. However preliminary experiments failed to show any binding of either protein in terms of the Laurell assay or by optical absorbance measurements.

3. Lotus tetragonobulus lectin-Sepharose

Initial analysis of X_3 indicated that it might contain fucose. This would distinguish it from Factor X and as fucose is normally a terminating sugar residue it is usually exposed (Spiro 1970). A survey of fucose-binding lectins showed that the lectin from Lotus tetragonobulus (asparagus pea) was relatively easy to isolate by means of a fucose-Sepharose column. A lectin-Sepharose column specific for fucose was therefore prepared (see Appendix 3) but was found not to bind any Factor X_3 .

4. Reduction and Carboxymethylation

SDS-gel analysis shows that Factors X and X_3 are of similar size but Factor X is composed of two chains. On this basis it might be expected that X and X_3 could be separated after carboxymethylation by gel filtration. Various gel filtration media were tried in this attempt - Ultrogel AcA44, Sephadex G-200 and BioGel P60 together with various solvents - 0.1M NH_4HCO_3 , 0.2M citric acid/NaOH pH6.9, 100mM Tris/HCl pH7.5 with and without 0.5M NaCl and 1mM alpha methyl glucoside (to reduce the interaction between protein and the dextran matrix). In none of these experiments was the resolution of X from X_3 any better than that achieved by the recycling ion exchange method. Attempts to absorb the carboxymethylated Factor X_3 to barium citrate

TABLE 6.1.

Purification of Factor X ₃ .						
Volume	units/ml.	Total units.	Yield.	Absorbance	units/OD	Purification.
	(mls)		(%)	(OD)		
Plasma	24x10 ³	1.2x10 ⁶	100	70	0.7	1
Barium eluate	2200	3.3x10 ⁵	27.5	8.2	18.3	26.1
DEAE-1	533	1.25x10 ⁵	10.4	0.253	929	1327
DEAE-2	44	6.3x10 ⁴	5.2	0.213	6718	9598
DEAE-3	33.5	5.7x10 ⁴	4.7	0.156	10879	15541

(X_2 heavy chain should not absorb as the calcium binding site of Factor X is located in the light chain) were also unsuccessful.

As a sequitur to these experiments gel filtration of the unreduced X_2/X_3 pool was tried on the basis that if X_3 and heavy chain of X have the same apparent size by gel filtration complete Factor X should have a different size despite the SDS-gel results. Again the separation achieved was only minimal.

5. Russell's Viper venom

As Factor X_3 is not affected by RVV in the normal assay for Factor X (see below) the Factor X_2/X_3 pool was digested with RVV as described by Jesty and Esnouf (1973). As this process results in the formation of a large molar excess of Factor Xa, it was felt necessary to inhibit this enzyme before proceeding further so as to prevent non-specific proteolysis. This was done by adding phenyl methyl sulphonyl fluoride (PMSF) to the incubation mixture. This inhibitor is insoluble in water and the addition of the isopropanol, required for PMSF solution, resulted in the precipitation of some of the protein. Following dialysis to remove calcium, as described by Jesty and Esnouf, the precipitate was dissolved up by the addition of GuHCl . As Xa is considerably smaller than Factor X, separation was then attempted by gel filtration on Ultrogel AcA44 without success. Subsequently chromatography on DEAE-Sephadex was tried and resulted in X_3 appearing in the breakthrough of the column, while Xa was bound to the column. The yield by this method was low, perhaps due to the accidental precipitation of some protein or maybe due to the accidental precipitation of some protein or maybe due to the brief exposure of X_3 to a 2 to 3-fold molar excess of Factor Xa. The latter possibility is supported by the altered behaviour of X_3 on DEAE-Sephadex following RVV digestion, it no longer binding to the column.

In the end therefore the procedure of recycling ion exchange chromatography was adopted for the remainder of the work described here. Table 6.1 shows the results of one such

FIGURE 6•2.

SDS-gel analysis of purified Factor X₃.

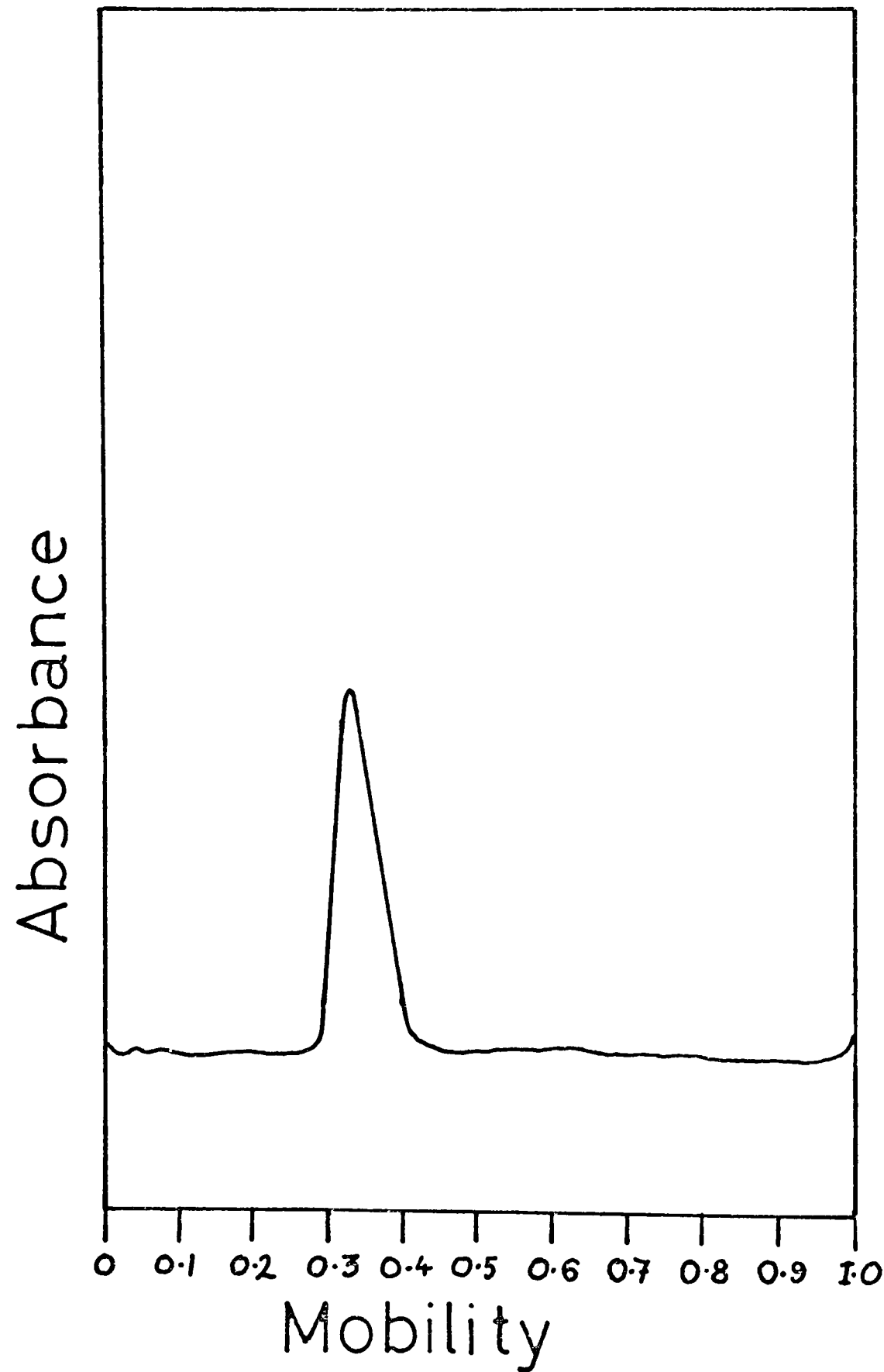
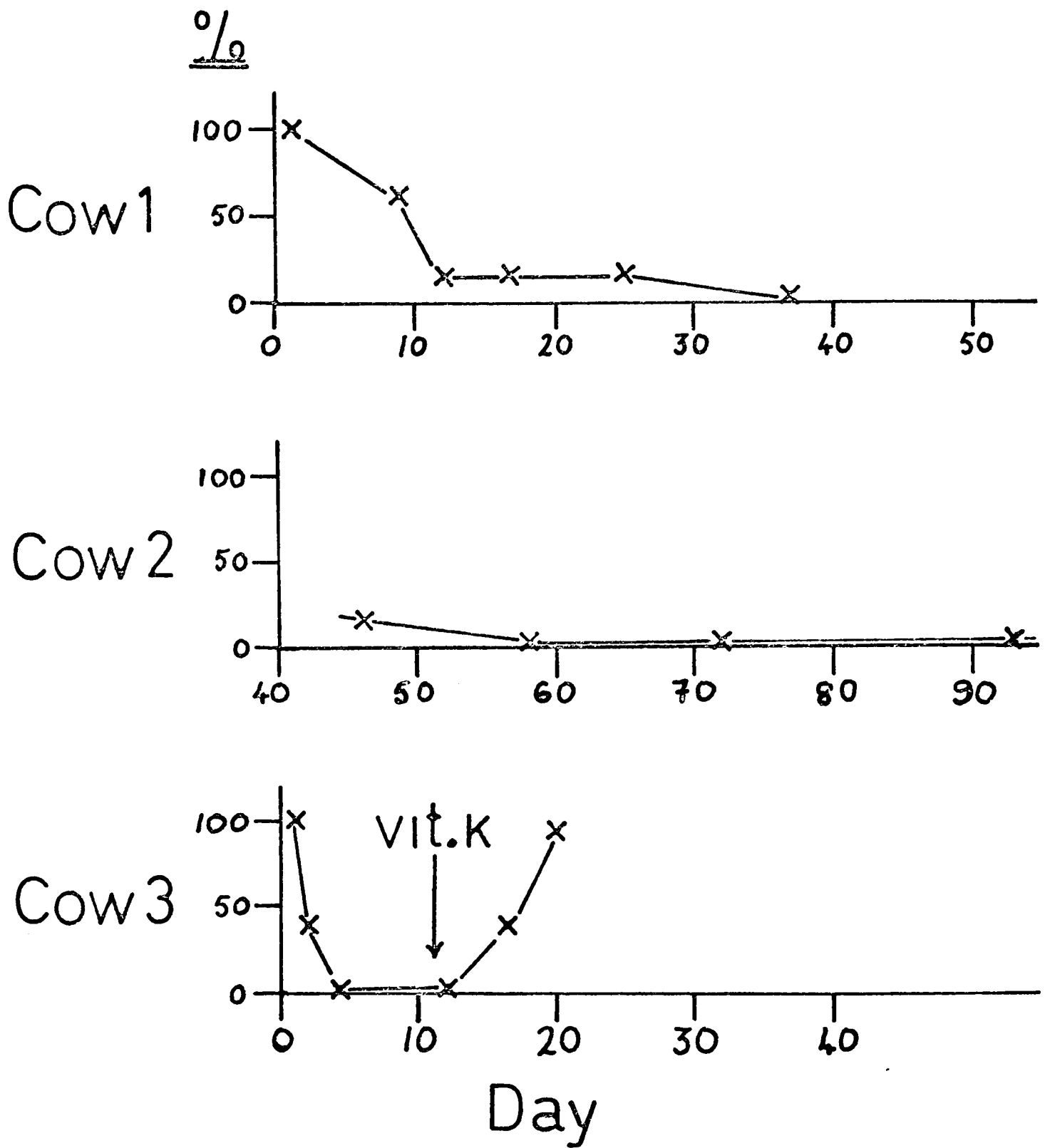


FIGURE 6.3.

Effect of Warfarin on the plasma level of Factor X_3 .



the immunological level of Factor X_3 is expressed in terms of percentage of that in normal plasma.

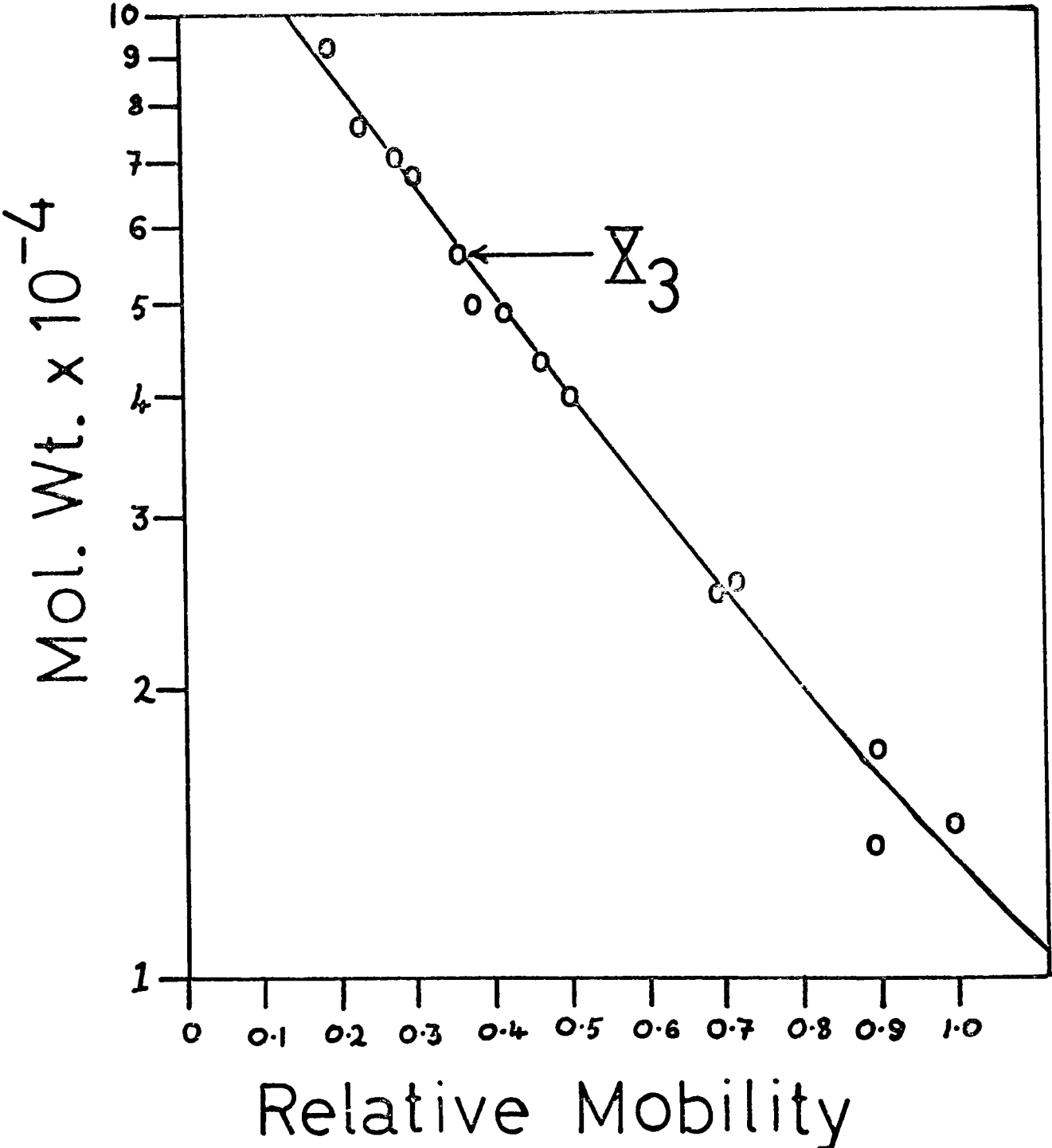
preparation the assay being the electroimmunoassay of Laurell. The standard for this assay was a single fraction from the trailing edge of one of these preparations arbitrarily defined as containing 1000 units of X_3 /ml. Figure 6.2 shows an SDS-gel of this fraction which was one of the purer samples of X_3 obtained. These results also indicate a X_3 plasma concentration of about 10 mg/ml i.e. about the same as the plasma Factor X level.

The effect of Warfarin on plasma X_3 levels was studied on serial samples from the two cows described in Chapter 3 and also in a third cow which was only given Warfarin for 10 days, followed by administration of vitamin K at 10 mg/day (this reduced the level of plasma prothrombin activity to 5% but this quickly returned to normal following vitamin K administration). Assay of plasma X_3 is difficult as it is present at a level only just detectable by the Laurell technique; however the method is sufficient to demonstrate that Warfarin results in a reduced level of plasma Factor X_3 and that administration of vitamin K reverses this effect (Figure 6.3).

Molecular weight determinations were carried out on Factor X_3 by SDS-gel analysis and by sedimentation equilibrium in the analytical ultracentrifuge. Comparison of SDS-gel runs on Factor X_3 with a calibration curve constructed from runs on proteins of known molecular weight (see Chapter 2 and Figure 6.4) showed that the reduced protein has a MW_{app} of 55,000 daltons while the unreduced protein had MW_{app} 56,000. This shows that the protein is composed of a single polypeptide chain, a result confirmed by the finding of only one N-terminal residue, alanine. A sedimentation equilibrium run, according to Chervenka gave a value of MW_{app} of 44,000 daltons in 20mM Tris/HCl pH7.5, 1mM dithiothreitol (Figure 6.5). A second sedimentation equilibrium run was also attempted by a conventional technique. The sample for this run unfortunately contained some aggregated material which persisted as the run was carried out in 0.1M NH_4HCO_3 (the choice of buffer being required by other experiments).

FIGURE 6.4.

Determination of the molecular weight of Factor X_3
by SDS-gel analysis.



The mobility of Factor X_3 is shown on a plot derived from the mobilities of standard proteins as described in chapter 2.

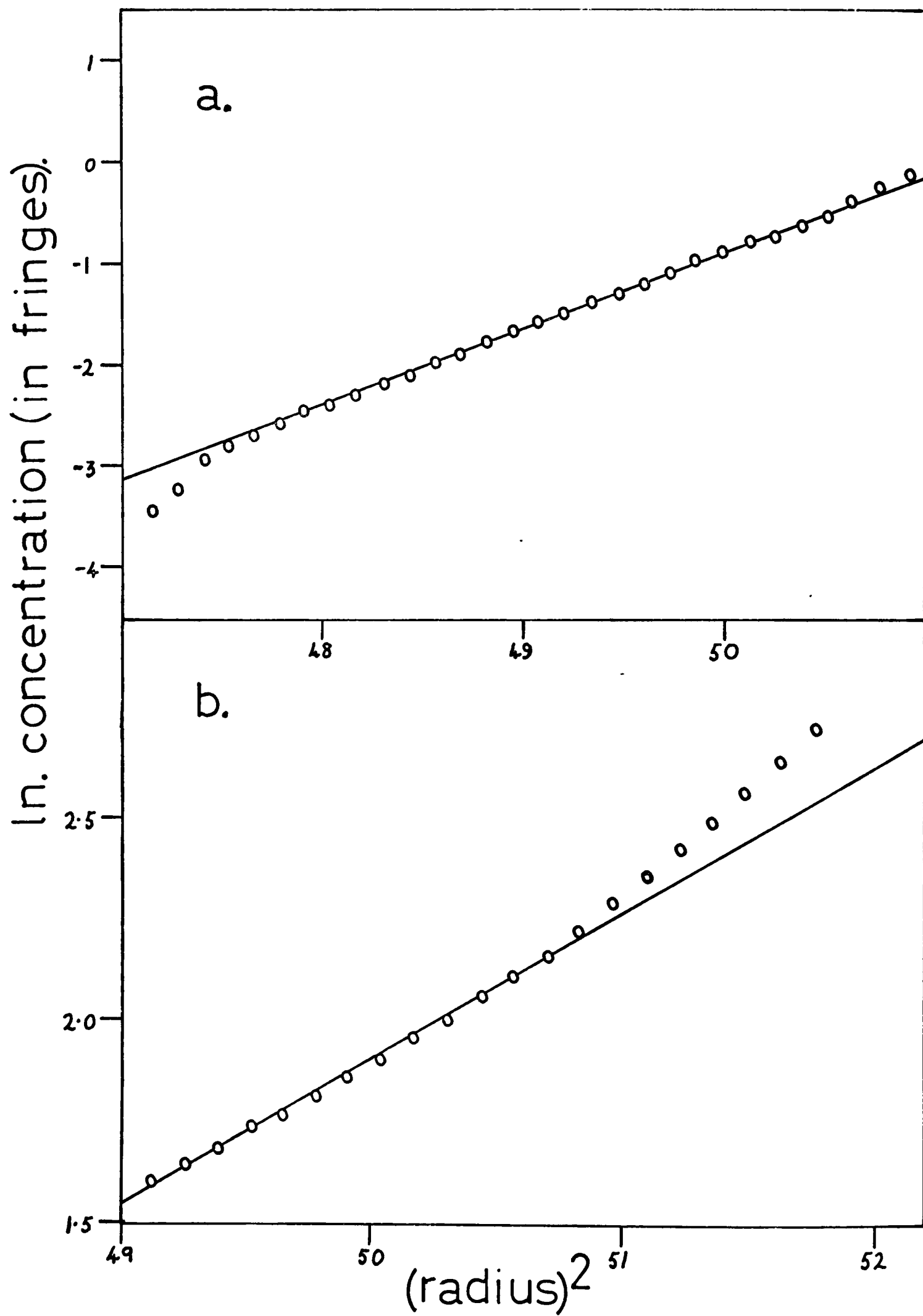


FIGURE 6·5

Determination of Factor X₃ molecular weight
by sedimentation equilibrium methods

- (a) - by the method of Chervenka (1970)
Factor X₃ at 0·15mg/ml in 20mM
Tris/HCl pH7·5, 1mM in
dithiothreitol was sedimented at
16,200 rpm for 20 hours at 20°C.
Apparent molecular weight = 44,000.
- (b) - by the method of van Holde and
Baldwin (1958)
Factor X₃ at 2·4mg/ml in 0·1M
NH₄HCO₃ pH8·1 was sedimented at
11,272 rpm for 18 hours at 20°C.
Apparent molecular weight = 50,000.
- in both cases a partial specific volume
of 0·718ml/g was assumed for the
calculation of apparent molecular
weight.

