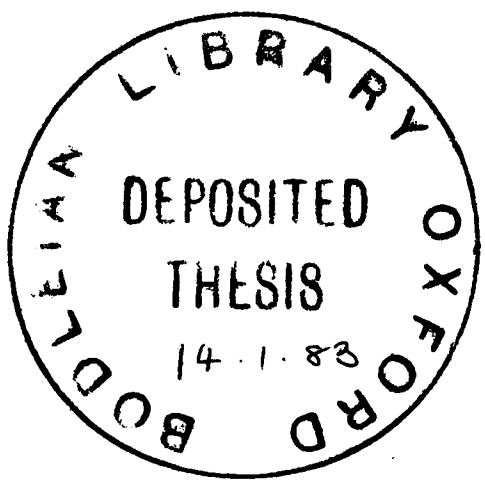
The Role of Factor VIII in Blood Coagulation.

A Thesis Submitted for the Degree of

Doctor of Philosophy

by

G G Neal



New College,

Oxford.

February 1982.

"'Sblood, there is something in this more than natural, if philosophy could find it out."

Hamlet. Act II, Sc ii.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr M P Esnouf, for all his encouragement and assistance during the time when this work was being carried out. His patience when progress was slow, and his understanding of my desire to pursue leather spheres and ovoids around green or muddy fields were particularly appreciated.

I would also like to thank Dr Stephen Chavin for introducing me to the field of factor VIII research and for collaborating in my early attempts to purify the activity. In addition to Steve, everyone else - Alex, Ann, Annette, Bent, Brent, Dave, Ed, Jean, Marwan, Mike, Modris, Rachel, Steve W, and T C - made the laboratory a pleasant, happy place to work; my thanks to them all.

The support of my parents has been a source of inspiratio throughout my education, and especially during my time in Oxford. My gratitude to them is immeasurable.

Jean Smith, who typed this thesis, deserves the highest praise, not only for her skill with the typewriter, but also for her accomplished transcription of my much-altered hand-written draft into a legible format.

I must also thank Prof P J Randle in whose department this work was carried out.

The financial support for this work was provided by the Medical Research Council, to whom I am most grateful.

ABSTRACT

THE ROLE OF FACTOR VIII IN BLOOD COAGULATION

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submitted for the Degree of Doctor of Philosophy
Hilary Term 1982

Factor VIII, a component of the intrinsic pathway of blood coagulation, has yet to be purified to homogeneity. It appears that, *in vivo*, the factor VIII coagulant protein is closely associated with one or more other proteins (factor VIII-related antigen and platelet aggregating factor). The material normally isolated from bovine plasma as 'factor VIII' possesses all three activities and is therefore either a mixture or a complex of the various proteins. In the present study, bovine factor VIII:C was purified approximately five-thousand-fold by a combination of ion-exchange chromatography and fractional precipitation. The factor VIII coagulant activity can be separated from the other activities of the 'factor VIII complex' but the procedures involved are not suitable for preparative use as the factor VIII:C which is obtained is unstable.

During coagulation, factor VIII:C is required during the activation of factor X. Studies with purified bovine clotting factors indicate that factor IX_a is the enzyme responsible for the cleavage of factor X, in a calcium-dependent reaction which is stimulated by phospholipid. Factor VIII:C further accelerates the rate at which factor X_a is generated. Preliminary investigations of the kinetic parameters of the reaction indicate that the stimulation by factor VIII:C occurs through a marked increase in the V_{max} of the reaction; factor VIII:C does not affect the K_m for factor X.

The coagulant activity of factor VIII is enhanced by exposure to thrombin, but the 'activated' factor VIII:C which is produced is not itself capable of activating factor X in the absence of factor IX_a. Thus, the 'activation' of factor VIII:C, in contrast to the activation of, for example, factors IX and X, does not appear to result in the formation of an enzyme. That is, factor VIII:C is a non-enzymic, high molecular weight cofactor for factor IX_a.