TABLE 6.2.

Analyses of vitamin B₆-dependant proteins.

	$\bar{X}_3^{***}$	$\bar{X}_1$	$\bar{X}_2^c$	$\bar{X}_4^d$	$\bar{X}_5^e$
LYS	8.2	22.2	9.5	27.6	19.9
HIS	9.3	11.3	8.7	8.1	7.7
ARG	25.1	25.7	25.4	17.0	26.0
ASP	31.2	40.3	28.4	36.3	41.5
THR	22.1	27.7	14.2	20.0	18.9
SER	17.3	30.3	17.0	29.0	28.4
GLU	41.8	54.8	47.5	46.6	52.3
PRO	24.1	20.3	23.5	13.1	22.2
GLY	49.3	41.0	39.8	29.7	43.1
ALA	37.5	32.0	27.7	18.8	23.4
CYS	23.2*	20.4**	28.0**	17.2**	26.4**
VAL	19.2	23.5	25.8	24.9	34.0
MET	1.8	5.6	1.3	2.6	7.5
ILEU	5.1	10.3	7.7	19.3	16.9
LEU	34.3	31.0	33.1	18.7	33.6
TYR	9.3	11.4	7.3	9.2	13.1
PHE	13.9	19.9	16.0	15.2	14.4
TRYP	—	<u>9.6^a</u>	<u>5.0</u>	<u>11.4</u>	<u>13.2</u>
	372.9	437.3	365.9	364.7	442.5
Fucose	(2)	0	—	—	—
Mannose	12.5	3.8	8.4	14.1	32.6
Galactose	8.3	4.6			
Galactosamine	0	2.1	8.3	6.2	16.4
Glucosamine	17.6	6.2			
Sialic Acid	13.0	10.8	7.9	15.6	14.8
GLA	6.3	14 ^b	>2 ^e	>2 ^e	
Free carboxyl	38.4	—	—	—	—

TABLE 6.2 (continued).

	<u>X</u> ₃ ^{***}	<u>X</u> ₄	<u>VII</u> ^c	<u>IX</u> ^d	<u>'C'</u> ^e
Mol. Wt.					
a. ultracentrifuge.					
	>44,000	55,000 ^a	45,500	55,400	-
b. SDS-gels	55,000	63,000	54,000	65,000 ^e	56,000
		(44,000+16,000)			(35,000+21,000)
%protein	79	89	87	74	
%carbohydrate					
	21	11	13	26	assumed 15.
N-terminal	Ala	Ala+Tryp	Ala	Tyr	Ala

*-determined as cysteine.

** - determined as cysteic acid or carboxymethyl cysteine.

***- calculated on basis of Mol. Wt. 50,000.

a.- Fujikawa et al (1972)

b.- Howard and Nelsestuen (1975), but see next section.

c.- Kisiel and Davie (1975).

d.- Fujikawa et al (1973)

e.- Stenflo (1976).

However this run did show that the major component of this sample had an apparent molecular weight of 50,000 daltons. This figure was calculated on the assumption that the partial specific volume was the same as that for Factor X (ie. 0.718) as it was found impossible to obtain a reasonable measurement of v in this buffer in a Paar digital densimeter. Supplementary to these observations of X_3 it was found that the specific absorption coefficient of Factor X_3 , $E_{280}^{1\%} = 11.64$. A value of 50,000 has been assumed for the calculation of the results of chemical analyses. The discrepancy between the results by the sedimentation methods and those obtained by SDS-gel analysis would indicate a fairly large carbohydrate content for this molecule (see Chapter 2).

Chemical analysis of Factor X_3 showed that it contained approximately 21% carbohydrate. Results of chemical analysis are shown in Table 6.2, together with data for the other vitamin K dependent proteins for comparison. Alkaline hydrolysis of the protein showed a Gla content of 6.4, substantiating the vitamin K dependence of Factor X_3 (Gla has only been found in such proteins). This value is not corrected for losses during hydrolysis; if these are similar to those for prothrombin the corrected Gla content would be 7.4 moles/mole of Factor X_3 . Free carboxyl content was also determined but the results are uninterpretable without a knowledge of the amide content of this species. If the above Gla analysis is taken this would indicate that about 40 of the acid residues occur as amides. Other points of interest in these analyses are that X_3 has N-terminal alanine in common with all the other vitamin K dependent proteins except Factor IX and the low content of lysine, valine and isoleucine when compared to the other proteins. Of these X_3 is most similar to Factor VII. It is however easily distinguished from this protein by the chemical analyses, by the lack of VII activity and by an apparently much higher plasma concentration than Factor VII (10 mg/ml as compared to about 1 mg/ml).

As Factor X_3 cochromatographs with Factor X and was initially thought to be a single chain form of Factor X it was tested

for activity and degradation in the presence of RVV/calcium/phospholipid and Factor Xa/calcium/phospholipid (the latter mixture converts Factor X to a smaller beta-X form - Jesty et al 1974, Fujikawa et al 1974). In neither experiment was any Xa activity formed, nor was any degradation found by SDS-gel analysis of the mixtures. The disappearance of the Factor X_3 peak in the activation experiments of Mattock and Esnouf is due to the formation of a calcium/SDS/protein complex. A similar phenomenon is seen for Fragment 1 of prothrombin and appears to be characteristic of certain calcium binding proteins and may be taken as preliminary evidence that Factor X_3 binds calcium, as do the other vitamin K-dependent proteins. The formation of the complex may be prevented by the addition of EDTA to the SDS-gel sample solution.

At the time of this work all known vitamin K dependent proteins were clotting factors. X_3 was therefore tested for clotting factor activity using the methods described by Denson (1972) for Factors II, V, VII, IX, X, XI and XII. These tests were carried out at the Oxford Haemophilia Centre through the kindness of Dr.D. Austen. None of the above activities were detected in the X_3 preparation, although a slight inhibition of the two stage IX assay was observed. X_3 was also assayed for gross effects in the complement system by the sensitised erythrocyte haemolytic assay of Mayer et al (1946). This was also negative, a result confirmed by the full complement activity of barium sulphate absorbed normal plasma (X_3 absorbs to insoluble barium salts).

The results of the attempted sequence analysis of Factor X_3 have been slightly disappointing to date due to the presence of contaminants in the sample despite its apparent purity by SDS-gel analysis. However a certain amount of the N-terminal sequence was deduced from the available results on the basis of the major amino acid obtained by back hydrolysis of the organic layer of the cyclisation mixture. The results shown in Figure 6.6, together with the corresponding region of the other vitamin K-dependent proteins, shows that this protein is not homologous with the others. In addition it was possible to deduce from the

FIGURE 6.6.

N-terminal sequences of vitamin K-dependant proteins.

II	Ala-Asn-Lys-Gly-Phe-Leu-Gla-Gla	-	Val-Arg-Lys-Gly-Asn-Leu
VII	Ala-Asx - Gly-Phe-Leu- ? - ?	-	Leu-Leu - -Pro-Gly-Ser-Leu
IX	Tyr-Asn-Ser-Gly-Lys-Leu-Glx-Glx-Phe	-	Val-Arg - Gly-Asn-Leu
X ₁	Ala-Asn-Ser - Phe-Leu-Glx-Glx	-	Val-Lys-Gln-Gly-Asn-Leu
C	Ala-Asn-Ser - Phe-Leu-Gla-Gla	-	-Leu-Arg-Pro-Gly-Asn-Val
X ₃	Ala-Leu-Thr-Tyr-Leu-Leu *		

* Factor X₃ contains no Gla or glutamic acid residues
in the sequence up to residue 11.

sequenator results that no glutamic acid or Gla residues are present at positions 7, 8, 9, 10 or 11, although the actual residues present at these positions were not assignable due to multiple peaks in the amino acid analysis. Due to the highly acid character of Gla it is possible that PTH-Gla remains in the aqueous phase rather than the organic phase after the cyclisation reaction. To check this back hydrolysis was also performed on this phase and showed that no appreciable amount of amino acid was retained in this phase in any cycle up to residue 11.

d) Discussion

In common with the classical vitamin K-dependent proteins and the newly discovered K-dependent protein C of Stenflo, Factor X_3 is absorbed to insoluble barium salts and DEAE-Sephadex. Antiserum raised against the purified protein was used to confirm that this protein is a constituent of starting plasma and that administration of Warfarin reduces the plasma level of Factor X_3 . The behaviour of X_3 on SDS-gel in the presence of calcium suggests that this protein also binds calcium, as do the other vitamin K dependent proteins.

Further confirmation of the vitamin K dependence of Factor X_3 is given by the demonstration of the presence of Gla in this molecule. This amino acid has only been found in vitamin K dependent proteins; the Gla-containing bone protein (Hauschka et al 1976) has not yet been characterised in this respect.

The evidence presented is sufficient to show that Factor X_3 is distinct from other vitamin K dependent proteins. The single chain protein of molecular weight 50,000 has no clotting or complement activity and does not cross react with antiserum to protein C. Chemical analysis and N-terminal sequencing of this factor also distinguish it from the other K-dependent proteins.

The results of the sequence analysis of X_3 are especially unusual in view of the high degree of homology of the other vitamin K-dependent proteins in the N-terminal region. As Gla is present in the molecule it must occur at other positions. In view of the N-terminal restriction of Gla in the K-dependent proteins so far characterised the most probable

position of carboxylation would be those glutamic acid residues nearest to the N-terminus (these occur after residue 11). If this is so Factor X₃ has extra residues on the N-terminal side of the "Gla region" when compared to the other vitamin K-dependent proteins.

B. FACTORS X_1 and X_2

a) Introduction

Factor X may be separated into two components (X_1 and X_2) by chromatography on DEAE-Sephadex (Jackson and Hanahan 1968, Fujikawa et al 1972, Esnouf et al 1973, present study). Jackson (1972) ascribed this behaviour to small differences in the carbohydrate contents of the two species. However work in this laboratory using the glc method (Mattock- personal communication) and the work of Fujikawa et al (1972) has failed to confirm this observation. On the other hand Fujikawa et al (1972) and Esnouf et al (1973) have shown that there is a difference in the specific activity, as assayed in the RVV system, of Factors X_1 and X_2 ; Factor X_1 having 86% and 76%, respectively, of the activity of Factor X_2 .

The chromatographic behaviour of Factor X on DEAE-Sephadex is most easily explained in terms of Factor X_2 having a more negative charge than Factor X_1 at pH 6.9. Such a difference in charge might be expected to be revealed by electrophoresis on polyacrylamide gels. No such difference is found (Jackson et al 1972, Fujikawa et al 1972, Esnouf et al 1973). However the difference in charge between prothrombin-super and normal prothrombin is not revealed in this system either (Stenflo and Ganrot 1972). This charge difference in prothrombin is due to differences in the Gla content of the two forms of the protein. Recently Gla has also been indirectly identified in the N-terminal portion of the light chain of Factor X (Howard and Nelsestuen 1975). We have undertaken analysis of the Gla content of the light chains of Factors X_1 and X_2 , by the alkaline hydrolysis method, on the basis that the difference in chromatographic behaviour may be due to differing degrees of carboxylation of the two proteins. Part of this work was performed in this laboratory by G. Neal towards part II of his degree in biochemistry.

b) Methods

1. Preparation of Factor X

Factors X_1 and X_2 were prepared as described in Chapter 2, followed by rechromatography ^{on} as a (1.5 x 20)cm DEAE-Sephadex column, eluting with a linear gradient from 200 to 400 mM citric acid/

NaOH pH6·9, 1mM BZ

2. Warfarin/Vitamin K treatment

- (i) Plasma samples were taken from the cow, described in the section on Factor X_3 , which was given Warfarin for 10 days and then vitamin K.
- (ii) Two cows were each given 10mg vitamin K_1 per day intramuscularly, for ten days prior to collection of blood.

3. Preparation of light chains

Route a

Pools of Factor X_1 (40mg) and X_2 (20mg) were each treated as follows: after concentration to 5mg/ml the protein was dialysed overnight into 6M GuHCl, 0·2M Tris/HCl pH8·0. The solution was then made 5% in 2-mercaptoethanol and incubated at 37°C for 1 hour; and the reduced protein then carboxymethylated by addition of iodoacetic acid to 0·1M. The pH was maintained at 8 during carboxymethylation by addition of 1M Tris. After 15 minutes excess iodoacetic acid was neutralised by the addition of a second aliquot of mercaptoethanol. The carboxymethylated heavy and light chains were separated by gel filtration on a (2·5 x 80)cm column of Ultrogel AcA44 in 0·1M NH_4HCO_3 .

Route b

A pool containing both Factors X_1 and X_2 was concentrated to about 60mg/ml (total about 300mg) and made 4M in GuHCl by addition of the solid salt. After incubation at 37°C for 30 minutes a 3-fold molar excess (assuming 20moles cysteine/mole protein) of dithiothreitol was added. The mixed (X_1 and X_2) heavy and light chains were separated on a (2·5 x 100)cm column of Ultrogel AcA44 in 0·1M citric acid/NaOH pH6·9, 1mM dithiothreitol. The mixed (X_1 and X_2) light chains were concentrated and separated on a (1·5 x 20)cm column of DEAE-Sephadex eluting with a linear gradient from 0·2 to 0·6M citric acid/NaOH pH6·9, 1mM dithiothreitol at 40ml/hour (Figure 6·7).

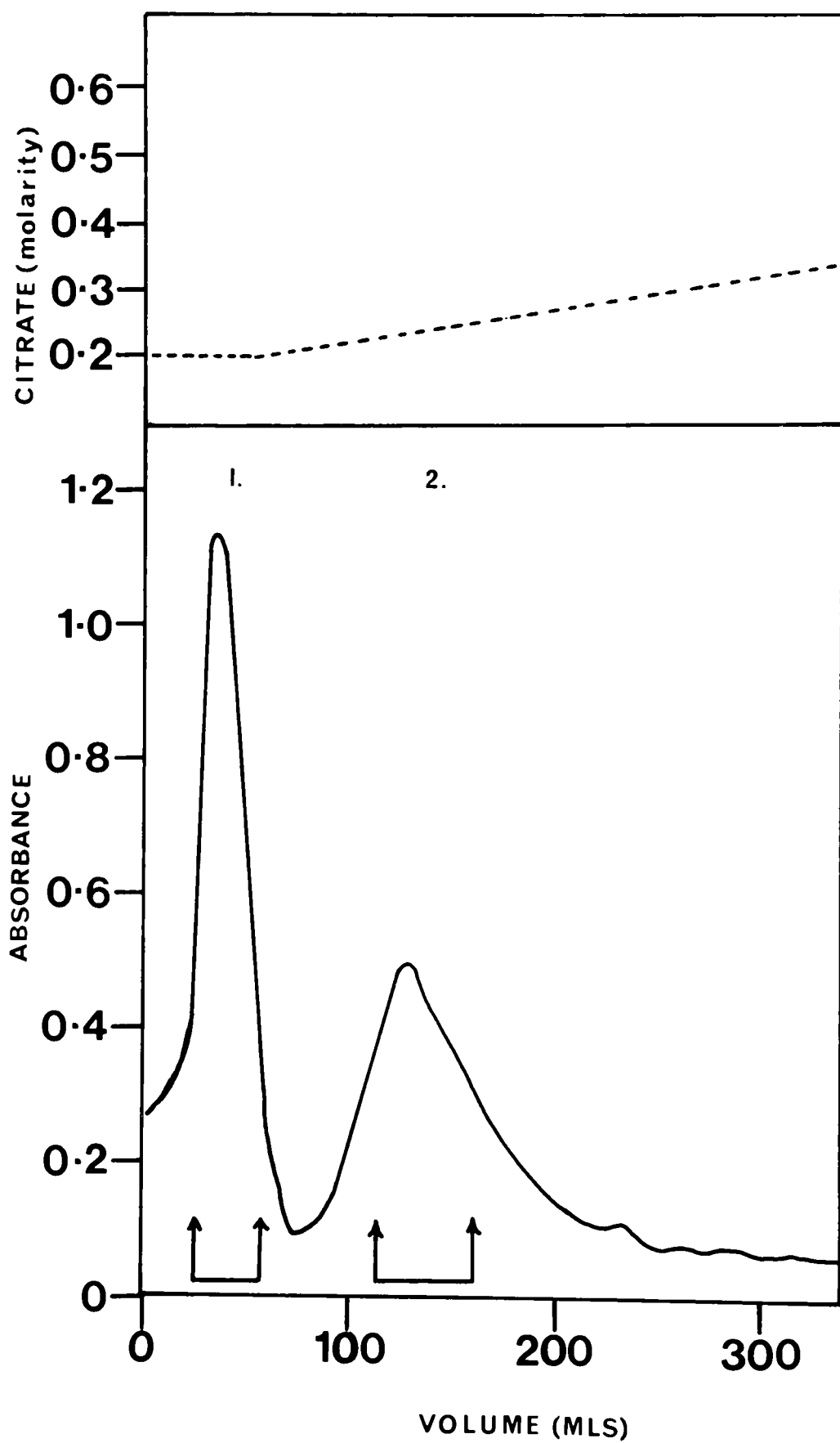


FIGURE 6·7

Separation of the light chains of Factor X_1 and X_2 on a column of DEAE-Sephadex A-50 (1·5 x 20)cm. Protein was eluted at 40ml/hr with a linear gradient from 0·2 to 0·6M citric acid/NaOH pH6·9 containing 1mM dithiothreitol.

c) Results

The light chain of Factors X_1 and X_2 were isolated by two different routes and from two different 50 l preparations:

- (a) X_1 and X_2 were separated on DEAE-Sephadex, carboxymethylated and the light chains isolated by gel filtration.
- (b) A mixed X_1 and X_2 pool was reduced, the mixed light chains recovered by gel filtration and separated by ion exchange chromatography.

The use of two routes of preparation ensured that the techniques employed did not result in decarboxylation of the Gla residues and also allowed demonstration of the separation of X_1 and X_2 light chains by ion-exchange chromatography. Figure 6·7 shows that the light chains are resolved better by this method than are the complete X_1 and X_2 molecules (see Chapter 2). This supports the hypothesis that the differences between X_1 and X_2 reside in the N-terminal portion of the light chain.

Amino acid analysis of the acid hydrolysed light chains revealed no difference in their compositions (Table 6·3) confirming the results of Jackson (1972) and Fujikawa et al (1972). However under alkaline hydrolysis conditions a considerable difference in the Gla content of the two light chains was apparent.

Alkaline hydrolysis results (Table 6·3) show that although the sum of Gla and glutamic acid released by this procedure corresponds to the glutamic acid released by acid hydrolysis in both proteins, the Gla content of X_1 is only about half that of X_2 . The resultant difference of 5 negative charges between the two proteins might well explain the difference in their chromatographic behaviour.

The low degree of carboxylation of Factor X_1 might be ascribed to a vitamin K deficiency resulting in a partially carboxylated protein, as seen in the case of prothrombin-Ba. This possibility was excluded by giving two cows a course of vitamin K treatment prior to preparation of Factor X from their blood. In both cases the Factor X was still recovered in two separate peaks, as in the case of untreated cattle, and the activity of X_1 was found to be 64% that of X_2 in the RVV

TABLE 6.3.

Amino acid composition of light chains of Factor X₁ and X₂.
(moles per 17,000 α).

Acid hydrolysates.

	X ₁	X ₂	X ₁ *
LYS	6.8	6.6	7
HIS	2.9	2.8	3
ARG	7.1	7.0	8
ASP	13.4	13.2	14
THR	5.8	6.2	6
SER	8.6	8.6	11
GLU	23.6	23.7	27
Gly	13.6	14.0	14
ALA	6.3	6.3	6
VAL	4.4	6.1	5
ILEU	1.7	2.9	2
LEU	6.5	7.3	7
TYR	1.9	2.7	3
PHE	6.3	5.4	8

Alkaline hydrolysates.

	<u>route a.</u>	<u>route b.</u>	<u>route a.</u>	<u>route b.</u>
GLU	15.8	14.9	10.9	10.9
GLA	6.8	8.1	12.8	13.3

*- figures of Enfield et al (1975).

assay. This result is confirmed by an experiment in which a cow was given Warfarin for 10 days, followed by vitamin K for the next 10 days. This treatment resulted in a rapid fall in the plasma Factor X activity to about 5% of normal followed by a rapid rise back to 100% on administration of the vitamin K. Preparations of Factor X from plasma taken at intervals throughout this treatment all exhibited two peaks of Factor X, although the total amount of protein was reduced.

d) Discussion

The finding that two different forms of Factor X, with distinct Gla contents and RVV assayable activities, circulate in the plasma is difficult to understand and the possibility cannot be eliminated that the effect is an artefact of the preparation procedure. However the Glu:Gla ratio in X_1 and X_2 and the relative amounts of the two proteins are not altered by rechromatography on DEAE-Sephadex suggesting that if this is so it must take place during the initial stages of purification. As barium citrate absorption was used to purify the Gla-containing peptides of prothrombin (Magnusson et al 1974b, Fernlund et al 1975, Nelsestuen and Suttie 1973) it is unlikely that decarboxylation of Gla takes place at this stage; this leaves only the actual collection of the blood as a possible source of any artefact. This also seems very unlikely in view of the prothrombin results and we are forced to conclude that X_1 and X_2 exist as circulating proteins in vivo.

The 13 Gla residues found in Factor X_2 in the present study must be compared with the 11 or so suggested for X_1 and X_2 by Enfield et al (1975) on the basis of homology with prothrombin and poor yields at certain steps in sequence analysis; and with the 14 proposed by Howard and Nelsestuen (1975) for a barium-absorbable peptide obtained by tryptic digestion of a mixed Factor X_1 and X_2 pool. The latter conclusion is based on analyses using the diborane and glycine ethyl ester assays (see Chapter 5). The alkaline hydrolysis technique provides a much more easily interpretable result than either of the above methods as it yields Gla directly in yields of about 90% (on the basis of the prothrombin results).

In view of the finding that partially carboxylated prothrombin has a low biological activity due to its reduced calcium (and calcium mediated phospholipid) binding ability it is not unreasonable to ascribe the differences in activity of Factor X_1 and X_2 , in the RVV assay system, to a similar effect, resulting from the lower extent of carboxylation of the Factor X_1 . Such a deficiency should also result in a low activity of the X_1 in other systems requiring its calcium binding activity e.g. its activation by physiological activators such as the IXa/VIII/calcium/phospholipid complex.

CHAPTER 7

GENERAL DISCUSSION

a) Introduction

It is now commonly accepted that during the administration of coumarin drugs to cattle and man the level of prothrombin activity in the plasma is reduced disproportionately in relation to the prothrombin antigen. The low activity of such abnormal prothrombin results from a slow rate of activation rather than from the formation of low activity thrombin. This has been explained in terms of a failure to carboxylate certain glutamic acid residues in the N-terminal portion of the abnormal prothrombin which reduces the ability of the protein to bind calcium ions.

Other groups working in this field have characterised an abnormal bovine prothrombin that does not bind to insoluble barium salts and has negligible biological activity, corresponding to the prothrombin-super of the present study, it being suggested that the loss of activity is due to all ten specific glutamic acids being uncarboxylated. The barium-absorbable bovine coumarin prothrombin described in this work has properties intermediate between the former protein and normal prothrombin. Thus 'prothrombin-Ba' has a reduced ability to bind calcium ions, has significant physiological activity and does bind to insoluble barium salts. Prothrombin-Ba consists of a range of molecular species which may be partially resolved by chromatography on DEAE-Sephadex. On this evidence it has been proposed that the result of coumarin administration is the production of partially carboxylated prothrombins. This range of molecular species will not be obvious when the coumarin is given at doses high enough to completely block carboxylation.

Parallel studies on human prothrombin indicate a similar condition during prophylactic coumarin therapy. These conclusions are not applicable to all species as it has been shown that no abnormal prothrombin is released into the plasma of Warfarin treated rats, rather it accumulates in the liver (Carlisle et al 1975). Thus in proposing a general mechanism for vitamin K action the effect of the extent of carboxylation on the release of prothrombin (and other proteins) should be taken into account. The model that we propose would indicate a more generalised effect of the coumarins and vitamin

K on protein synthesis than has generally been supposed. This is supported by the recent discovery of vitamin K-dependent proteins that are not involved in blood clotting.

b) Evidence for a range of abnormal prothrombins

In 1963 Hemker and his colleagues found, using coagulation tests, that plasma from patients on coumarin therapy contained an inhibitor of prothrombin activation, PIVKA-II. They proposed that the inhibitor might be a precursor of normal prothrombin. This early hypothesis was apparently confirmed by the demonstration of abnormal prothrombin in coumarin plasma. The more recent demonstration that purified barium non-absorbable prothrombin has no effect on the assay of normal prothrombin (Stenflo 1972a, present study) makes Hemker's conclusion untenable, moreover as the addition of normal prothrombin to prothrombin-super promotes the formation of thrombin from the latter species (Figure 4.2C) the inhibition effect is even more difficult to explain.

In cattle the immunological level of prothrombin is not appreciably changed during Warfarin treatment, in agreement with the results of Stenflo (1968) and Malhotra (1972). Morrison and Esnouf (1973) and Brozovic and Gurd (1973) have reached a similar conclusion with regard to human coumarin plasma although Josso et al (1968) and Denson (1971) found prothrombin antigen levels only 60% of normal; in all cases the coagulant activity was considerably depressed. In the present study an intermediate value for the level of prothrombin antigen in human coumarin plasma was found. These differences may reflect the various methods of immunoassay used by different investigators but can also be explained in terms of the varying types and extents of coumarin treatment used at different centres.

During the Warfarin treatment of cattle a low coagulant activity prothrombin can be recovered from both barium-absorbable and non-absorbable fractions of plasma; the former may be further fractionated by chromatography on DEAE-Sephadex. This suggested that a range of abnormal prothrombins was produced under a low dose coumarine regime. The finding that purified prothrombin-Ba is

absorbed to barium citrate eliminated the possibility that the prothrombin-Ba was a mixture of normal prothrombin and prothrombin-super. The direct demonstration of partially carboxylated prothrombin confirmed this conclusion.

Malhotra's observations (1972) that coumarin prothrombin may be fractionated into three or more types by sequential absorption to various inorganic salts supports the existence of such a range of molecules and moreover the more abnormal forms of prothrombin were found to have a higher isoelectric point than normal indicating these species are less negatively charged than the normal protein.

Barium-absorbable prothrombin was also demonstrated in human coumarin plasma, in agreement with Morrison and Esnouf (1973), where it accounts for the majority of prothrombin. This prothrombin-Ba may correspond to the 'coumarin prothrombin' isolated by Cesbron et al (1973) who carried out their preparation by absorption to barium sulphate after a preliminary absorption to remove 'normal prothrombin'. In both this study and that of Cesbron et al it was found that the normal proteins were eluted from DEAE ion exchange columns prior to the normal prothrombin suggesting that, in this case also, the normal protein is more negatively charged.

c) Nature of the defect in abnormal prothrombins

Any defect in the C-terminal, thrombin portion of coumarin prothrombin has no effect on the activity of the thrombin formed from this species. It is assumed in this discussion that the only defect exists in the N-terminal 'pro portion' of the molecule.

In 1973 one of the major contenders for the 'prosthetic' group of normal prothrombin was the carbohydrate portion of the molecule (Pereira and Couri 1971, Morrison and Esnouf 1973). Careful analysis of this portion of the molecule from normal and abnormal prothrombins revealed no significant differences between the various forms, even when a number of different methods were used to determine the amount of protein present. In this context it should be noted that the human coumarin prothrombin samples often gave a high degree of light scattering. Two other groups have also failed to find

any difference in the carbohydrate content of normal and abnormal (bovine) prothrombin - Stenflo (1972), Nelsestuen and Suttie (1971 and 1972b - see also Henricksen et al 1976).

The reduced calcium binding ability of abnormal prothrombin, together with its early elution from anion exchange columns (Cesbron et al 1973, present study) and the higher isoelectric point of the more abnormal proteins (Malhotra 1972) suggested that the 'prosthetic group' is an anionic group responsible for calcium binding. The continued work of Stenflo's and Suttie's groups located the calcium (and barium) binding site in small acidic peptides, since located in the N-terminal portion of Fragment 1 (Nelsestuen and Suttie 1973, Stenflo 1973, 1974). Magnusson's work on the sequence of prothrombin has revealed the presence of unusually acidic peptides in this portion of the molecule (Magnusson 1972). Concurrently Connor-Johnson's group demonstrated that labelled bicarbonate could be introduced into glutamic acid residues of prothrombin during its synthesis by an in vitro system (Girardot et al 1974). This experiment has been criticised on the grounds of the low level of prothrombin labelling and also the unexpected release of all the label from the glutamic acid on acid hydrolysis. The experiment has since been repeated under more rigorous control conditions such that a higher specificity of labelling was achieved and only the expected half of the label released by acid hydrolysis, the original conclusions were confirmed (Esmon et al 1975c). These various leads came together in the proposal that gamma-carboxy glutamic acid (Gla) is the acid 'prosthetic group' in normal prothrombin. The unambiguous assignment of this residue depends on the use of mass spectrometry (Magnusson et al 1974, Stenflo et al 1974, Nelsestuen et al 1974), although more or less indirect methods have now been designed for the assay of this novel amino acid.

Mass spectrometric demonstration of the number and location of all ten Gla residues has only recently been published (Morris et al 1976). Using conventional peptide chemistry techniques we have demonstrated the existence of a partially carboxylated form of bovine prothrombin. Isolation of the analogous protein from human coumarin plasma suggests that

partially carboxylated prothrombin is the major component of plasma during coumarin therapy. The results also show that the barium non-absorbable bovine coumarin prothrombin contains some Gla. This result is contrary to the general assumption that this protein is totally uncarboxylated, although the presence of glutamic acid instead of Gla has only been unequivocally demonstrated at residues 7, 8 and 15 of this prothrombin (Stenflo et al 1974).

Reduction in the amount of vitamin K thus results in incomplete carboxylation of prothrombin. If this effect is progressive then a range of partially carboxylated molecules will exist at all states of vitamin K availability. At any given vitamin K status there will be, therefore, a distribution of prothrombin in the various carboxylation states about a frequency mode determined by that status. This distribution will be skewed towards all glutamic acid residues in vitamin K deficiency or to all Gla when there is an adequate amount of the vitamin. As there are 10 sites of carboxylation there are a possible 11 different extents of carboxylation although the number of distinct sequences is 2^{10} . It is to be expected that certain of these sequences will occur more frequently. The characterisation of any such preferred sites would require knowledge of the distribution of the Gla residues in prothrombin under a variety of different coumarin dosages, with concomitant analysis of the concentration of reduced vitamin K in the liver (see below).

That normal Factor X exists in two forms with differing Gla contents shows that, in addition to the above vitamin K-dependent carboxylation, a second carboxylation limiting mechanism must exist. This may or may not correspond to the method whereby carboxylation is restricted to the N-terminal portion of prothrombin.

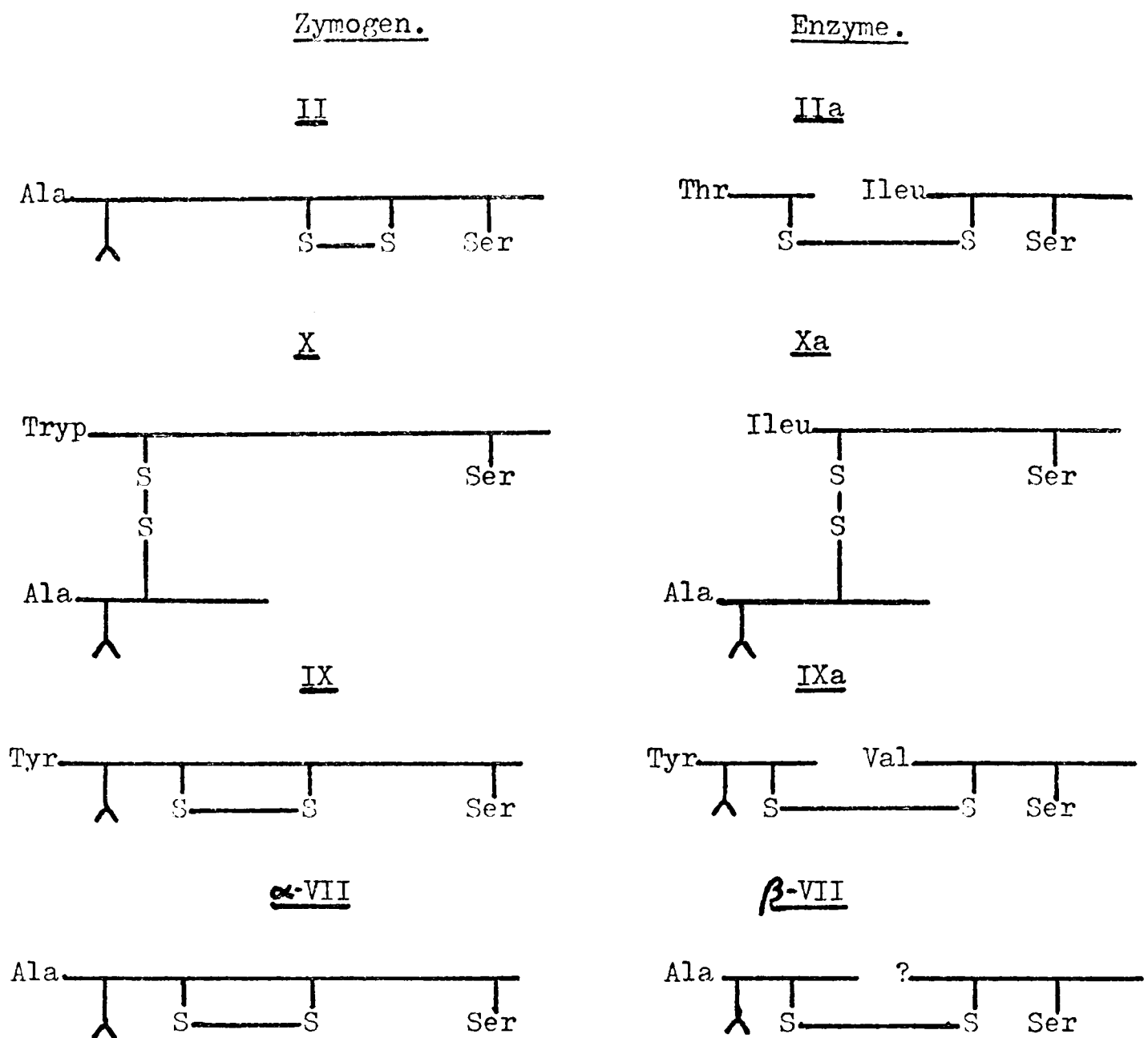
It should be pointed out that the mass spectral and chemical analyses do not preclude the existence of derivatives of Gla e.g. amides, esters, in prothrombin. However this is unlikely in view of the electrophoretic mobility of Gla-containing peptides obtained from digests of native prothrombin (Magnusson et al 1974b, present study) unless the acid prosthetic group is considerably larger and more charged than has been proposed.

d) The effects of carboxylation

In the present study it is shown that the failure to carboxylate prothrombin results in the loss of the ability to bind calcium ions by the Fragment 1 region of the molecule, the effect of which is to reduce the rate at which the protein is activated. More detailed work on these effects may be summarised:

- (1) Abnormal prothrombin has a reduced ability to bind calcium ions (Nelsestuen and Suttie 1972c, Stenflo 1973, present study).
- (2) This loss of calcium binding results in a loss of the calcium-mediated phospholipid binding of prothrombin (Esmon et al 1975b).
- (3) Scatchard plots of the binding of calcium by prothrombin are non-linear. This can be interpreted in terms of cooperative binding of the calcium (Nelsestuen et al 1975) or of the existence of non-identical binding sites (Benson and Hanahan 1975). This effect requires a larger portion of the molecule than the Gla-region alone (i.e. residues 1-35) as this portion of the molecule has different binding characteristics from the whole molecule (Nelsestuen et al 1975).
- (4) The binding of the Gla region to insoluble barium salts may be inhibited by blocking the carboxyl groups of the peptide with glycine ethyl ester (Howard and Nelsestuen 1974).
- (5) Optical rotatory dispersion and circular dichroism studies on normal prothrombin show that calcium binding results in a conformational change in the molecule. In the case of the barium non-absorbable bovine protein this effect is not seen, the spectra in this case corresponding to the calcium-free normal C.D. spectrum (Bjork and Stenflo 1973, Gabriel et al 1976).

FIGURE 7-1.



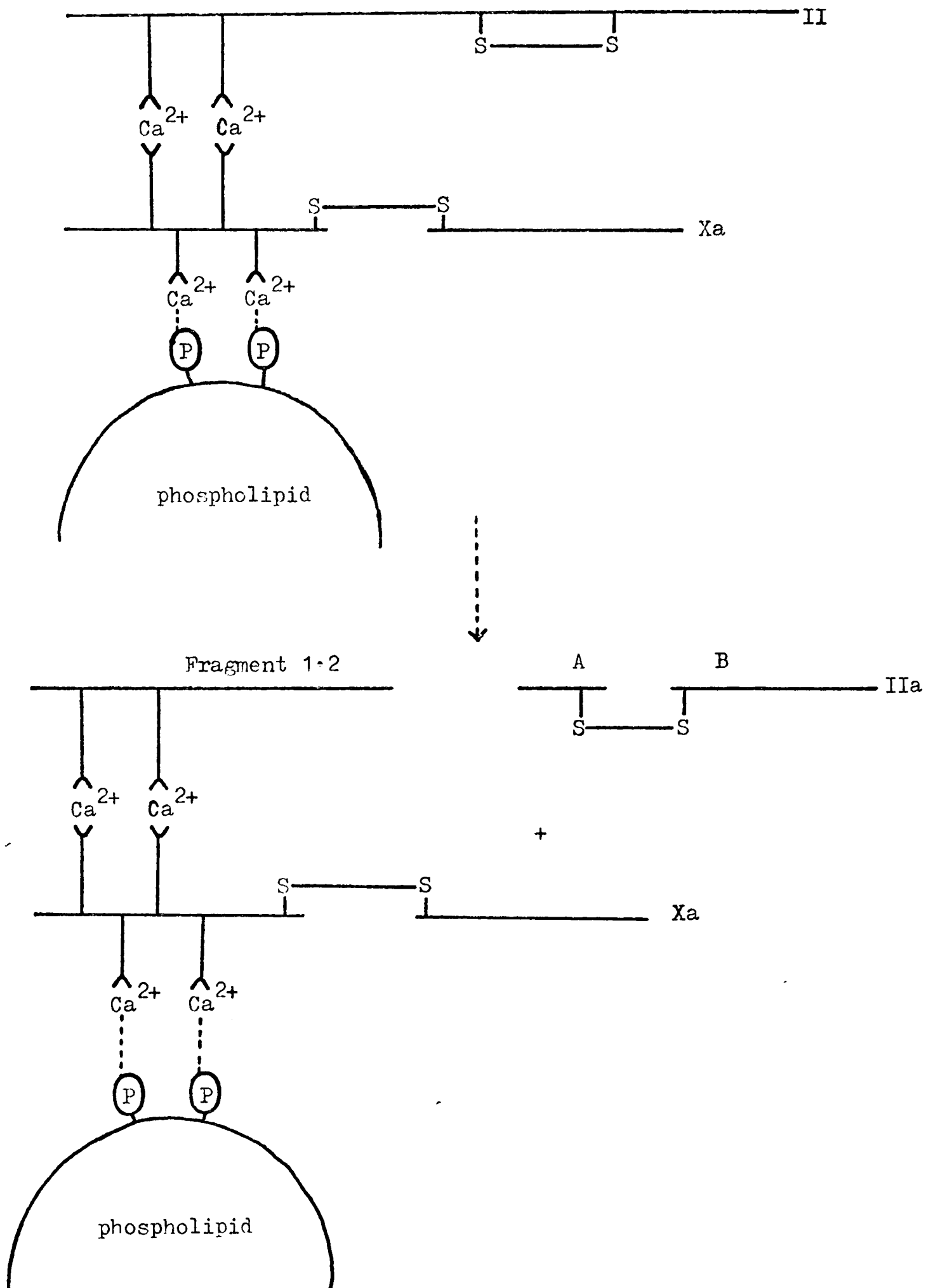
Line diagrams of vitamin K-dependant zymogens
and their enzymes.

- represents the Gla residues.
- N-terminal residues, active site serine residue and interchain disulphide bonds are shown.

The results of the present work show that prothrombin-super, with an average of 4 Gla, has a reduced ability to bind both calcium and barium, while prothrombin-Ba, with 6.6 Gla, retains barium but not calcium binding activity. Thus as the extent of carboxylation is decreased calcium binding, and the associated physiological activity, is quickly lost whereas barium binding ability is retained at lower degrees of carboxylation. Inspection of the Gla region sequence (Figure 5.2) shows that the Gla residues occur in three pairs - residues 7/8, 20/21 and 26/27, the other four residues being more widely spaced, occurring as residues 15, 17, 30 and 33. If the binding of calcium ions is dependent on these Gla pairs loss of the carboxyl groups from three residues e.g. 7, 20 and 26, would abolish the calcium binding ability of the prothrombin. Such a situation is seen in the case of prothrombin-Ba, which does however bind barium. In this model, therefore, the carboxylation requirement for barium binding must be less stringent than three Gla pairs.