The Role of Factor VIII in Blood Coagulation

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Chapter 1

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INTRODUCTION

Early Theories of Blood Coagulation

Scientific investigations of the process of blood coagulation have been in progress for over one hundred years. During the course of the Croonian lecture to the Royal Society, Lister (1863) noted that 'soon after blood has been shed from the body, it passes from the fluid into the solid form. This depends on the development in the blood of a solid material termed "fibrin".' He showed experimentally that the coagulation of the blood did not depend on exposure to the air, nor on the release of ammonia, nor on the flow being stopped and the blood coming to rest. Upon contact with a foreign solid, however, a reaction between the corpuscles and the fluid components of the blood was said to cause the latter to coagulate.

Much of the early work on blood coagulation was carried out in Germany. Schmidt (1872) was the first author to suggest that enzymes were involved in clotting, when he used the term 'fibrin ferment' to describe the coagulant material which was precipitated from serum by the addition of alcohol. Hammarsten (1879) separated fibrinogen from plasma and subsequently (1896) demonstrated that its conversion to fibrin did not require calcium. Thus, the observation of Arthus and Pagès (1890) that coagulation was a calcium-dependent process reflected the conditions necessary for the formation of thrombin rather than its action on fibrinogen.

Morawitz (1905) proposed a mechanism for coagulation involving two enzymes: thrombokinase and thrombin, the former

being liberated from damaged cells or platelets and producing the latter via a reaction with the inactive plasma precursor, prothrombin. The 'classical', four-factor theory of coagulation (Figure 1.1), based on this proposal was widely accepted. Although several authors (Mellanby, 1933; Eagle, 1935) agreed that the action of thrombin on fibrinogen was enzymic in nature, others (Pickering, 1928; Nolf, 1938) concluded on the basis of an analogy with the formation of complexes between antigens and antibodies, which was much in vogue at the time, that the final stage in coagulation involved a combination of thrombin with fibrinogen. The hypothesis that the formation of a clot depended on the combination of thrombin with fibrinogen was, however, generally rejected following the preparation of thrombin by Mellanby (1933) and Seegers (1940), who showed that the interaction of the proteins was not stoichiometric, and the demonstration by Lorand (1952) and Lorand and Middlebrook (1952) that thrombin cleaved the fibrinogen molecule.