The homologies between the N-terminal regions of the various vitamin K-dependent proteins, discussed in Chapter 6, indicate that this region may be responsible for the calcium binding ability of all these molecules. As they all require calcium and phospholipid either for their action or for their activation we can propose that the Gla region performs a specific function in mediating the binding of the vitamin K-dependent proteins to phospholipid surfaces, thereby permitting localisation and concentration of the components of the clotting cascade. Note that prothrombin is the only one of these proteins in which the Gla region is separated from the enzymic portion of the molecule upon activation. This allows the end product of the cascade, thrombin, to be released into the aqueous phase, while the other activated clotting factors remain associated with the phospholipid surface (Figure 7.1). According to this scheme the entire clotting cascade is located on the phospholipid surface via calcium/Gla interactions. The accelerating factors, V and VIII, will also be localised due to their binding to the enzymic factors Xa and IXa respectively.

FIGURE 7•2.



Possible mechanism of product inhibition of the prothrombinase complex.

It is also possible that protein/calcium/protein complexing takes place via the Gla regions of two of these factors and a model for the Factor Xa/calcium/phospholipid/prothrombin complex may be proposed on this basis - Figure 7·2. A mechanism of this kind would also explain the calcium-dependent Fragment 1 inhibition of Factor Xa more easily than one in which prothrombin is bound directly to the phospholipid surface. The demonstration of such a protein/protein complex requires a study of the binding between Factor Xa and prothrombin, with and without calcium present. Such a study necessitates the complete inhibition of the Factor Xa, a protein which is particularly resistant to the usual active site inhibitors (Jackson and Hanahan 1968, Leveson and Esnouf 1969).

Such a model would also explain the anomalously low activity of partially carboxylated prothrombin (5% activity at 66% carboxylation). As partial carboxylation has a disproportionate effect on the calcium binding of the molecule a similar relationship will hold for the calcium mediated protein/phospholipid and protein/protein interactions, with a resultant disproportionate loss of prothrombinase binding by the abnormal protein. This in its turn will make the abnormal prothrombin more susceptible to the 'thrombin-mediated feedback control' of prothrombin activation, whereby the prothrombin is cleaved to Intermediate 1, as the coumarin prothrombin will not absorb to the prothrombinase complex normally and will be exposed to the thrombin in the aqueous phase. All effects will combine in reducing the rate at which abnormal prothrombin can be activated in the normal manner.

As a footnote Nelsestuen et al (1975) have shown that S-100 protein of brain, which binds phospholipid in the presence of calcium, contains no Gla. Thus the formation of calcium-mediated phospholipid binding sites is not exclusive to vitamin K-dependent carboxylation of glutamic acid residues.

e) Determinants of the carboxylation site

Sequence studies on the vitamin K-dependent proteins have revealed extensive homology between the Gla regions of these molecules. As free glutamic acid occurs in other areas of these

molecules, carboxylation of the N-terminal Gla residues must be specified in some way. The possibilities for restricting the carboxylation mechanism appear to be:

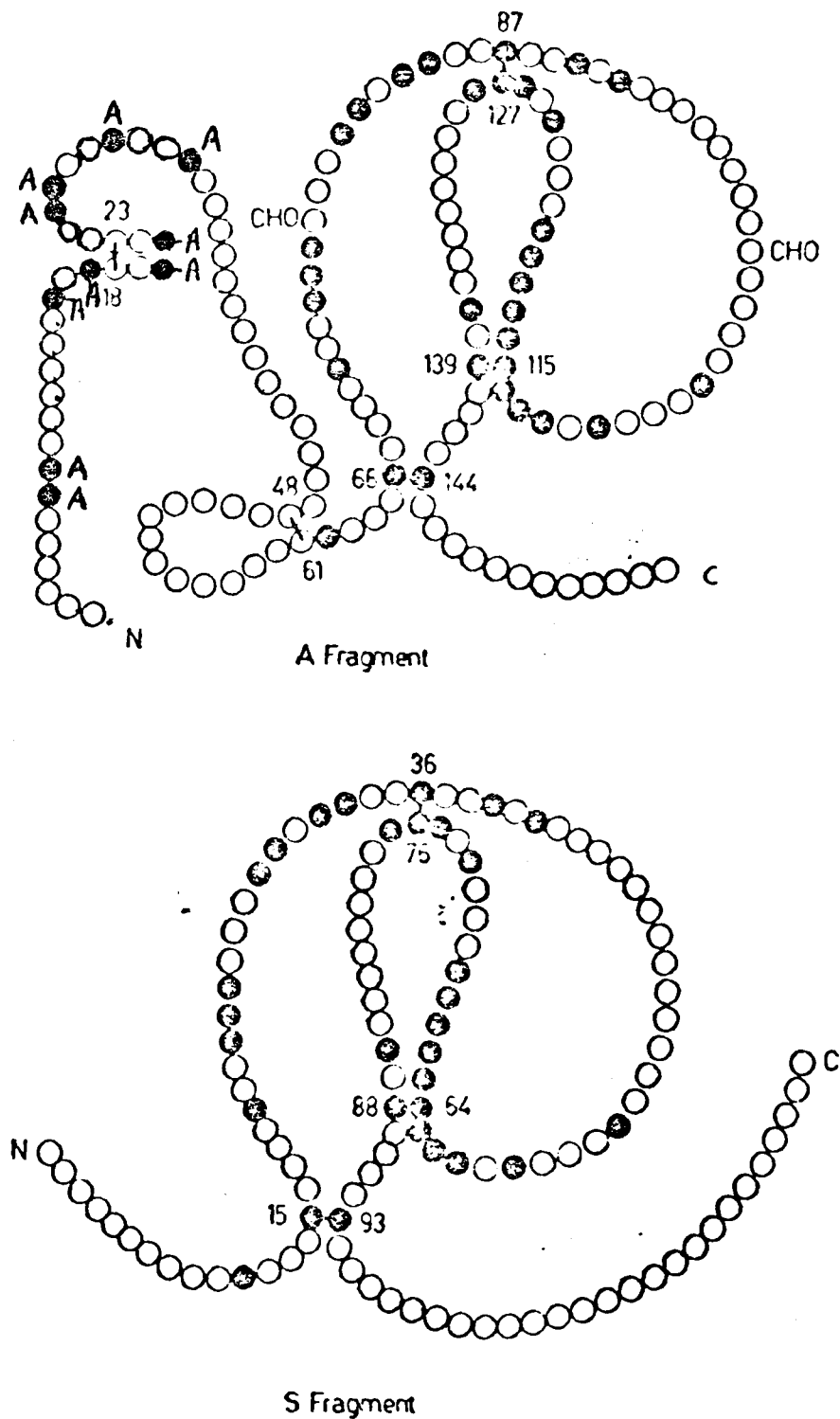
- (1) Carboxylation proceeds until a specific rejection sequence is reached which results in the release of the carboxylation enzyme. Alternatively a specific acceptor sequence for the enzyme may occur at each carboxylation site. Either of these mechanisms could be due to a particular spacing of the potential Gla's.
- (2) The carboxylation enzyme acts in a concerted fashion on the whole of the Gla region. This would require a specific binding of the whole N-terminal portion of the vitamin K-dependent proteins.
- (3) The conformation of the vitamin K-dependent proteins is such that carboxylation proceeds sequentially but is sterically limited to the prescribed positions. This implies that the tertiary structure of the protein is formed prior to carboxylation.

The second proposal is unlikely as, despite the homologies in the various Gla regions, the number of Gla residues per molecule varies from a minimum of about 7 in Factor X_3 to 14 in X_2 (no figures available for Factors VII, IX and 'C'). This mechanism is also insupportable on the grounds that it requires an enzyme with a much larger binding site than any proposed to date.

In view of the sequence homologies between the C-terminal enzymic portions of the vitamin K-dependent zymogens and the pancreatic serine proteases (Fujikawa et al 1974, Enfield et al 1974, Titani et al 1975) it is probable that these portions of the zymogens have essentially the same tertiary structure, corresponding to that of chymotrypsin. If this conformational homology could also be shown for the pro portions of the vitamin K dependent proteins, responsible

FIGURE 7.3.

The kringle structure.



- from Magnusson et al (1974a)

- each circle represents an amino acid residue. In the terminology of these authors Fragment 1 is known as A Fragment (A for N-terminal alanine) and Fragment 2 as S Fragment (N-terminal serine). Filled circles indicate sites of homology between the two prothrombin kringles. Filled circles marked A indicate Glu residues present in the A Fragment. Kringle structures have also been found in plasminogen (Sottrup-Jensen et al 1975).

for the calcium-mediated phospholipid binding and accelerator factor binding of these molecules, the third proposal of steric hindrance might be reasonable. Inspection of the known sequences of vitamin K-dependent proteins, those of Factor X_1 and prothrombin, shows that such homology is unlikely. The sequencing of prothrombin (Magnusson et al 1975) has shown that the so-called 'kringle' structures account for most of the proportion of prothrombin (two kringles occur in prothrombin) and are thus the major conformational determinant of this portion of the molecule (see Figure 7·3). Inspection of the sequence of the light chain of Factor X_1 , which accounts for the majority of the molecule not homologous with chymotrypsin, shows that there is very little correspondence with the proportion of prothrombin, apart from the N-terminal Gla region (Figure 7·4). Another unique feature of prothrombin is the potential thrombin A chain. In the active enzyme A chain appears to function in substrate binding as its loss, to form beta-thrombin, leads to a preferential loss of clotting over esterase activity (Mann et al 1971a). Against this it has been recently shown that immobilised B-chain of thrombin, the A-chain having been removed by partial reduction, retains at least some of the substrate specificity of the complete molecule (Hageman et al 1975).

Thus although the Gla region and the enzymic portions of prothrombin and Factor X_1 are homologous, other portions of the molecules show no obvious similarity. Although this does not preclude the possibility that progressive carboxylation is limited by steric hindrance, such a mechanism requires a similar restriction on 'Gla region availability' from at least two distinct structures.

The most acceptable mechanism for carboxylation seems to be one in which the extent of carboxylation is controlled by specific sequences in the substrate molecule; - either a rejection or acceptor sequence or perhaps a particular spacing of potential Gla residues in the molecule. Figure 7·4 shows that the Gla region of prothrombin and Factor X_1 have a number of features in common, the most obvious of which are the frequent occurrence of the sequence -Leu-Gla-Gla- or -Leu-Gla-?-Gla- (which do not occur elsewhere in the molecule)

FIGURE 7.4.

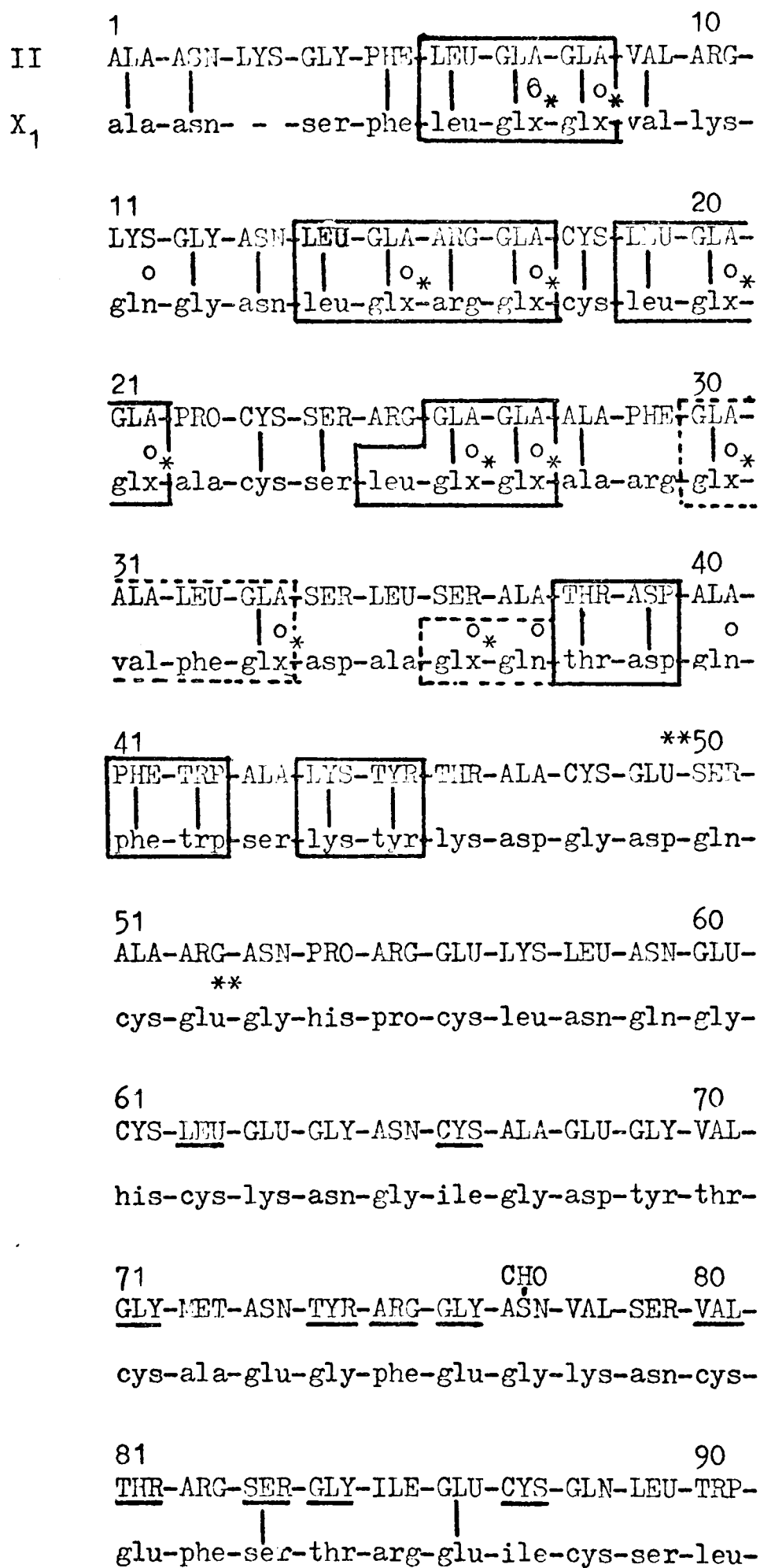


FIGURE 7.4

Comparison of the N-terminal sequences of prothrombin and Factor X₁.

- ° - assigned as Gla by Howard and Nelsestuen (1975).
- * - assigned as Gla by Enfield et al (1975)
- ** - first uncarboxylated glutamic acid in sequence from N-terminal.
- vertical lines show sites of identity between X₁ and prothrombin.
- underlining shows residues characteristic of the kringle structure in prothrombin.
- boxing shows some of the following points of interest in the Gla region:
 - (1) Gla residues occur in pairs or 'pseudo-pairs' and are frequently preceded by a Leucine residue.
 - (2) The Gla region contains no aromatic residues (except Phe 29 in prothrombin and Phe 32 in X₁) or threonine or aspartic acid residues, but immediately following this region we find the conserved sequence:

-Thr-Asp- ? -Phe-Trp- ? -Lys-Tyr-
 - (3) The spacing of Gla residues shows no pattern, apart from pairing. It is possible that such a pattern exists in the tertiary structure of the Gla region. The spacing between Gla residues is 7 or less in all cases whereas the space between the last Gla and the first uncarboxylated glutamic acid residue is 16 residues.
 - (4) No sequence homologous to the kringle structure is found in the light chain of Factor X₁.

and the presence of a highly aromatic sequence immediately following the Gla region. Normally such an aromatic region would be buried in the interior of a plasma protein. The tyrosine and tryptophan residues in this part of the molecule are the closest aromatic residues to the Gla region, apart from the phenylalanine residues actually included in it, and are probably the major contributors to the change in CD spectrum of prothrombin on binding calcium (Bjork and Stenflo 1973). This shift indicates that the aromatic residues are reorientated on calcium being bound to the molecule and could be moved out of their normal environment.

The Gla region shows no obvious pattern of Gla residue spacing, although the space between Gla's is always small when compared to the 16 residue gap between the last Gla residue and the first uncarboxylated glutamic acid residue. These features of spacing and sequence need not be of significance in the carboxylation process but could be related to the function of the completed molecule, either as substrate or enzyme.

The above observations permit the hypothesis that carboxylation occurs only in the N-terminal region of vitamin K-dependent proteins due to the existence of a specific sequence in this portion of the molecule. The best candidate for a specifying sequence is -Leu-Gla-Gla-, although the requirement for this cannot be absolute as carboxylation also occurs at other sites. In addition to this the termination of the Gla region is characterised by a highly aromatic sequence and by a glutamic acid free sequence which may limit the efficacy of the carboxylation enzyme. The latter hypothesis presupposes that carboxylation commences at or near the N-terminal of the protein.

If this mode of carboxylation is accepted the proviso must be made that restriction of carboxylation may also occur due to the lack of sufficient reduced vitamin K e.g. during Warfarin therapy, so as to account for the existence of partially carboxylated prothrombin (and other vitamin K-dependent proteins).

f) The carboxylation mechanism

Rats differ from men and cattle in that during Warfarin treatment they do not form abnormal plasma prothrombin. (Carlisle et al 1975). Using *Echis carinatus* venom to assay the abnormal protein Suttie (1973) showed that instead a corresponding protein accumulates in the liver, and that a similar accumulation occurs in vitamin K deficiency or during treatment with chloro-vitamin K (Cl-K). This abnormal prothrombin has been isolated from Warfarin-treated rat liver (Esmon et al 1975a) and exhibits an amino acid composition corresponding to the normal plasma protein, although it has a higher isoelectric point. This protein has also the same molecular weight as plasma prothrombin and is cleaved by thrombin to products of the same size. On administration of vitamin K to the deficient rat this protein disappears and normal plasma prothrombin appears, first in the microsomal fraction of the liver and then in the plasma. These results are interpreted as demonstrating that during Warfarin treatment uncarboxylated prothrombin accumulates in the liver of the rat and can then be carboxylated and released into the plasma in a vitamin K-dependent manner.

A rat microsomal system that exhibits this completion activity requires bicarbonate, vitamin K and an energy source (Shah and Suttie 1974) and is inhibited by Cl-K but not by cycloheximide or puromycin. Thus the completion of prothrombin in this system is due to a post-translational step. By the use of $^{14}\text{C HCO}_3^-$ it can be shown that the bicarbonate in this system is incorporated into the Fragment 1 region of the prothrombin precursor present in the microsomes. This process also requires proteins from post-microsomal supernatant. Although this reaction is again inhibited by Cl-K it isn't by Warfarin thereby showing that these two anticoagulants act at different sites (Esmon et al 1975b).

A similar in vitro system for the completion of Factor X has been described by Chung et al (1975) who have used Factor X completion as an assay in the fractionation of the postmitochondrial supernatant and attempted purification of the carboxylase enzyme. Some success (250 fold purification at 50% yield) has been achieved in isolating this enzyme

from the post-microsomal supernatant, but this work has been hampered by the extreme lability of the protein.

The advantage of using a Factor X completion system is that the clotting assay for this protein is much more sensitive than that for prothrombin. However this also requires much stricter control experiments as the activation of Factor X to Xa, possibly aided by the probable Factor VII carboxylation in this system, will have the same end effect as simple Factor X completion.

In this laboratory work has been commenced on the purification of the bovine carboxylation enzyme. Using rat liver microsomes as a source of incomplete Factor X it has been shown that the bovine post-microsomal supernatant provides at least two components to the carboxylation system; an enzyme of about 60,000 daltons, that has been partially purified (about 1000 fold) and also a 3,000 dalton cofactor (Y). The requirement for this cofactor can be overcome by using a very large excess of bicarbonate (Esnouf and Walter - unpublished observations).

Following the demonstration of vitamin K 2,3 epoxide accumulation during Warfarin treatment (See Chapter 1) it was shown that this is due to the inhibition of the reductase, which recycles this epoxide to the active reduced vitamin, by the coumarin (Matschiner et al 1974). This is in contrast to the action of two new classes of anticoagulant, the chloro-vitamin K's (Lowenthal and Roy-Chowdhury 1970) and the tetrachloropyridinols (Marshall 1972), which inhibit the epoxidase required for the conversion of the active reduced vitamin to the epoxide (Willingham et al 1974). Recently Sadowski (1975) has reported that amino glutethimide also inhibits this enzyme, presumably at a different site from the above vitamin K-analog inhibitors.