The Recognition of the Other Clotting Factors

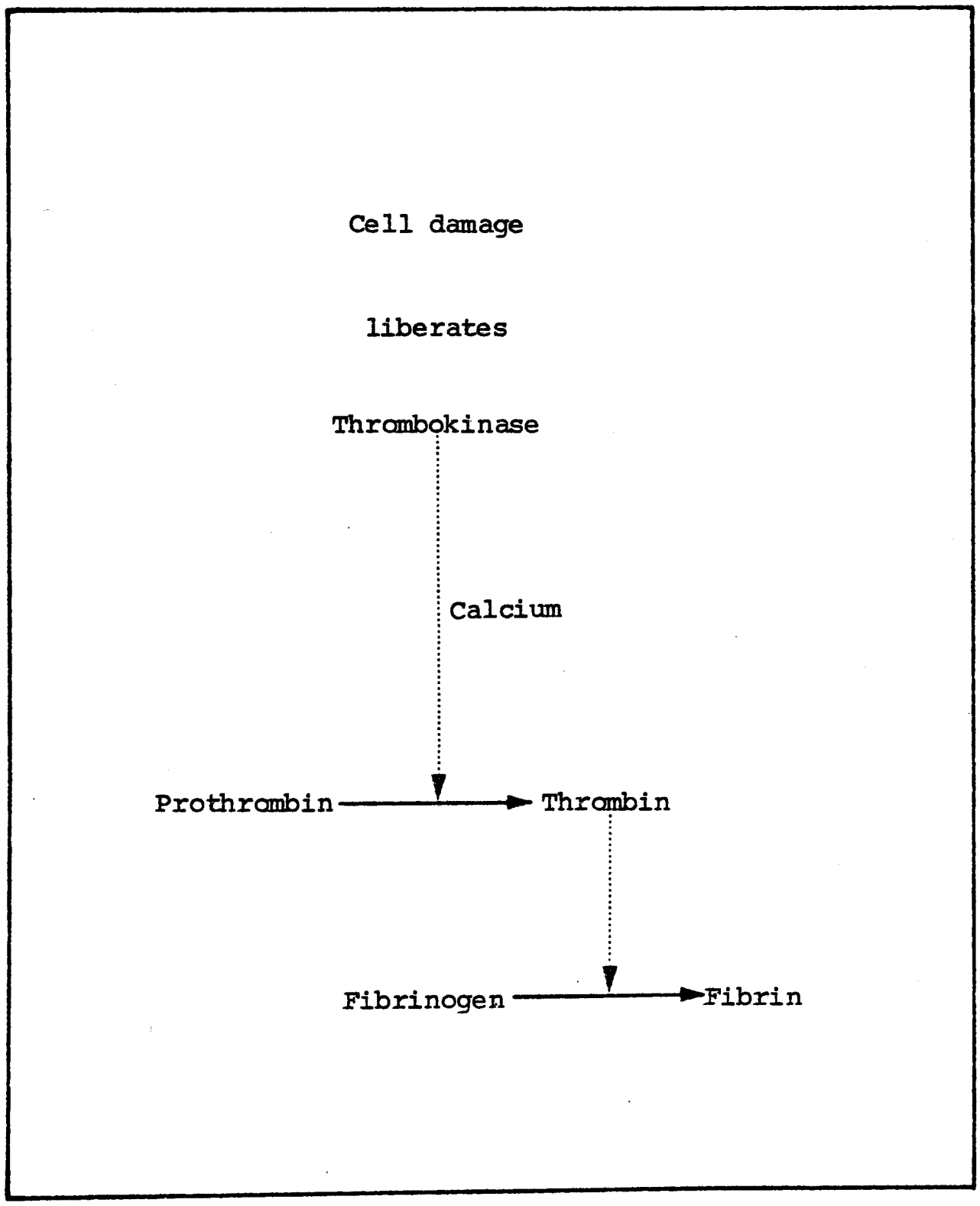
It had been established by 1940 that the final stages of clotting required one enzyme (thrombokinase) and calcium ions to generate a second enzyme (thrombin) which converted soluble fibrinogen into insoluble fibrin. This four-factor theory did not, however, account for the observation that blood coagulated rapidly following contact with a foreign surface such as glass (Lister, 1863).

The introduction of the one-stage prothrombin time assay (Quick *et al*, 1935), which was based on the classical four-factor

FIGURE 1.1

The Classical Four-Factor Theory of Blood Coagulation

(After Morawitz, 1905)



theory, led to the extension of the scheme. This assay required the addition of rabbit brain thromboplastin and calcium chloride to plasma samples containing oxalate as an anticoagulant. It was assumed that the time taken for the plasma fibrinogen to clot, in the presence of an excess of the other components, would be proportional to the concentration of prothrombin in the plasma. Several anomalies in the results of prothrombin time assays of plasma samples could be explained only on the basis of the existence of more factors than the four envisaged in the classical theory. Owren (1947), for example, found that the prothrombin time of normal plasma increased as it was aged, and that this could be corrected by the addition of fresh plasma from which the 'prothrombin' had been removed by adsorption with alumina. He also described a patient whose plasma had a prolonged prothrombin time which was shortened by the addition of alumina-adsorbed normal plasma. Owren (1947) therefore proposed that plasma contained a labile factor, which he termed factor V; he suggested that factor V was converted to an enzyme (factor VI) by thromboplastin, and that factor VI subsequently activated prothrombin.

The investigation of the coagulation of blood following its exposure to a foreign surface produced a number of radical developments. Many reports of previously undescribed clotting factors appeared in the literature and much confusion arose as a result of the use by different groups of their own terminologies. Thus, the same protein might be referred to by a different name in several laboratories, or in some cases, similar names were applied to different proteins. The situation was clarified by

the introduction (Wright, 1959) of a system of Roman numerals for the nomenclature of the clotting factors. Table 1.1 lists the factors involved in coagulation, together with many of the common synonyms.