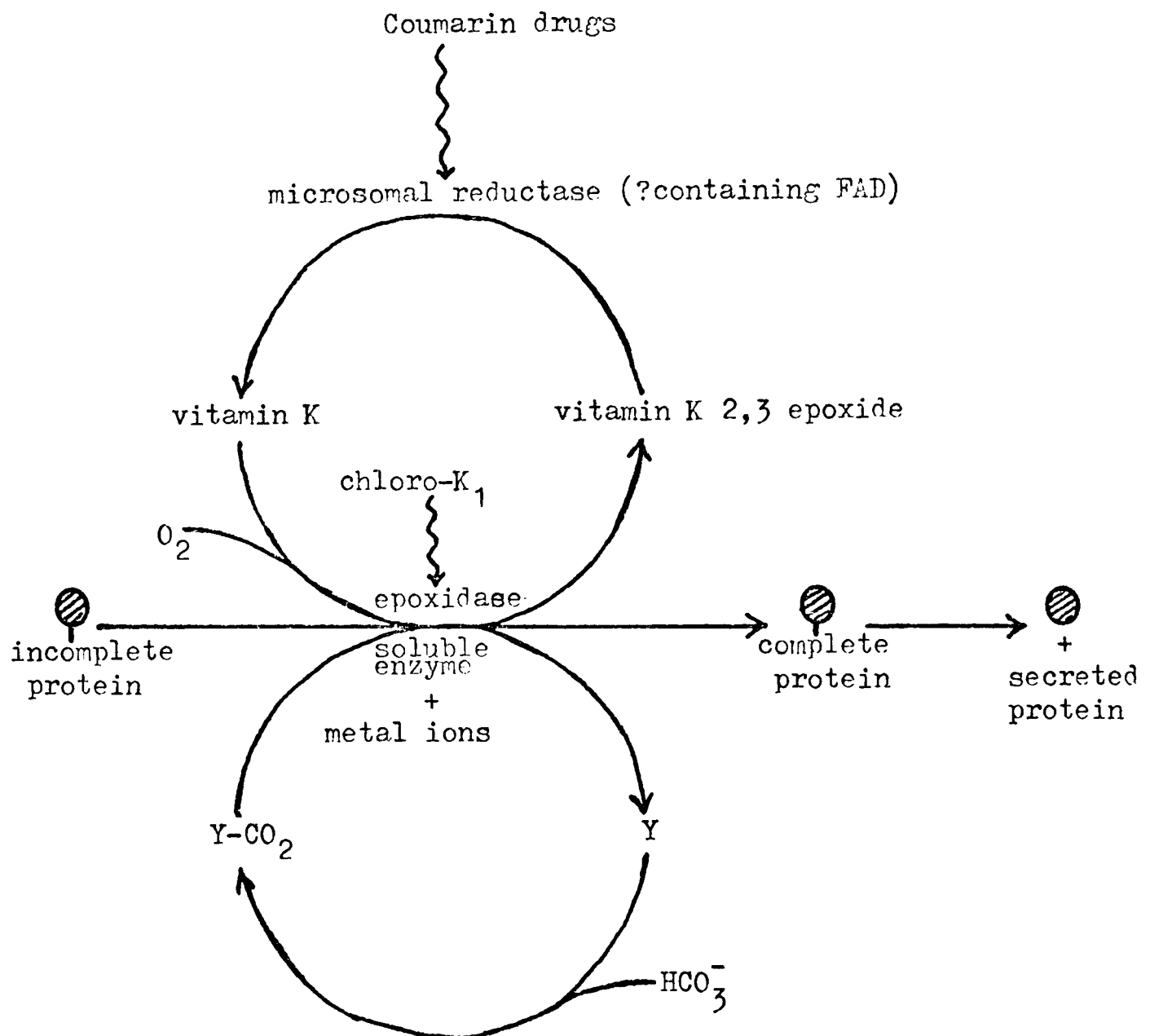
The final elucidation of coumarin action depends on the isolation of the reductase. There is very little information available on this enzyme although Matschiner et al (1974) showed that dithiothreitol enhances reductase activity. Early work by Marki and Martius (1960) describes the isolation of a coumarin-inhibitable 'vitamin K reductase', but this enzyme is apparently responsible for a quinone/quinol reaction

rather than a quinone epoxide/quinone one. The flavin-containing enzyme described by these authors requires that vitamin K₁ or K₂ be incorporated into liposomes before they are reduced, whereas the water soluble vitamin K₃ can be reduced in the aqueous phase (Martius et al 1975). This enzyme has been implicated in bacterial oxidative carboxylation in a similar manner to the benzoquinones ubiquinone (CoQ) in animal oxidative phosphorylation and phylloquinone in photosynthetic phosphorylation (Brodie 1961). In this context it is interesting to note that vitamin K will transfer reducing equivalents across a lipid membrane (Anderson et al 1975) and that Warfarin treatment produces marked changes in the structure of rat liver endoplasmic reticulum (Noonan et al 1975). As the substrate for the carboxylation reaction (e.g. incomplete prothrombin) is located in the microsome fraction of liver it may well be that carboxylation is at least partly membrane localised, in which case vitamin K could act as a redox cofactor in transferring reducing equivalents from either side of such a membrane.

It has been proposed that the action of Warfarin is due to the accumulation of vitamin K epoxide, due to inhibition of the reductase, which then acts as a direct competitive inhibitor of the active vitamin K, in a similar manner to the other epoxidase inhibitors. By studying the effect of a variety of coumarins on the vitamin K/K epoxide ratio Sadowski and Suttie (1974) have shown this to be untrue. Warfarin action is therefore presumably due to its blocking of the vitamin K cycle with a resultant lowering of the level of active reduced vitamin. Such an effect is supported by the lack of effect of Warfarin in the in vitro system, described above, in which sufficient active vitamin is supplied exogenously.

Warfarin therapy does however have an effect on epoxidase activity, which may be assayed as a direct conversion of vitamin K to its epoxide (in the presence of sufficient Warfarin to inhibit reductase activity). Willingham and Matschiner (1974) showed that besides increasing the amount of *Echis carinatus* venom assayable prothrombin precursor and decreasing the level of plasma prothrombin, Warfarin treatment of rats increases epoxidase activity, all these effects being simultaneously

FIGURE 7.5.



● = microsomal fraction with bound precursor protein.

Proposed pathway for vitamin K-dependant protein carboxylation.

reversed by the administration of vitamin K. Using the assay it was shown that epoxidase activity requires microsomes, soluble protein, a heat stable soluble factor and oxygen. The Warfarin-induced activity changes are due to effects on the microsomal component. If it is accepted that epoxidation is coupled to protein carboxylation this component may well comprise the uncarboxylated protein. The Warfarin induced rise in activity would then be due to the increased substrate availability for the epoxidase/carboxylase.

These various observations may be utilised in proposing a mechanism for the involvement of vitamin K and the coumarins in the completion of the vitamin K-dependent proteins (Figure 7.6⁵). This model requires that the precursor protein is located in a microsomal particle and that carboxylation of this protein is accompanied by the epoxidation of vitamin K and the decarboxylation of cofactor Y. This step is catalysed by epoxidase/carboxylase, which may or may not be the same enzyme, from the soluble fraction. In vivo the enzyme(s) may well be located on the membrane and only be solubilised by the purification procedure. The fact that this enzyme is not inhibited by the coumarin drugs, which are close structural analogs of vitamin K, shows that it has a greater specificity for vitamin K than the reductase. In this connection it is probable that the epoxidase inhibitor amino glutethimide acts at the substrate binding site, rather than acting as a vitamin K antagonist. If this is so it requires a tight coupling between epoxidation and carboxylation. This is supported by the anticoagulant effects of the Cl-K inhibitors and by the effects of Warfarin on epoxidase activity. That no such coupling occurs on the reductase side of the K cycle is shown by the in vitro carboxylation experiments, the Warfarin-sensitivity in vivo reflecting the low endogenous levels of vitamin K.

The energy required for carboxylation of precursor proteins could most easily be fed into the proposed pathway either at the stage of vitamin K reduction or during the carboxylation of cofactor Y, or more probably both. It may also be said that the above mechanism requires that endogenous vitamin K is located in the microsomal fraction of liver and that carboxylase and epoxidase activity should

co-purify. Further in any in vitro vitamin K-dependent protein protein carboxylation system protein carboxylation and vitamin K epoxidation should occur concomitantly.

Janson and Helgeland (1975) have suggested that the carboxylase reaction takes place in the smooth endoplasmic reticulum (SER) on the basis that the prothrombin activity in rough endoplasmic (RER) is lower than in the smooth. The results presented, however, could also be explained in terms of a difference in the total amount of zymogen rather than a specific activity difference. The stages through which a secreted protein passes following the initiation of polypeptide synthesis may be listed as:

- (1) Translation, polypeptide synthesis and folding on the ribosomes of the RER.
- (2) Sequential glycosylation during passage through the RER and then the SER.
- (3) Secretion via the Golgi apparatus.

Carboxylation could occur at any of these sites and there is little evidence for preferring any particular one. A possible approach to such localisation would be the use of fluorescently labelled antibody either to the epoxidase/carboxylase or the reductase, following isolation of the enzyme, on sections of liver. The earlier work of Noonan et al (1976) is suggestive of a site in the RER.

g) Other vitamin K-dependent proteins

Until recently it was thought that the coumarin drugs and vitamin K had a very specific effect on the four enzymic clotting factors. The high specificity of this action is difficult to explain in terms of any model requiring translational control and led to the proposal of schemes based on the operon model. These have had to be rejected with the demonstration of the post-ribosomal site of vitamin K action. This still left the problem of the high apparent specificity of vitamin K action, until the recent discovery of other proteins affected by the coumarin drugs - Factor X₃ and protein 'C', which have no clotting activity. Recently Hauschka et al (1976) have presented preliminary evidence for the occurrence of Gla in an EDTA-soluble protein of adult chicken bone.

This suggests that this protein is also vitamin K-dependent, although the appropriate coumarin treatment experiments have yet to be done. However there is some evidence that coumarin treatment of pregnant women (so treated due to heart valve replacements) may result in foetal bone abnormalities (Tejani 1973, Pettifor and Benson 1975), suggesting an involvement of vitamin K in bone calcium deposition.

In this context it is interesting that during purification of bovine prothrombin-super it was noticed that Warfarin plasma gave rise to a much heavier cryoprecipitate, following deep freezing prior to the preparation, than from a corresponding normal plasma sample. Preliminary work on this protein using the SDS-gel technique revealed no obvious differences between the two samples, both being mixtures, the major component of which was fibrinogen.

A common clinical experience is the delay of wound healing in patients on coumarin drugs and in addition it is worth remembering that the classical work of Dam showed mucous membrane degeneration in vitamin K deficient hens.

It thus appears that vitamin K action, and the related effects of the coumarin drugs, are more extensive than has been assumed and that the apparent high specificity of vitamin K action is probably due to a high turnover rate of the clotting proteins and the obvious manifestations of the reduction of clotting ability.

h) Conclusion

It is now obvious that the major effect of the administration of coumarin drugs on plasma glycoproteins is to decrease the extent to which certain glutamic acid residues in certain of these proteins are carboxylated, with a resultant loss in calcium binding ability. In the case of the clotting zymogens this results in a reduced ability to be activated to the enzymic form, with resultant haemorrhagic diathesis. We have shown that therapeutic Warfarin administration only partially reduces the extent of this carboxylation in the case of prothrombin.

With the increased understanding of the method of oral anti-coagulant and vitamin K action it is becoming increasingly clear that the carboxylation mechanism may be a general one and therefore have

effects in areas distinct from blood clotting. This has been borne out by the discovery of two vitamin K-dependent proteins without clotting activity and of Gla in a bone protein. As the clotting proteins have a very high turnover rate and are therefore quickly affected by interference with their synthesis, it may be expected that new effects of coumarin administration will be discovered with study of the effects of longer term therapy. Under these conditions vitamin K dependent proteins that turn over more slowly will be affected. To the clinician such effects would be of obvious import as they would have a prolonged latent period such that remedial vitamin K administration would have no immediate effect.

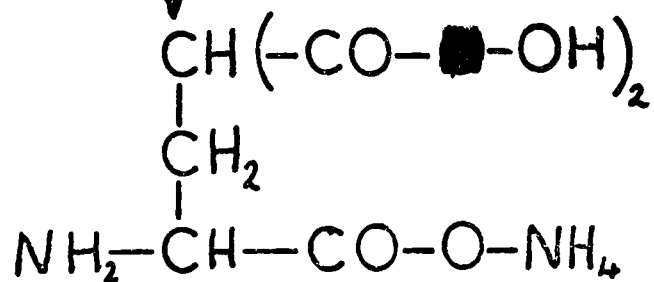
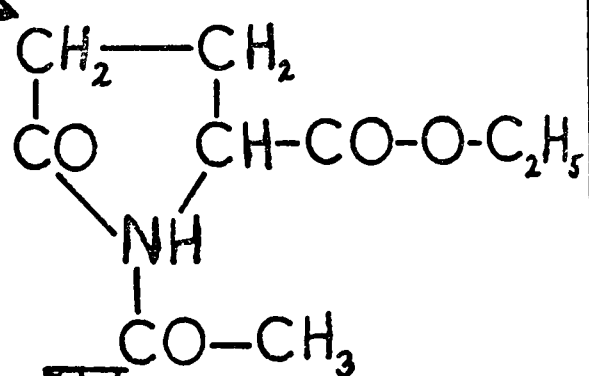
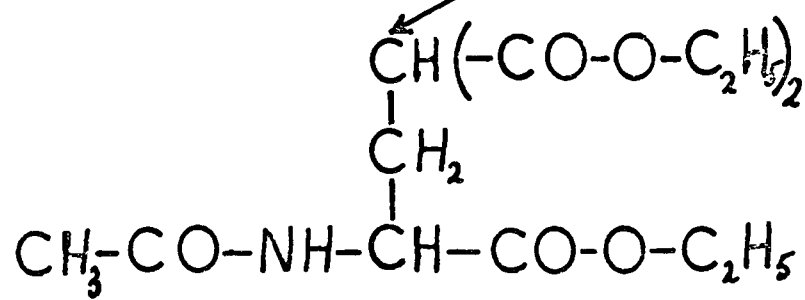
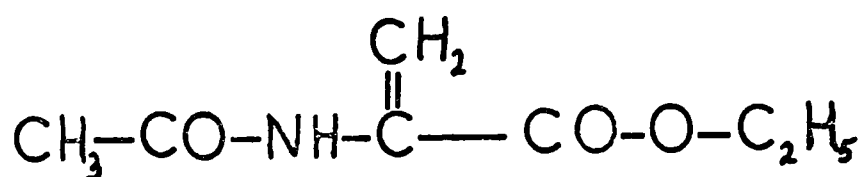
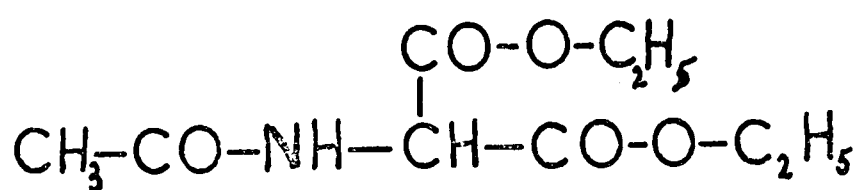
The other major area of future interest with respect to the coumarin drugs, apart from the search for effects distinct from those on blood clotting, is that of the site of their action at the enzymic level. To date such work has shown that a series of coupled reactions occur during the vitamin-K-dependent carboxylation of clotting proteins. At the moment the enzymes used in such studies are still crude, effectively being a microsomal fraction of liver. Despite this it has already been shown that at least two enzymic sites exist at which anticoagulant drug action may occur, one sensitive to the coumarins and the other to a new class of inhibitors which, unlike the coumarins, are direct inhibitors of vitamin K action. Further research in this area may well reveal further sites for possible 'anti vitamin K' drug action. However such drugs would presumably have pleiotropic effects rather than being restricted to coagulation proteins. In the search for antithrombotic drugs it may well be more fruitful to look for inhibitors of the action, for example based on the Fragment 1 inhibition of prothrombin activation, rather than of the synthesis of the coagulation zymogens.

APPENDICES

APPENDIX 1

Preparation of Factor Xa-Sepharose

1. Factor Xa was prepared from Factor X, isolated as described in Chapter 2, by activation with the coagulant fraction from Russell's Viper venom as described by Jesty and Esnouf (1973).
2. Sepharose was activated with cyanogen bromide by the method of Cuatrecasas (1970). 5mls of packed Sepharose 4B (Pharmacia) was added to an equal volume of water and the pH adjusted to 10 with 2M NaOH. 1.5g of cyanogen bromide, dissolved in a minimum volume of dimethyl formamide, was then added and the pH maintained at 10 with the NaOH. The temperature was kept below 20°C by the addition of ice chips in order to minimise the formation of side products. After about 15 minutes the mixture was filtered, by suction, on a sintered glass funnel and washed, on the funnel, with cold water and then 0.2M citrate/NaOH pH 6.9. Washing time was less than 90 seconds in all.
3. The activated Sepharose was quickly resuspended in 8 mls of the citrate buffer containing Factor Xa at 0.8 mg/ml. Reaction was allowed to proceed for 6 hours at 4°C. Excess imino-carbonate was then neutralised by filtering the Sepharose and by resuspending the Sepharose in 0.1M glycine, pH 8.6 (Porath 1970). This was left overnight and then well washed on a sintered funnel with 50mM Tris/HCl pH 8.0.
4. The Xa-Sepharose was packed into a (1 x 8)cm column ready for use.
5. This preparation retains both esterase and prothrombin activating activity. No activity was detectable in the supernatant of the coupling mixture. Acid hydrolysis of the Factor Xa-Sepharose and amino acid analysis of the released amino acid showed a Factor Xa content of 0.13mg Factor Xa/g of Sepharose; not all of this is available to prothrombin (Merson, R. 1976 - personal communication).



APPENDIX 2

Synthesis of gamma-carboxy glutamic acid

Initially this was prepared by the method of Morris et al (1975), however this method is prolonged and the reagents are unpleasant. As a result later preparations of gamma-carboxy glutamic acid (Gla) were carried out essentially as outlined by Fernlund et al (1975) as follows:

0.2 moles (43.44g) of acetamidomalonic acid diethyl ester (I) in 80 mls of ethanol was added, dripwise, over a period of 2 hours, with stirring, to 200 mls of ethanol containing 0.2 mole KOH. This was allowed to stand overnight at room temperature and yielded a clear solution. This was cooled to 0°C and neutralised with 17.4mls of conc. HCl. The precipitated KCl was removed on a sintered glass funnel and the filtrate rotary evaporated and dried in vacuo. Yield 38g of ~~monoester~~.

0.2 moles (38g) of the monoester was added to 0.22 moles (18mls) of 37% formaldehyde solution and 0.22 moles (40mls) 25% dimethylamine solution and 400mg of hydroquinone added to the mixture, with shaking (CO₂ was evolved violently at this stage). This mixture slowly dissolved to yield a clear pink solution which was then rotary evaporated at 90°C, under vacuum, to yield an oil after about 20 minutes. This oil was vacuum distilled at 0.3mm Hg and the 95°C fraction collected. On storage overnight at -25°C this fraction crystallised. The crystals were squeezed on a sintered glass disc and then recrystallised with difficulty from 40/60 petroleum ether. This stage yielded 15g of 2-acetamidoacrylic acid ethyl ester (II). This ran as a single spot with R_f=0.52 on tlc on silica plates developed with 1:1 heptane:ethyl acetate.

0.3g of sodium in 30 mls of dry ethanol (dried with freshly regenerated Linde 4A molecular sieve) was added to 18 mls of malonic acid diethyl ester in a flask, fitted with a side arm filled

with KOH and open to the atmosphere. 30 mls of dry ethanol containing 15g acetamidoacrylic acid ethyl ester was added to the flask, dropwise, through a funnel, over a period of 15 minutes and then stirred overnight. The mix was then neutralised with 0.9 ml acetic acid and rotary evaporated to a light brown oil. This was washed with 250 mls of water and then 2 x 50 mls of diethyl ether. The water wash was re-extracted with ether (50 mls). The combined ether phases were dried over fused sodium sulphate and rotary evaporated to another brown oil. This was vacuum distilled at 0.15mm Hg. Excess diethyl malonate distilled off at about 60°C and the product at 170°C as a colourless oil. This gave two spots on tlc in the above system with Rf values of 0.18 (III) and 0.51 (IV).

The product was placed in two rubber-bunged polypropylene tubes and the contents of each tube hydrolysed, with 0.4g NaOH in 8 mls of water, at 110°C overnight. The tubes were shaken at intervals until the phases no longer separated. Each hydrolysate was diluted to 25 mls with 1 M NH_4OH and desalted on Dowex-50 x8 (20-50 mesh, NH_4^+ form), eluting with 1 M NH_4OH . Fractions containing fractions were taken to dryness on the rotary evaporator and vacuum dessicated to remove residual ammonia. The residue was taken up in 30 mls of water and the pH adjusted from about 6 to 2.8 with conc. HCl and the monoammonium salt of Gla then precipitated by slow addition of 40/60 petroleum ether. Yield 3.2g of V.

Unprotected Gla was found to become ninhydrin-negative fairly rapidly at room temperature. This is presumably due to spontaneous cyclisation to an analog of pyroglutamic acid. In later preparations the synthesis was therefore stopped at the penultimate stage and the final deprotective hydrolysis carried out on aliquots as required.

APPENDIX 3

Preparation of Fucose-Lectin-Sepharose

A. Preparation of Fucose-Sepharose

1. Preparation of beta-L-fucopyranosylamine

60 millimoles (10g) of L-fucose dissolved in 80 mls of methanolic ammonia (methanol saturated with ammonia gas) was stood in a stoppered flask for 8 days at room temperature. A heavy precipitate formed which filled the original solvent volume. This was filtered off and washed with methanol and then diethyl ether.

Yield 3.2g of white powder

2. Preparation of CBZ-NH-(CH₂)₆-COOH

0.75 moles of carbobenzoxy chloride (CBZ-Cl) was dissolved in an equal volume of diethyl ether and added, together with 1 mole of 4N NaOH to 0.08 moles epsilon-amino caproic acid dissolved in 1 mole of 1N NaOH. After stirring for 1 hour the mixture was stood overnight at room temperature. The mixture was extracted twice with ether and then acidified and left to stand at 4°C over the weekend.

Yield 15.2g of white powder

3. Preparation of NH₂-(CH₂)₆-CO-NH-Fucose

8 g of CBZ-NH-(CH₂)₆-COOH was dissolved in 50 mls of DMF and 4.22mls triethylamine added. This mixture was cooled to -5°C and 4.1 mls of isobutylchloroformate added slowly. After 20 minutes the mixture was filtered to remove triethylamine hydrochloride and immediately a suspension of 3.2g of fucopyranosylamine in 50 mls DMF added. The mixture was stirred for 16 hours at room temperature (the fucose had completely dissolved after 1½ hours). The product was rotary evaporated at 60°C in vacuo to dryness, redissolved in a small amount of methanol and reprecipitated by addition of diethyl ether. The damp product (32g) was dissolved in

100 mls 80% aqueous methanol and hydrogenated overnight in the presence of palladium/charcoal catalyst to remove the protecting CBZ group. Tlc showed that the removal was complete. The product was taken up in methanol and reprecipitated with ether, twice.

Yield 3.2 g of dry white powder

4. Preparation of Fucose-Sepharose

100g of packed Sepharose 4B, previously treated with epichlorohydrin/ NaBH_4 to increase the extent of cross-linking and reduce the slow leakage of carbohydrate residues from the matrix, was suspended in an equal volume of water, taken to pH 10 with 5N NaOH and chilled. 10 g of CNBr in 15 mls DMF was added to the Sepharose and the pH maintained at 10 with the NaOH for 15 minutes, the temperature being kept below 20°C. After this the Sepharose was quickly washed on a sintered glass funnel with 1 l of cold 0.1M borate buffer pH10 and the activated Sepharose added to 620mg (2mmoles) of epsilon-amino-caproyl-beta-L-fucopyranosylamine and left stirring at 4°C overnight. The fucose-Sepharose was then washed with 100mM HCl/Tris pH7.5 to ensure no reactive groups were left.

B. Preparation of Fucose-binding Lectin from Lotus tetragonobulus seeds

52g of asparagus pea seeds (Thompson and Morgan Ltd., Ipswich) were ground to a fine powder in an electric coffee grinder and extracted with 1 l 0.15M NaCl, 20mM NaOH/ H_3PO_4 pH6.8. The extraction was carried out in a blender for about 3 minutes, the temperature being kept low by the addition of ice chips. The gelatinous extract was centrifuged to remove debris, ammonium sulphate added to 30% saturation and stood overnight at 4°C before centrifuging again. The ammonium sulphate concentration in the supernatant was increased to 60% saturation and again stood overnight at 4°C. The precipitate was suspended in a minimum volume of 50mM NaOH/ H_3PO_4 pH6.8 and dialysed against many changes of the same buffer (volume increased to a final 70 mls). The lectin was assayed throughout the preparation by its ability to agglutinate group O human red blood cells.

The extract was applied to the fucose-Sepharose column (2x25)_{cm} and the column washed with the above buffer until no further protein was eluted. The lectin was then eluted with the same buffer containing 40mM fucose as a very sharp peak. This method is based on various techniques described in Methods in Enzymology XXVIII (1972).

Yield 130mg of lectin

C. Preparation of Fucose-binding lectin-Sepharose

The lectin was dialysed into 0.5M NaHCO₃ and coupled to 150g Sepharose 4B : 150g of Sepharose 4B in 150 mls water was adjusted to pH10 with 5N NaOH and cooled to 10°C. 15g CNBr in 15 mls DMF was added and the pH maintained at 10 for 15 minutes, the temperature being kept below 20°C. The Sepharose was washed quickly with cold 0.5M NaHCO₃ and then added to the dialysed protein in 150 mls of buffer, stirred overnight at 4°C, sucked dry on a sintered glass funnel and suspended in a saturated solution of glycine, to neutralise unreacted iminocarbonate groups (Porath 1970). The column was washed in the appropriate buffer prior to use.

This column did not, as it had been hoped, bind Factor X₃. It did however bind Factor VIII (high purity material obtained from the Oxford Haemophilia Centre by courtesy of Dr. Dennis Austen). This was attempted as it had been reported that Factor VIII has a very low sialic acid content relative to the total amount of carbohydrate present (Legaz et al 1973) and may therefore be expected to have fucose as an exposed terminating sugar residue on the carbohydrate moieties (Spiro 1970). Unfortunately only low amounts of Factor VIII activity have been recovered from this column to date by attempted elution with fucose and/or high salt concentrations.

APPENDIX 4

A Variant of Prothrombin induced in Cattle
by prolonged Administration of Warfarin

BBA 37323

A VARIANT OF PROTHROMBIN INDUCED IN CATTLE BY PROLONGED ADMINISTRATION OF WARFARIN

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SUMMARY

Cows on long term Warfarin therapy produce a form of prothrombin which, although it binds to barium citrate, has a low biological activity.

Activation experiments on this form of prothrombin show that it is only slowly converted to thrombin in the presence of Ca^{2+} , although the thrombin produced has normal activity. Further experiments show that the Fragment 1 region of the molecule has a reduced calcium binding capacity. The results indicate the existence of a partially carboxylated form of prothrombin.

INTRODUCTION

It is well known that vitamin K antagonists are effective as anticoagulants because they interfere with normal production of the so-called prothrombin group of clotting factors [1-4]. So far prothrombin has been the most extensively studied and it has been shown that in cattle, during dicoumarol treatment, abnormal prothrombin is released into the plasma [5, 6]. This abnormal prothrombin is characterised by its failure to be adsorbed by insoluble barium salts [7] and also by its inability to be converted normally to thrombin by the prothrombin-converting complex [5]. In contrast, human patients on long term anticoagulant therapy produce in addition to the non-barium-absorbable prothrombin a form of prothrombin, which although it can be absorbed onto barium citrate, has a low specific activity [8]. Since large amounts of plasma from patients on long term therapy cannot be readily obtained, we have treated cattle with Warfarin for a prolonged period in an attempt to isolate large amounts of the low specific activity barium citrate-absorbable prothrombin.

MATERIALS AND METHODS

Administration of Warfarin. Two cows were given 100 mg sodium Warfarin (B.D.H. Chemicals Ltd., Poole, England) daily. Cow 1 (475 kg) and Cow 2 (650 kg) were given the Warfarin in a drench of 1% NaHCO_3 and milk.

Preparation of antiserum. Rabbits were immunized against purified bovine prothrombin by multiple site intradermal injections of a total of 1 mg of antigen in 1 ml of Freund's Complete Adjuvant (Difco Labs., Mich., U.S.A.). A booster dose was given after 2 weeks and blood collected from an ear vein about 10 days later. The antiserum was stored in 1-ml aliquots at -20°C .

Quantitative immunoelectrophoresis. This was performed by the one-dimensional method of Laurell [9]. 17.5 ml of a 1% (w/v) agarose (Serva, Heidelberg, W. Germany) solution containing the antiserum was poured on to a 10×20 cm glass plate. 19 wells were cut in the agarose and $5 \mu\text{l}$ of a sample containing up to $0.5 \mu\text{g}$ of antigen was added to each well. The plates were then electrophoresed at 5 V/cm overnight. The "rocket" heights of the precipitin peaks were measured after staining with Coomassie Brilliant Blue (Sigma Chemical Co., Mo., U.S.A.).

Bioassay of prothrombin. The biological activity of prothrombin samples diluted in 1% bovine serum albumin (Armour Pharmaceutical Co., Ltd., England) was determined by the one-stage procedure of Jobin and Esnouf [10] using crude tiger snake venom (*Notechis scutatus occidentalis*). 1 ml of plasma is defined as containing 1000 units of activity.

Bioassay of Factor X. The biological activity of Factor X samples diluted in 1% bovine serum albumin was determined by the method of Denson [11] using a coagulant fraction from Russell's viper venom purified by the method of Williams and Esnouf [12].

Zone electrophoresis. Zone electrophoresis in 60 mM barbitone, pH 8.65, containing 1% agarose with either 2 mM calcium lactate or 2 mM EDTA was performed essentially according to Johansson [13] except that fixing was carried out in 20% trichloroacetic acid for 15 min followed by two 5-min washes in ethanol.

Analytical polyacrylamide gel electrophoresis in sodium dodecyl sulphate. This was performed by the method of Weber and Osborn [14]. Before use, acrylamide (B.D.H. Ltd.) was re-crystallised from chloroform, *N,N'*-methylene-bis-acrylamide (B.D.H. Ltd.) was re-crystallised from acetone. 7.5% acrylamide gels were electrophoresed at 4 mA/tube overnight before fixing in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid for 24 h and then staining with 0.1% Coomassie Brilliant Blue in the same solvent for 2 h. The gels were destained in 14% (v/v) methanol, 7% (v/v) acetic acid and stored in 7% (v/v) acetic acid. Prior to electrophoresis samples were added to an equal volume 0.2 M $\text{H}_3\text{PO}_4/\text{NaOH}$ buffer, pH 7.1, containing 8 M urea, 10 mM EDTA, 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol and bromophenol blue as a tracking dye.

Carbohydrate analysis. This was performed by the method of Chambers and Clamp [15]. Protein solutions (1 mg/ml) were dialysed against 0.1 M NH_4HCO_3 before the addition of mannitol as an internal standard, and the solutions containing 1–2 mg of protein dried at 60°C for 1–2 days and then in vacuo for 1 day. 1 ml of methanolic HCl was added to each sample and methanolysis was allowed to proceed in screwcapped vials under N_2 for 18 h at 85°C . Excess acid was neutralised with Ag_2CO_3 and N-acetylation was performed for 8 h by the addition of 10 mg of silver acetate followed by $50 \mu\text{l}$ of acetic anhydride. The supernatant was decanted and rotary evaporated at 40°C . After drying in vacuo overnight, trimethylsilyl derivatives were prepared by the addition of $100 \mu\text{l}$ of a mixture of trimethylchlorosilane, hexamethyldisilane (B.D.H. Ltd.) and pyridine in the ratio of 1:1:5. Typically, 2–4 μl

of each sample was analysed using an F and M Hewlett Packard gas-liquid chromatograph fitted with a 2 m U-shaped column packed with 3% OV-1 on Diatomite CQ 85–100 mesh [J.J.'s (Chromatography) Ltd., King's Lynn, Norfolk, England].

The oven temperature was programmed to rise at $1^{\circ}\text{C}/\text{min}$ from 150 to 230°C after an initial 10-min delay. The carbohydrate residues were estimated from the recordings by measuring the areas under the appropriate peaks and comparing them with standards. All estimations were carried out at least in duplicate.

Purification of barium citrate-absorbable prothrombin. Blood, (usually 2 l) was collected from the jugular vein of the cow by gentle suction into 0.1 volume of 4% trisodium citrate containing 1 g benzamidine hydrochloride (R. Emanuel, England) per l of blood. The container was packed in ice until the red blood cells could be removed by centrifugation at $2000 \times g$ for 45 min. 30 g BaCl_2 in 150 ml distilled water was added to 1 l of plasma and stirred for 20 min at room temperature. The insoluble barium citrate was removed by centrifugation at $5000 \times g$ for 20 min and was washed three times with 100 ml of cold distilled water before being suspended in 100 ml of 0.1 M sodium citrate, pH 6.9, containing 10 mM benzamidine. Solid $(\text{NH}_4)_2\text{SO}_4$ was then added slowly with stirring to 10% saturation to precipitate excess barium which was removed by centrifugation at $5000 \times g$ for 30 min. The clear supernatant was dialysed overnight into 0.02 M sodium citrate, pH 6.9, containing 1 mM benzamidine, and then concentrated to 20 ml by ultrafiltration.

A column was packed with DEAE-Sephadex A-50 (1×20 cm) (Pharmacia Fine Chemicals, London) equilibrated with 0.02 M sodium citrate containing 1 mM benzamidine, pH 6.9. The concentrated sample was loaded onto the column at 15 ml/h and a linear gradient (400 ml) applied from 0.02 to 0.4 M Na citrate (pH 6.9) containing 1 mM benzamidine and the column eluted at 15 ml/h. 5-ml fractions were collected and those containing the purified proteins were pooled, concentrated to approx. 1 mg/ml and stored at -20°C .

Isolation of prothrombin-super. The supernatant remaining from the adsorption of 1 l of plasma with barium citrate was adjusted to pH 6.0 with 1 M acetic acid and diluted four times with distilled water. 200 ml of DEAE-Sephadex A-50 equilibrated in 0.02 M sodium citrate, pH 6.0, was added and stirred for 30 min before the Sephadex was removed by filtration. The Sephadex was then packed into a column (20×4 cm) and washed with 0.02 M sodium citrate, pH 7.0, at 100 ml/h until no further protein was eluted. A 3 l linear gradient was then applied from 0.02 to 0.15 M sodium citrate, pH 7.0, and the column eluted at 75 ml/h. 25-ml fractions were collected and those containing prothrombin-super were pooled and solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly with stirring to 40% saturation. The precipitate was discarded and the $(\text{NH}_4)_2\text{SO}_4$ concentration of the supernatant increased to 70% saturation. After 30 min the precipitate was removed by centrifugation at $500 \times g$ for 30 min, redissolved in 20 ml 0.02 M Na citrate (pH 7.0) and applied to a Sephadex G-200 column (2.5×114 cm) previously equilibrated in the same buffer and eluted at 20 ml/h; 8-ml fractions were collected.

The fractions containing prothrombin-super were again pooled, concentrated to about 40 ml, and dialysed against 0.23 M Tris/ ϵ -amino caproate buffer, pH 8.9. To 10-ml aliquots was added 0.4 ml ampholine, pH 5–8 (LKB Products Ltd.) and a little sucrose, the mixture was layered onto a polyacrylamide gel. The gel, 10 cm high, containing 5% acrylamide (acrylamide:bisacrylamide ratio 35:1) and buffered with

0.02 M Tris/glutamate, pH 5.0, was polymerized in a Canaleo preparative electrophoresis apparatus (Canal Industrial Corp; Md., U.S.A.) fitted with a 2.3 cm diameter column. The cathode compartment was filled with 0.23 M Tris/ ϵ -amino caproate, pH 8.9, and the anode compartment and eluting buffer was 0.03 M Tris/sulphate, pH 7.1. Preparative isotachopheresis was carried out at 20 mA, the apparatus being cooled with circulating tap water. The proteins electrophoresed from the gel were removed with buffer at flow rate of 100 ml/h. Fractions were collected at 2-min intervals and those containing prothrombin-super were pooled, dialysed against 0.02 M sodium citrate, pH 6.9, and applied to a DEAE-Sephadex column (1×15 cm) at 20 ml/h. The column was eluted with a 500 ml linear gradient from 0.02 to 0.1 M citrate, pH 6.9, at the same flow rate and 8-ml fractions were collected. The prothrombin-super was at least 90% pure by this method. The protein was stored at -20°C until required.

Measurement of protein concentration. Protein concentrations of solutions were calculated from optical measurements at 280 nm and were corrected for large particle light scattering from measurements made at 320 nm. The specific extinction coefficient $E_{1\%}^{1\text{cm}}$ at 280 nm for prothrombin was taken as 13.8 [16], for Intermediate I as 16.0 [17] and for thrombin as 19.5 [18].

Activation of prothrombin. Incubation mixtures contained 0.2 mg/ml prothrombin and 60 $\mu\text{g/ml}$ purified activated Factor X (X_a), purified by the method of Jesty and Esnouf [19], dissolved in 0.05 M Tris·HCl, pH 8.0. In experiments in which calcium was added the incubation mixture contained 0.2 mg/ml prothrombin, 3 $\mu\text{g/ml}$ Factor X_a and 5 mM CaCl_2 . In experiments both with calcium and phospholipid the incubation mixtures contained 0.2 mg/ml prothrombin, 0.01 $\mu\text{g/ml}$ Factor X_a , 5 mM CaCl_2 , and 0.3 mg/ml of Inosithin (Associated Concentrates Inc., Woodside, N.Y.) emulsified in 10 mM Tris·HCl buffer, pH 8.4. The mixtures were incubated at 37°C in plastic tubes and 0.1-ml aliquots were withdrawn at intervals for the assay of thrombin or for analytical polyacrylamide gel electrophoresis in sodium dodecyl sulphate.

Isolation of prothrombin activation products. Profragments of prothrombin were isolated essentially as described by Jesty and Esnouf [19]. Thrombin was purified from scaled-up activation mixtures as described above. Normal and barium citrate-absorbable prothrombin were activated using Factor X_a and Ca^{2+} , while prothrombin-super was activated with Factor X_a alone. The esterase activities of the mixtures were followed and on reaching a plateau 0.1 M EDTA, pH 7.5, was added to a final concentration of 10 mM. The activation mixtures were then concentrated by ultrafiltration and applied to a column 20×1.6 cm of SP Sephadex C-50 (Pharmacia Fine Chemicals, London) equilibrated in 50 mM Tris·HCl, pH 7.0. The profragments are washed through the column and thrombin is eluted with a salt gradient (0–0.35 M NaCl) at flow rate of 20 ml/h. The thrombin was detected both by the measurement of esterolytic activity and fibrinogen clotting time.

Esterolytic activity of thrombin. This was measured by the method of Schwert and Takenaka [20]. 1 ml of 3 mM *N*- α -benzoyl arginine ethyl ester (Koch-Light Laboratories Ltd., Colnbrook, England) in 0.05 M Tris·HCl, pH 8.0, was mixed with 0.1 ml of the incubation mixture and the volume made to 3.0 ml with 0.05 M Tris·HCl buffer, pH 8.0, in 1 cm cuvette. The decrease in absorbance was measured at 25°C in a recording spectrophotometer. The rate of hydrolysis of the substrate by

the incubation mixture was compared against a control incubation mixture which contained only Factor X_a .

RESULTS

The level of plasma prothrombin during Warfarin therapy may be measured either in terms of the total amount present or by activity measurements which only detect the active species. One-dimensional immunochemical methods may be used to determine the total amount of prothrombin since they do not distinguish between the active and inactive species [6]. Similarly, assays using *Echis carinatus* venom [5] or staphylocoagulase [3] include both active and inactive prothrombin. Biologically active prothrombin may be distinguished from the inactive forms using assays based on physiological activators such as Factor X_a , or substances which have Factor X_a -like activity such as tiger snake venom [10].