In addition to the Quick prothrombin time test, two further clotting assays yielded results which led to the recognition of new clotting factors. Witts and Hobson (1942) introduced a modified version of the prothrombin time test in which coagulation was initiated with Russell's viper venom rather than brain thromboplastin. The thromboplastin generation test (Biggs and Douglas, 1953) permitted the study of the components involved in the production of the activator of prothrombin, and made it possible for those factors present in alumina-adsorbed plasma to be distinguished from those remaining in serum.

The existence of factor VII was deduced by Owen and Bollman (1948) who observed that the Quick one-stage prothrombin time was prolonged in dogs treated with the anticoagulant dicoumarol, and that the time could be shortened by normal plasma or serum. They therefore suggested that dicoumarol reduced the plasma level of both prothrombin and a 'prothrombin conversion factor' which - unlike prothrombin - was present in serum. Koller *et al* (1951) advocated the use of the term factor VII for this material.

Factor VIII had long been recognised as the antihaemophilic factor, but it was rigidly defined only when true haemophilia A was distinguished from Christmas disease by Biggs *et al* (1952), who used the thromboplastin generation test to differentiate

TABLE 1.1

Synonyms for the Coagulation Factors

Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue factor (TF) Tissue extract Thromboplastin
Factor IV	Calcium Ions
Factor V	Thrombogène Accelerator globulin Plasma prothrombin conversion factor (PPCF) Proaccelerin Labile factor
Factor VII	Proconvertin Serum prothrombin conversion accelerator (SPCA) Autoprothrombin I Co-thromboplastin Stable factor
Factor VIII	Antihaemophilic factor A (AHF) Antihaemophilic globulin (AHG) Platelet cofactor I
Factor IX	Christmas factor Plasma thromboplastin component (PTC) Platelet cofactor II Antihaemophilic factor B Autoprothrombin II
Factor X	Stuart-Prower factor Prothrombokinase Autoprothrombin III

TABLE 1.1 (CONTINUED)

Factor XI	Plasma thromboplastin antecedent (PTA)
Factor XII	Hageman factor
Factor XIII	Fibrin stabilising factor (FSF) Fibrinoligase Plasma transglutaminase
-	Prekallikrein Fletcher factor
-	HMW Kininogen Contact activation cofactor Flaujeac factor Fitzgerald factor Reid factor Williams factor

between factor VIII and factor IX.

Two groups discovered factor X as a result of independent investigations of patients with mild haemorrhagic disorders. Telfer *et al* (1956) studied a Miss Prower, whose prolonged one-stage prothrombin time was corrected by factor VII-deficient plasma from people being treated with the anticoagulant phenylindandione. Hougie *et al* (1957) discovered that plasma from a Mr Stuart, who had been diagnosed previously as factor VII-deficient, would correct the prolonged one-stage prothrombin time of true factor VII-deficient plasma. In addition, the thromboplastin generation test was abnormal in both of these patients, and Mr Stuart's plasma had a slow clotting time with Russell's viper venom. These results provided further evidence for the existence of factors VII and X as separate entities.

Factor XI (Rosenthal *et al*, 1953) and factor XII (Ratnoff and Colopy, 1955) were originally recognised when plasma from patients with deficiencies of these factors gave abnormal results in *in vitro* assays. The involvement of prekallikrein (Hathaway *et al*, 1965) and high molecular weight kininogen (Schiffman and Lee, 1974) in blood clotting was discovered in a similar manner.

Robbins (1944) observed that clots formed by the action of thrombin on fibrinogen are soluble in dilute acids and alkalis, whereas those formed in the presence of calcium and serum are insoluble. Laki and Lorand (1948) suggested that a thermolabile component of serum was necessary for the production of a fibrin clot that was insoluble in urea and Lorand and Jacobsen (1958) showed that this component - factor

XIII - was responsible for the cross-linking of fibrin.

Haemophilia

Haemophilia A, which is caused by a deficiency of factor VIII, is the most common of the hereditary coagulation defects (Macfarlane, 1978). In the British population, approximately 60 people per million are affected by haemophilia A, whereas only five per million suffer from factor IX deficiency, and the total number affected by other hereditary defects of the coagulation system is less than two per million.