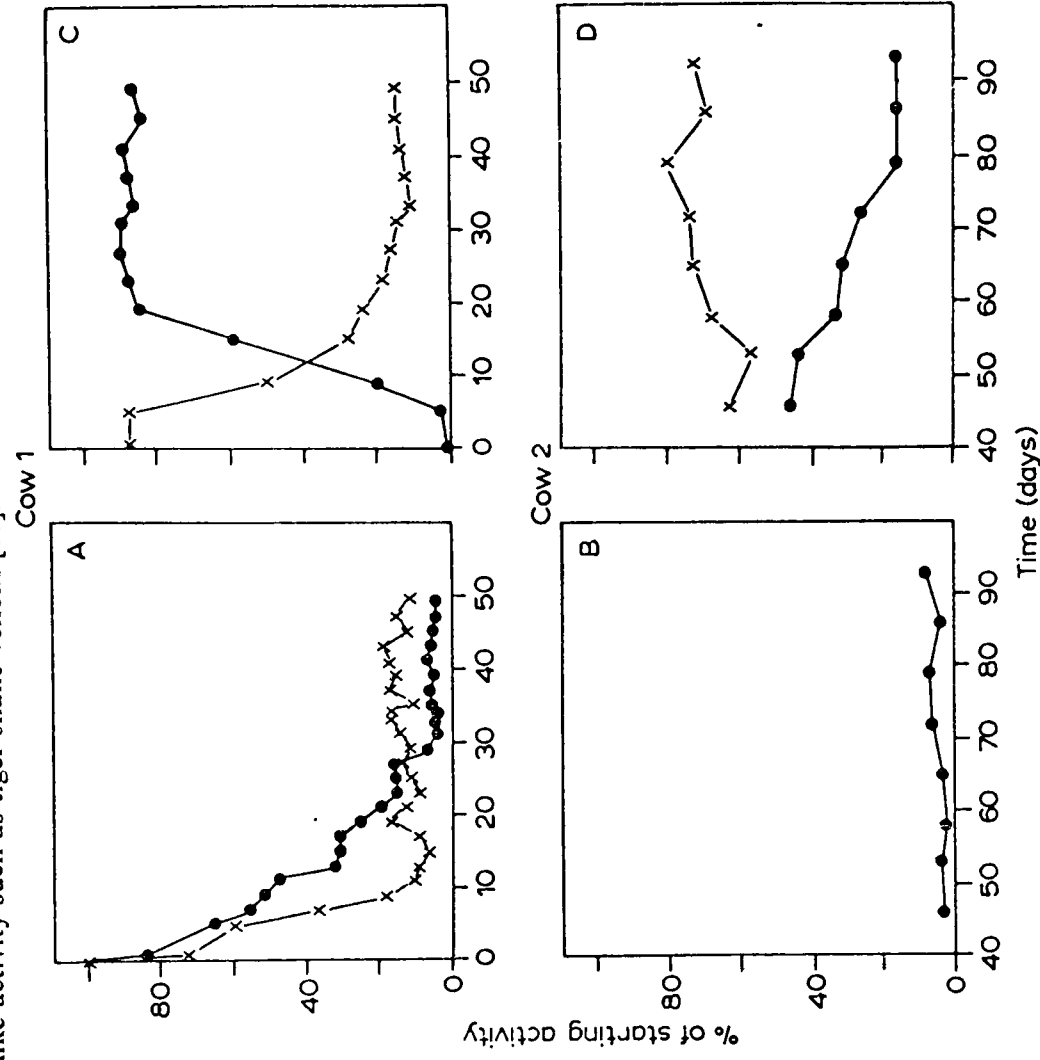


Fig. 1. The effect of administration of warfarin on the biological activity of plasma prothrombin and the level of barium-absorbable and non-absorbable prothrombin assayed by a one-dimensional immunoassay. A and B, bioassay: $\bullet$ — $\bullet$, prothrombin; $\times$ — $\times$, Factor X. C and D, immunoassay: $\times$ — $\times$, barium absorbable; $\bullet$ — $\bullet$, barium non-absorbable.

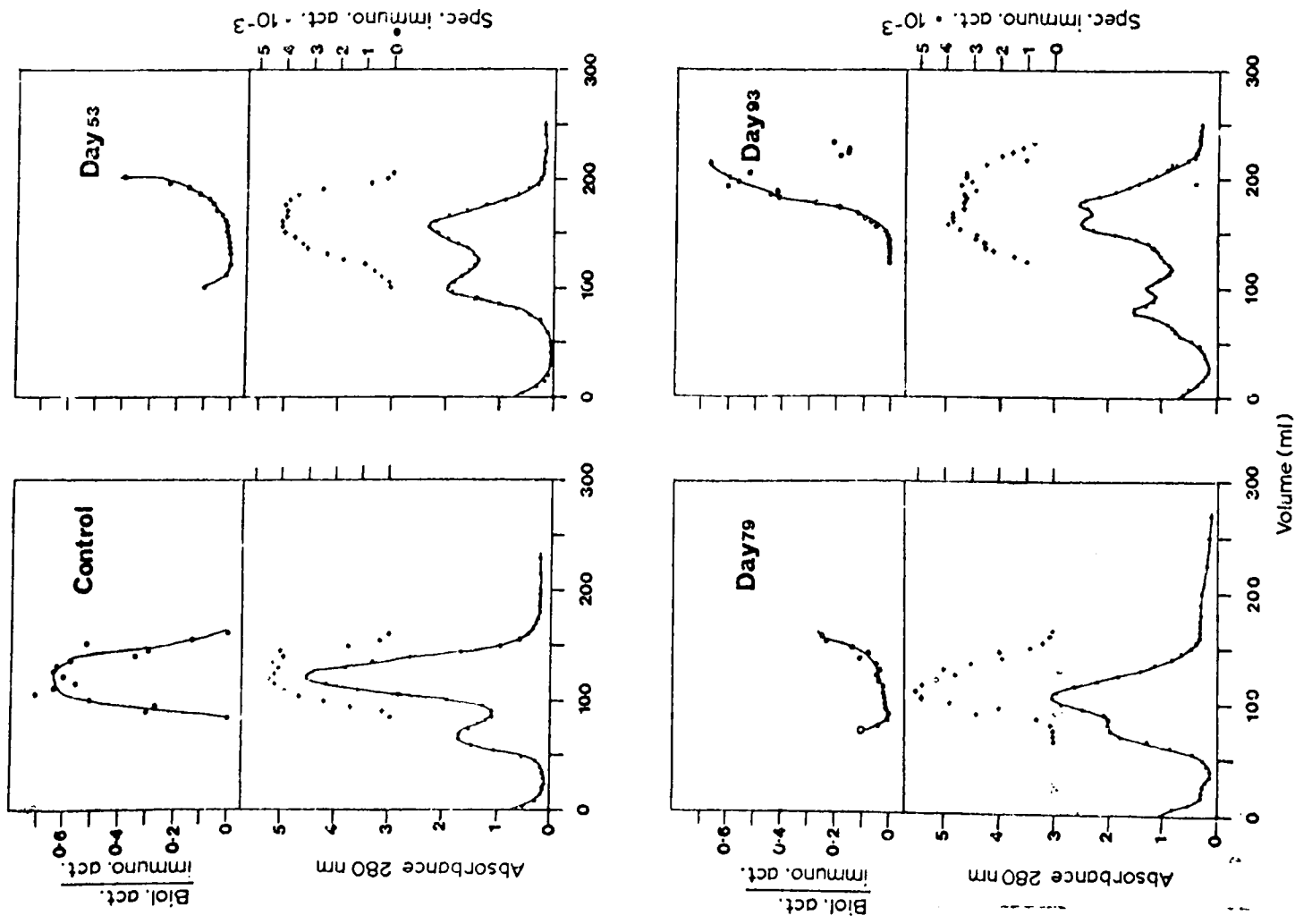


Fig. 2. Elution profiles from DEAE columns of control and Warfarin-treated preparations. Immunological activity according to Laurell [9] is based on a standard of 1000 arbitrary units per ml in a selected sample of normal bovine-citrated plasma. Biological activity is based on a one-stage tiger snake venom assay of prothrombin, the units being defined in the same way as for immunoassay.

Thus active, partially active and inactive species can be determined by a combination of the immunochemical method and the tiger snake venom assay.

As the result of our experience with patients a cow (Cow 1) was given a daily dose of Warfarin (1 mg/4.75 kg) and the biological activities of prothrombin (and Factor X) were followed (Fig. 1A). This showed biological activity fell to a low level after 4 weeks. Assays of the same plasma samples by immunochemical methods before and after absorption with barium citrate distinguishes between the barium-absorbable prothrombin (prothrombin-Ba) and non-absorbable prothrombin (prothrombin-super). Fig. 1C shows that during Warfarin treatment there is a fall in biological activity which is paralleled by a fall in barium-absorbable prothrombin and accompanied by a corresponding increase in non-absorbable prothrombin, which corresponds to the abnormal prothrombin described by others [5, 6]. Isolation of the prothrombin-Ba showed that it had a reduced biological specific activity, as had been noted already with the human patients [8].

As only small amounts of prothrombin-Ba were obtained from the first cow, a second cow (Cow 2) was treated with Warfarin (1 mg/6.5 kg) and 2 l samples were withdrawn at 7-day intervals after sufficient time had elapsed, indicated by Cow 1, for the biological activity to reach a low level. Fig. 1B shows that biological activity of the prothrombin remained at a low level until day 93 when the animal was slaughtered and 15 l of blood collected.

In contrast to Cow 1, Fig. 1D shows that the level of prothrombin-Ba is much higher in Cow 2 despite the low biological activity, supporting the idea prothrombin-Ba has a low biological activity.

The different forms of prothrombin were isolated from the plasma as described in Materials and Methods. The elution profiles, from the DEAE-Sephadex columns,

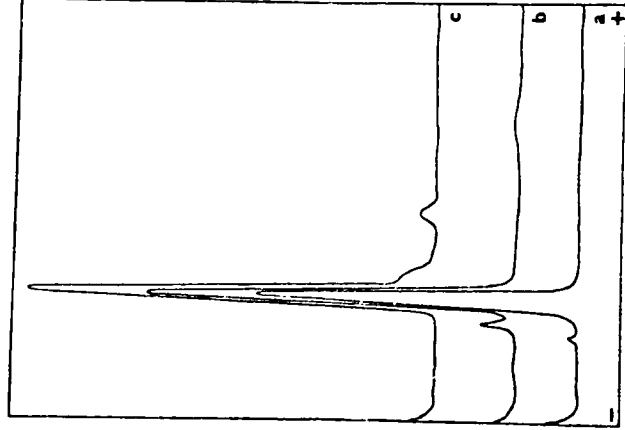


Fig. 3. Scans of absorbance vs. migration of stained polyacrylamide sodium dodecyl sulphate gels of the three forms of prothrombin purified as described in Materials and Methods. a, normal; b, prothrombin-Ba; c, prothrombin-super.

of the barium citrate-absorbable proteins obtained on day 0, 53, 79 and 93 are shown in Fig. 2. The ratio of biological to immunological activity in the prothrombin peak was characteristically 0.6 for normal prothrombin, and during Warfarin treatment this ratio is considerably lower, only approaching normal values with the protein eluted in the trailing edge of the prothrombin peak. Similar results have been obtained for human patients undergoing Warfarin therapy [8]. This behaviour on DEAE-Sephadex would be consistent with the higher specific activity prothrombin having a higher negative charge than the low specific activity material. Sodium dodecyl sulphate gels (Fig. 3) show that the three forms of prothrombin were obtained in a substantially pure state.

To check whether prothrombin-Ba really differed from the prothrombin not adsorbed by barium citrate (prothrombin-super) reported by others [5, 6], samples of prothrombin-Ba from the leading edge of the prothrombin peak were reabsorbed with barium citrate and in each case over 90% of the prothrombin was reabsorbed, from which it can be concluded that the barium-binding sites are normal.

Zone electrophoresis of Fragment 1 derived from both normal prothrombin and prothrombin-Ba in the presence of Ca^{2+} (Fig. 4) showed that the Fragment 1 derived from prothrombin-Ba has a higher mobility than normal Fragment 1.

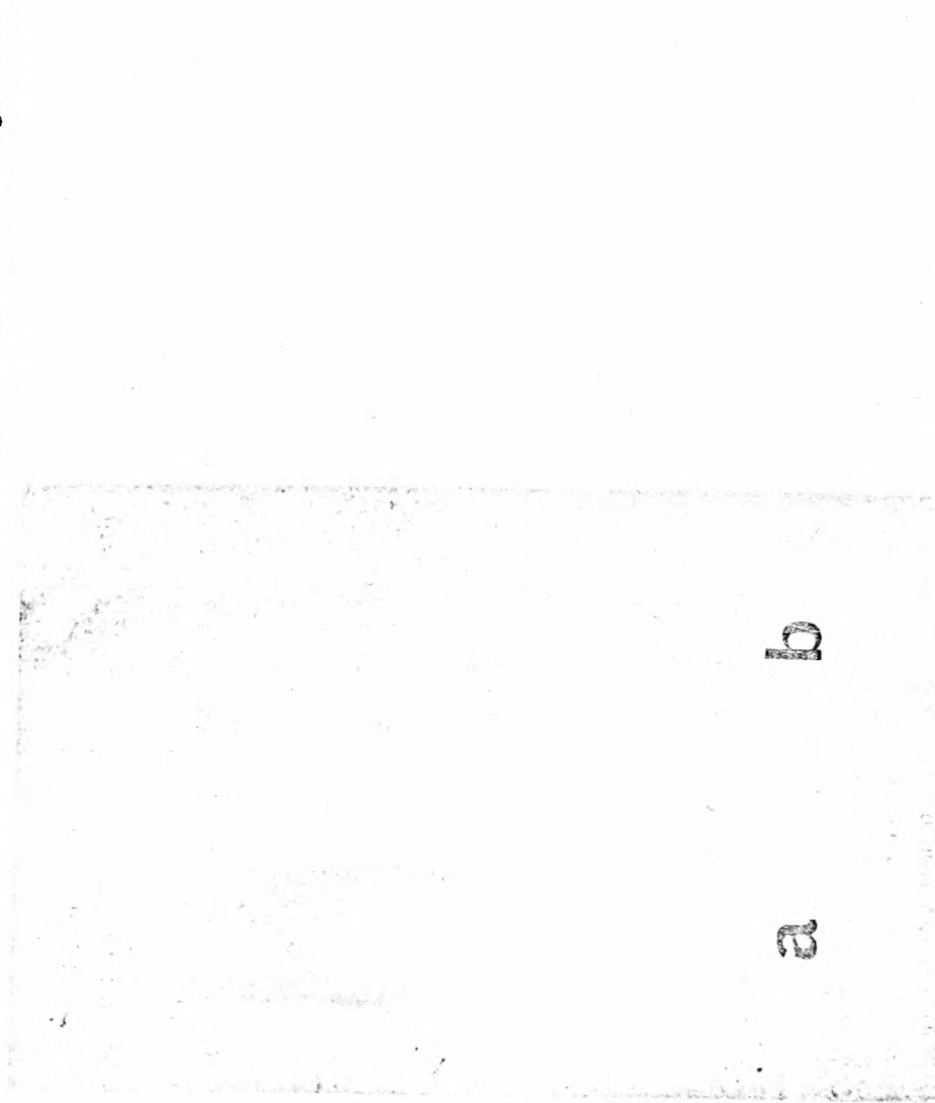


Fig. 4. Zone electrophoresis in agarose gel containing 2 mM calcium lactate/60 mM barbitone buffer, pH 8.65, of Fragment 1 derived from (a) normal prothrombin and (b) prothrombin-Ba.

Similarly prothrombin-Ba had a higher mobility than normal prothrombin. Stenflo and Ganrot [6] have already shown this difference in behaviour between normal prothrombin and abnormal prothrombin (prothrombin-super), we have also confirmed this observation (not shown). The difference in the mobility of Fragment 1 regions of the two prothrombins must arise from a difference in calcium binding as it is not observed when calcium is replaced with EDTA in the agarose.

Carbohydrate analysis of normal prothrombin and prothrombin-Ba gave very similar results (Table I), and, as was expected, no difference was found in the amino acid composition of acid-hydrolysed samples.

TABLE I

CARBOHYDRATE ANALYSES

Carbohydrate analyses of prothrombin during Warfarin treatment. Values are expressed as mol of carbohydrate residue/mol of protein, assuming a molecular weight of 70 000 for prothrombin

	Normal prothrombin	Prothrombin-Ba	Prothrombin-super
Mannose	8.3	8.2	8.5
Galactose	6.8	6.8	7.3
Glucose*	0.9	1.2	0.6
Glucosamine	14.1	13.8	12.1
Sialic acid	13.9	15.3	16.4

* Glucose is probably a contaminant derived from chromatography media.

TABLE II

ACTIVITIES OF THROMBIN PURIFIED FROM THE VARIOUS PROTHROMBINS, AS DESCRIBED IN MATERIALS AND METHODS

	Normal thrombin	Thrombin-Ba	Thrombin-super
Clotting assay NIHu/mg*	2750 ± 260	3244 ± 1170	2597 ± 380
Esterase assay μmol/min/mg	21.7 ± 1.3	18.0 ± 1.4	21.3 ± 2.9

* NIH_u = National Institutes of Health units.

The reduced specific activity of prothrombin-Ba could be accounted for, either in terms of a slow activation rate, or a poorly active thrombin. To test the latter possibility, thrombin was obtained from normal prothrombin, low activity prothrombin-Ba and prothrombin-super. The coagulant and esterase activities of the three thrombin preparations were compared (Table II). The results show that they have similar activities and hence the low specific activity of prothrombin-Ba must be related to the rate at which it can be activated.

Accordingly the activation of the three forms of prothrombin by Factor X_a was examined in the presence and absence of both calcium and phospholipid. The rate of thrombin formation was determined from the increase in esterase activity of incubation mixtures (Fig. 5) and from sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Figs. 6 and 7) on samples taken from the same activation mixtures.

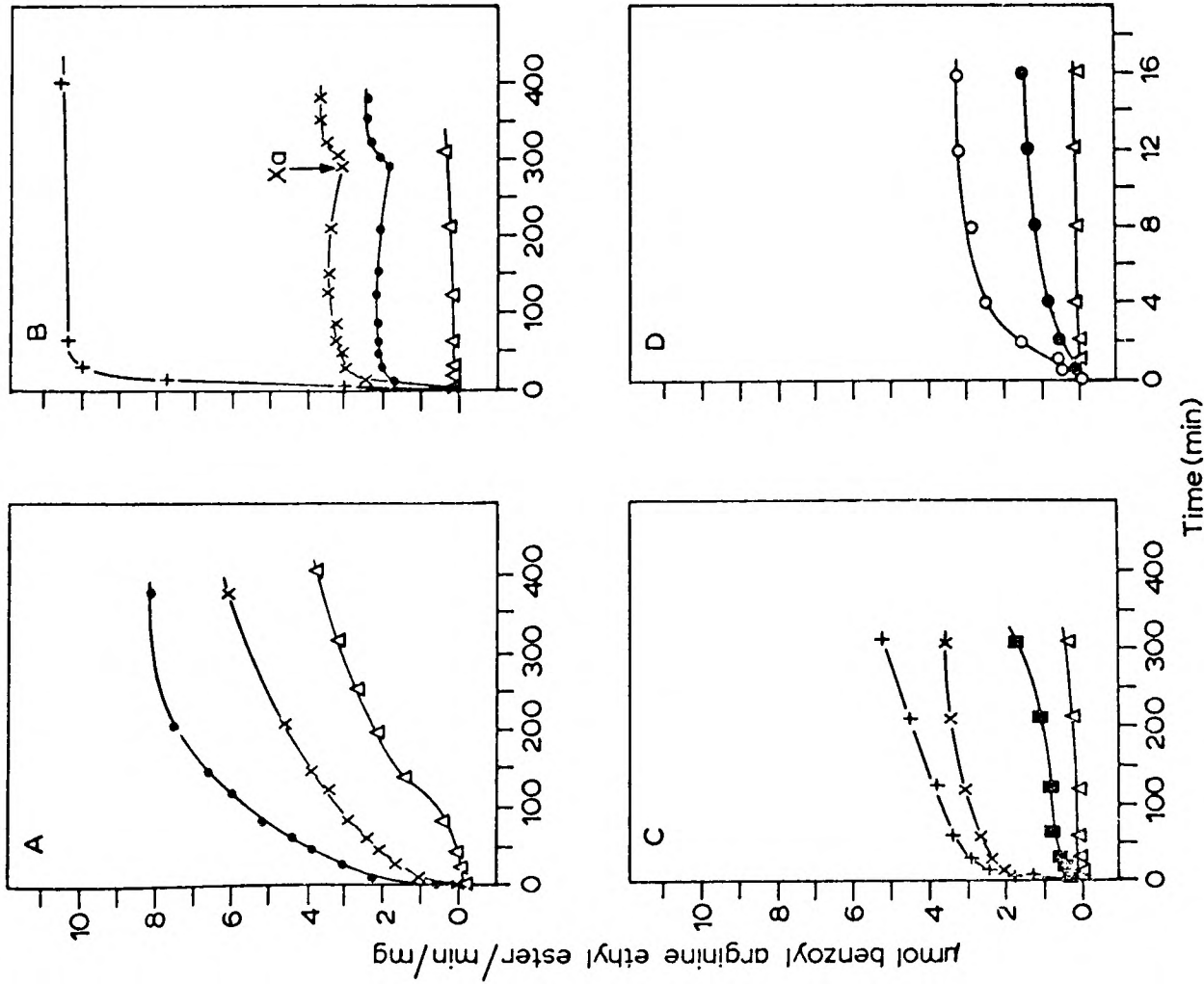


Fig. 5. Activation of various prothrombins; ester assay. (A) Activation by Factor X_a alone (prothrombin, 0.2 mg/ml; Factor X_a , 60 $\mu\text{g}/\text{ml}$) $\times$ — $\times$, normal; $\bullet$ — $\bullet$, -Ba; $\triangle$ — $\triangle$, -super (B) Activation by Factor X_a and calcium (prothrombin, 0.2 mg/ml; Factor X_a , 3 $\mu\text{g}/\text{ml}$; Ca^{2+} , 5 mM) $\times$ — $\times$, normal; $\bullet$ — $\bullet$, -Ba; $\triangle$ — $\triangle$, -super; $+$ — $+$, activation at equimolar Factor X_a . Sufficient Factor X_a to increase total concentration to 6 $\mu\text{g}/\text{ml}$ was added to normal and barium activations at the time shown. (C) Conditions as for B. $\times$ — $\times$, normal; $\bullet$ — $\bullet$, -super; $+$ — $+$, normal + super; $\blacksquare$ — $\blacksquare$, (normal + super) — normal (calculated). (D) Activation by Factor X_a , calcium and phospholipid (prothrombin, 0.2 mg/ml; Factor X_a , 0.01 $\mu\text{g}/\text{ml}$; Ca^{2+} , 5 mM and phospholipid, 0.3 mg/ml). $\times$ — $\times$, normal; $\bullet$ — $\bullet$, -Ba; $\triangle$ — $\triangle$, -super.

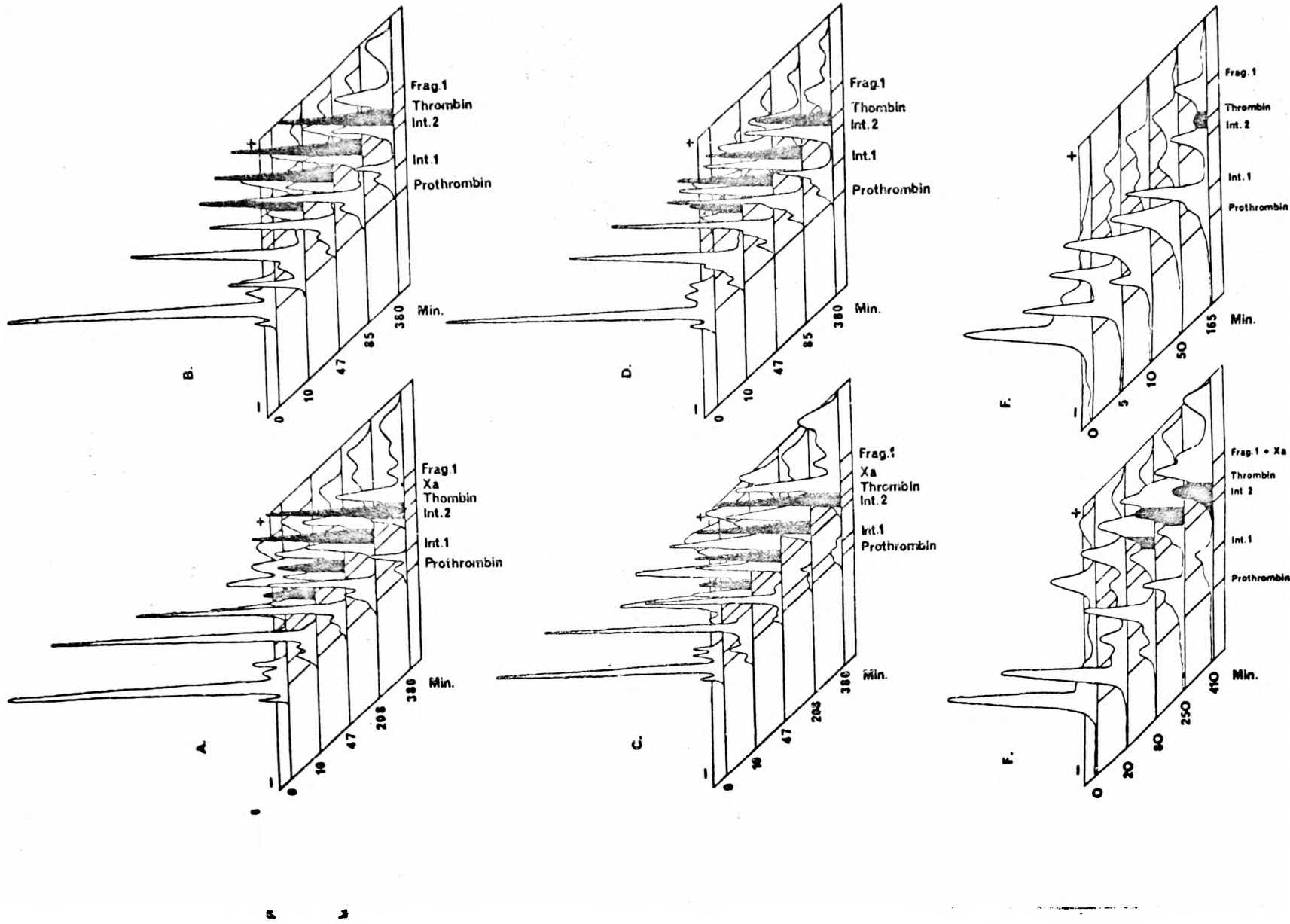


Fig. 6. Activation of various prothrombins; sodium dodecyl sulphate gel analysis of products. Conditions as described in Fig. 4. Factor X_a alone on normal (A), barium (C) and super (E) Factor X_a plus calcium on normal (B), barium (D) and super (F).

In all cases the dialysis of the prothrombin into 0.05 M Tris·HCl buffer resulted in a small amount of degradation, as can be seen from the zero time gel samples, and hence the time for dialysis was kept to a minimum. Inhibitors could not be used to reduce the degradation since we wished to measure esterase activities.

Incubation of normal prothrombin with Factor X_a in the absence of Ca^{2+} , results in a slow conversion of prothrombin to thrombin (Fig. 5A) the gels indicating the conversion to be almost complete after 380 min (Fig. 6A). The corresponding

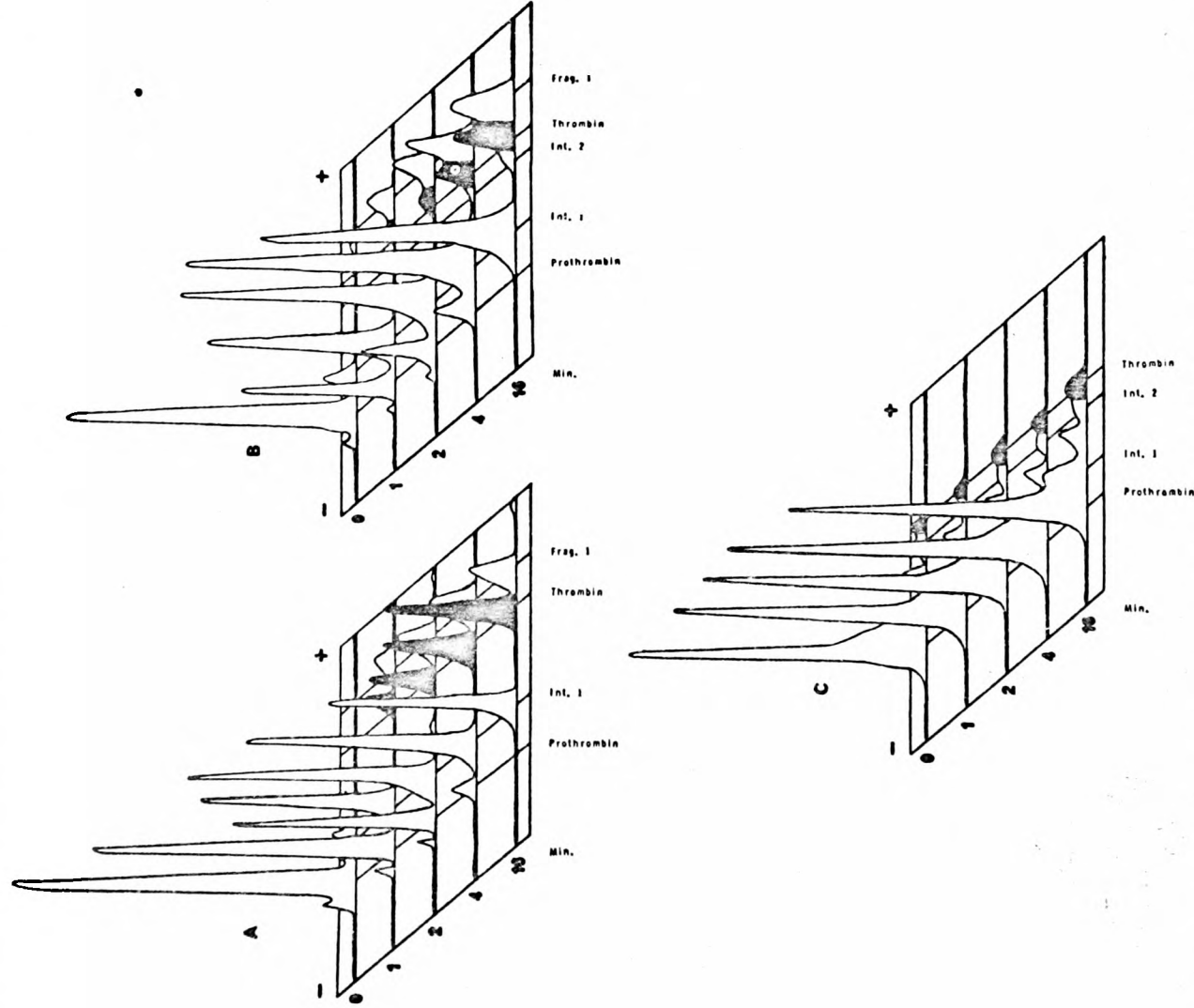


Fig. 7. Activation of various prothrombins by Factor X_a , calcium and phospholipid; sodium dodecyl sulphate gel analysis of products. Conditions as described in Fig. 4. A, normal; B, prothrombin-Ba; C, prothrombin-super.

activation of prothrombin-Ba is essentially similar (Figs. 5A and 6C) but apparently proceeds at a faster rate. Prothrombin-super, on the other hand, is cleaved more slowly and the activation plot is sigmoid in character. This may be due to the presence of a small amount of normal prothrombin in the prothrombin-super, an idea which is supported (Fig. 5C) by showing that the rate at which prothrombin-super is activated is enhanced by the addition of normal prothrombin. In these experiments relatively high concentrations of Factor X_a have been used and the heavy and light chains are clearly visible on the gels. The smaller peptides not identified in Fig. 5, include Factor X light chain, thrombin A chain, Fragment 2 and thrombin degradation products of Fragment 1.

In activation mixtures which include calcium the amount of Factor X_a used was reduced by 1/20 as the rate of activation of normal prothrombin could be conveniently followed. Factor X_a rapidly cleaves normal prothrombin until about a third of the potential thrombin has been formed (Fig. 5B) and then the activation stops, the remainder of the protein only being partially cleaved (Fig. 6B). Doubling the Factor X_a concentration after 300 min causes a further small burst in thrombin generation but this is soon inhibited. This implies that, at the concentration of Factor X_a used (3 μ g/ml) there is product inhibition [19]. If equimolar amounts of prothrombin and Factor X_a are incubated together then the conversion of prothrombin to thrombin is complete (Fig. 5B). The rate of conversion of prothrombin-Ba is also enhanced by the addition of calcium, and, as with normal prothrombin, the reaction does not go to completion (Figs. 5B and 6D). The rate of thrombin formation from prothrombin-super is not affected by the addition of Ca^{2+} (Figs. 5B and 6F) presumably due to the reduction in the calcium binding capacity in this protein [21].

One outstanding point from these activation studies is why the conversion of normal prothrombin by Factor X_a in the presence of calcium only goes to completion at a high concentration of Factor X_a . This is presumably due to product inhibition since the addition of further Factor X_a (Fig. 5B) does not yield much more thrombin. As this inhibition appears to be calcium dependent it would be reasonable to ascribe this effect to the calcium binding regions of prothrombin, i.e. Fragment 1 or Fragment 1:2 [22]. Thus the Fragment 1 formed during prothrombin activation competes for the Factor X_a , the effect of which is not seen when the prothrombin is activated with an equimolar mixture of Factor X_a .

The activation of the three forms of prothrombin in the presence of phospholipid and Ca^{2+} clearly show the gradation in activity (Fig. 4D) already shown by the tiger snake venom assay. The sodium dodecyl sulphate gels of these activations (Fig. 7) show that prothrombin-super (Fig. 7C) is not cleaved by Factor X_a , Ca^{2+} , phospholipid mixtures during the 16-min incubation period. The other two forms of prothrombin are cleaved, during a 16-min incubation, to Intermediate 1 and thrombin, but to different extents. This difference in rate of thrombin production between normal prothrombin and prothrombin-Ba is presumably due to a difference in calcium binding capacity of the two forms.

The Intermediate 1 arises from the degradation of the prothrombin by thrombin [22]. The principal difference, apart from rate, between activation in the presence of calcium with and without phospholipid is the accumulation of Intermediate 2 in the latter. In this case the Intermediate 2 would be cleaved to thrombin by Factor X_a [23] if it were not for the inhibition of the Factor X_a by the formation of a Fragment 1- Ca^{2+} -Factor X_a complex.

DISCUSSION

The evidence presented here shows that the prolonged administration of Warfarin to cows results in the production of a variant of prothrombin, in plasma, which, although it binds "normally" to insoluble barium salts, is only slowly activated physiologically. Similar results have been obtained with human prothrombin isolated from patients attending an anticoagulant clinic [8]. Our observations confirm those of Malhotra and Carter [7] who have also suggested that a low specific activity prothrombin is produced which has a "normal" reaction with insoluble barium salts.

The abnormal prothrombin isolated by Stenflo and Ganrot [6] and Nelsestuen and Suttie [5] is not only activated slowly by the physiological system, but is also not adsorbed onto insoluble barium salts. This defect has been correlated with a failure to carboxylate the γ -carbon atom of ten glutamic acid residues in the N-terminal region of the abnormal prothrombin [24, 25]. We suggest that prothrombin-Ba represents an intermediate state between normal prothrombin and that isolated by Stenflo and Ganrot [6] and Nelsestuen and Suttie [5]; and that Warfarin treatment can result in the formation of prothrombins with varying degrees of carboxylation rather than being an all or none effect.

In this context it is interesting to note that during chromatography on DEAE-Sephadex, the lower specific activity prothrombin is eluted first. This is consistent with this form of the protein having a less negative charge and hence a lower degree of carboxylation than normal. The DEAE-Sephadex elution profile of the prothrombin-Ba obtained on day 93 shows partial resolution into two components, which suggests that more than one form of prothrombin-Ba is present. On the basis of this hypothesis we have now obtained chymotryptic peptides from the Fragment 1 region of this material which are now being analysed. Preliminary results indicate that there are at least two peptides whose electrophoretic mobility indicates that they have been only partially carboxylated.

Equilibrium dialysis experiments [26] have shown that prothrombin has in the Fragment 1 region six binding sites which have a high affinity for Ca^{2+} ($\log K_{\text{assoc}} 3.5$) and four or five weaker binding sites ($\log K_{\text{assoc}} 2.7$) elsewhere in the molecule. Stenflo et al. [25] have shown that during dicoumarol therapy these high affinity binding sites are absent from prothrombin which will not bind to insoluble barium salts, nor does this prothrombin have any appreciable biological activity.

If prothrombin-Ba is only partially carboxylated it would be reasonable to suppose that it had properties intermediate between those of normal prothrombin and prothrombin-super. This is borne out by its ability to bind to barium salts and its failure to be activated normally. Thus when prothrombin-Ba is activated using Factor X_a , phospholipid and Ca^{2+} (or with the tiger snake venom) the reaction proceeds more slowly than with normal prothrombin. The activation of normal prothrombin and prothrombin-Ba by Factor X_a in the absence of Ca^{2+} does not show this difference in behaviour; in fact if anything it is inexplicably reversed. However, differences in the activation rates in the presence of Ca^{2+} can be explained on the basis that the phospholipid/calcium binding site of prothrombin is located in the Fragment 1 region [27] and hence differences in the calcium binding capacity of this portion of the molecule will be apparent in activation mixtures in which calcium is used.

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MATERIALS AND ABBREVIATIONS

<u>MATERIAL</u>	<u>ABBREVIATION</u>	<u>SUPPLIER</u>
<u>Chapter 2</u>		
Acid citrate dextrose anticoagulant	ACD	
DEAE-Sephadex		Pharmacia Ltd
2-amino, 2-hydroxymethyl propane, 1,3diol.	Tris	Sigma Chemical Co
Benzamidine hydrochloride	BZ	Aldrich Chemical Co
Tiger snake venom, dried	TSV	Mr. A. Softly
(Notechis scutatus Occidentalis)		Royal Perth Hospital, Perth, W. Australia
Inosithin		Associated Concen- trates Inc Woodside, NY, USA
Factor X-deficient plasma, charcoal filtered, bovine substrate plasma		Diagnostic Reagents Ltd Thame, Oxon
Russell's Viper venom- cephalin reagent	RVV-cephalin	" "
Standard Bovine thrombin (50 NIH units/ml)		" "
Fibrinogen, Grade 1 (approx 50% salts)		Kabi Pharmaceuticals Ltd
Barium sulphate (X-ray grade)		General Chemical Pharmaceutical Co
Acrylamide		BDH
N, N' methylenebisacrylamide	Bis	BDH
Sodium dodecyl sulphate	SDS	Sigma Chemical Co
Coomassie Brilliant Blue R		Sigma Chemical Co
Basic Fuchsin (for Schiff reagent)		BDH
Guanidinium hydrochloride (Aristar)	GuHCl	BDH

<u>MATERIAL</u>	<u>ABBREVIATION</u>	<u>SUPPLIER</u>
Lissamine Green		G. T. Gurr Ltd
Sepharose 6B		Pharmacia Ltd
SP-Sephadex		Pharmacia Ltd
Ethylene diamine tetra-acetic acid	EDTA	BDH
Aminex A4 and A5 resins		BioRad Laboratories
1-Dimethylamino, naphthelene, 5, sulphonyl chloride	Dansyl chloride (DNS-Cl)	Koch Light Laboratories
N(1-dimethylamino naphthelene, 5 sulphonyl) L amino acid	Dansyl amino acid	BDH
Blue dextran M		Pharmacia Ltd
Dinitrophenylalanine	DNP-ala	BDH
Polyamide sheets		Cheng Chin Trading Co, Taiwan
Amino acid calibration mixture		Calbiochem

Chapter 3

Rompun		Bayer Ltd, Germany
Freunds adjuvant		Difco Laboratories, Detroit
Agarose		Serva Entwicklungs labor, Heidelberg
sodium Warfarin as Marevan or BP		BDH Ward, Blenkinsop & co Ltd, London
QAE-Sephadex		Pharmacia Ltd
Ultrogel AcA44		LKB
Sephadex G-200		Pharmacia Ltd

<u>M A T E R I A L</u>	<u>A B B R E V I A T I O N</u>	<u>S U P P L I E R</u>
<u>Chapter 4</u>		
N-alpha benzoyl arginine ethyl ester	BAEe	Koch Light Laboratories
<u>Chapter 5</u>		
Hexamethyldisilazane	HMD) Packed	BDH
Trimethylsilane	) under TMS) N ₂	
3% OV-1 on Diatomite CQ 85-100 mesh		J J's Chromatography Kings Lynn
N-acetyl neuraminic acid	NANA(sialic acid)	Koch Light Laboratories
Glycine ethyl ester hydrochloride (¹⁴ C-labelled	GEE	BDH New England Nuclear)
1 ethyl, 3(3 dimethylamino propyl) carbodiimide hydrochloride	EDC	Sigma Chemical Co
Dowex 50 x-8 (20-50)		BioRad Laboratories
Chymotrypsin		Worthington Biochemical Co Ltd, NJ, USA
Pepsin		" "
Soya bean trypsin inhibitor	SBTI	" "
Diphenyl carbamoyl chloride	DPCC	Eastman Kodak, NY, USA
DE-52 cellulose (Microgranular)		Whatman Ltd
Phenanthrenequinone		Fluka
Sephadex G-10		Pharmacia Ltd
Dithiothreitol	DTT	BDH

<u>MATERIAL</u>	<u>ABBREVIATION</u>	<u>SUPPLIER</u>
<u>Chapter 6</u>		
Vitamin K ₁ as Konakion 10		Roche Products Ltd Welwyn Garden City
Biogel P-60		BioRad Laboratories
Conconavalin A linked to Sepharose	con A- Sepharose	Pharmacia Ltd
alpha-methyl-D-glucoside	α-Me-Glu	BDH
Russell's Viper venom (Vipera Russellii Russellii)	RVV	Prof. P.M. Dalal Haffkine Institute Bombay
2,5 bis (5' tert butylbenz oxazdyl(2')) Thiophene	BBOT	Sigma Chemical Co
<u>Appendices</u>		
Cyanogen bromide	CNBr	Koch Light Laboratories
Sepharose 4B		Pharmacia Ltd
Acetamido malonic acid diethyl ester		Sigma Chemical Co
Malonic acid diethyl ester		Sigma Chemical Co
Dimethylamine		BDH
Hydroquinone		BDH
L-fucose		Sigma Chemical Co
Dimethyl formamide	DMF	Koch Light Labora tories
Carbobenzoxy chloride	CBZ-Cl	BDH
Triethylamine		BDH
Isobutyl chloroformate		BDH
Epichlorohydrin (1 chloro 2,3 epoxypropane)		BDH

<u>M A T E R I A L</u>	<u>A B B R E V I A T I O N</u>	<u>S U P P L I E R</u>
Sodium borohydride		BDH
Sodium hydride		BDH
<u>Lotus tetragonobulus</u> seeds		Thompson & Morgan Ltd, Ipswich
Linde molecular sieve 5A		BDH

All other chemicals were of the highest grade
available, usually Analar.

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