The existence of an hereditary bleeding disorder, which was inherited by males from asymptomatic females, and was probably haemophilia, was recognised nearly two thousand years ago in the Jewish Rabbinic writings. The fifth century Babylonian Talmud (Epstein, 1936) quotes two cases from the 'Mishnah', a second century A.D. compilation of the Jewish law. Firstly, Rabbi Judah the Patriarch ruled that if a woman had lost two sons as a result of bleeding after circumcision, she must not circumcise a third son. Secondly, Rabbi Simeon ben Gamaliel decreed that a woman from Sepphoris, whose three sisters' sons had died after the operation, should not circumcise her own child. Similar rulings on circumcision were quoted in subsequent Rabbinic writings from the eleventh century onwards (Rosner, 1969).

In other cultures, the famous Islamic surgeon of the tenth century, Khalaf ibn Abbas - known in the West as Albucasis - recognised a similar condition. In his encyclopedia of medicine and surgery entitled 'Al Tasrif', he described the males of a

certain village who bled to death from trivial wounds (Major, 1954).

The best-known examples of haemophilia arose in England in the nineteenth century, where Queen Victoria was a carrier of the condition and transmitted it to several European Royal families (Ingram, 1976). In particular, her daughter Alexandra married Tsar Nicholas II of Russia and their son, the Tsarevich Alexis, was a haemophiliac whose condition undoubtedly influenced Russian politics at the time of the Revolution.

Bulloch and Fildes (1911), in London, collated approximately one thousand reports in the European literature and classified the coagulation defects which these described. Prior to this time, the term 'haemophilia' had been used in a very general sense to describe a variety of bleeding disorders. Bulloch and Fildes (1911) proposed a more specific definition of haemophilia as 'an *inherited* tendency in *males* to *bleed*'. Genetically, haemophilia shows a classical X-linked recessive pattern of inheritance. Under normal circumstances, only males are affected clinically while females are asymptomatic 'carriers' who transmit the condition to their sons. Occasionally, however, haemophilic girls are born in families where the mother is a carrier and the father has haemophilia; the earliest case of this type appears to be that described by Israëls *et al* (1951).

In the absence of suitable laboratory data, Bulloch and Fildes (1911) were unable to differentiate between deficiencies of factors VIII and IX. The finding (Pavlovsky, 1947) that the mixing of blood from two unrelated haemophiliacs could result in the mutual correction of the clotting defects, indicated

that at least two types of haemophilia must exist. Patek and Taylor (1937) had already shown that the abnormal clotting time of most haemophilic plasmas could be shortened by the addition of the 'globulin' fraction of normal human plasma. Seven patients examined by Biggs *et al* (1952) appeared to be suffering from haemophilia, but had normal antihaemophilic globulin levels, and were therefore diagnosed as being deficient in 'Christmas factor' which is present in serum and can be adsorbed on to alumina. The use of the thromboplastin generation test (Biggs and Douglas, 1953) permitted a clear differentiation of haemophilia A, caused by factor VIII deficiency, from haemophilia B or Christmas disease, resulting from a deficiency of factor IX. Today, specific clotting assays can be used to define these bleeding disorders and replacement therapy with concentrates of the appropriate factor is available for the treatment of patients.

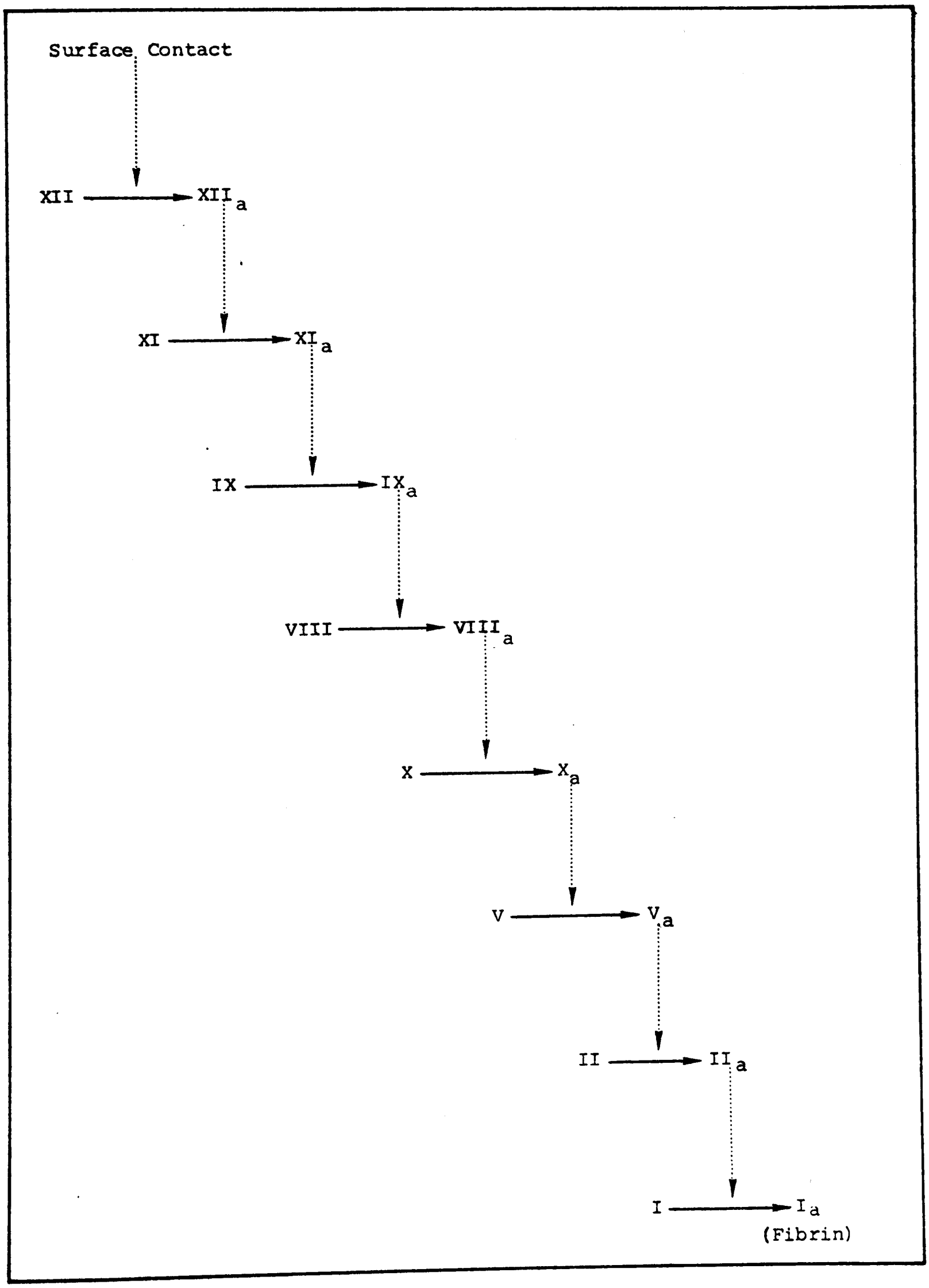
The 'Cascade' Hypothesis

It had been established by 1964 that at least ten proteins, in addition to 'tissue factor' and calcium ions, were involved in the coagulation process. It was also known that many of these proteins could be activated to give enzymes with esterase activity. Macfarlane (1964) and Davie and Ratnoff (1964) independently proposed that the clotting mechanism was a cascade of proenzyme-enzyme transformations, with each enzyme activating the next in the sequence until the final substrate, fibrinogen, was cleaved by thrombin. The 'cascade' schemes (Figure 1.2) consider only the sequence of reactions leading to the formation

FIGURE 1.2

The Cascade Model of the Blood Coagulation Mechanism

(Macfarlane, 1964)



of a clot following contact with a foreign surface. This 'intrinsic pathway' does not involve factor VII or tissue factor which are responsible for the activation of factor X via the 'extrinsic pathway' which is initiated by tissue damage (Macfarlane and Ash, 1964; Davie and Ratnoff, 1964).

The sequence of reactions between the factors of the intrinsic pathway provides a mechanism whereby a large quantity of fibrinogen can be converted rapidly into fibrin. The cascade functions as a biochemical amplifier, with the multiple stages giving a corresponding increase in the response to a small initial stimulus. This property is emphasised by the concentrations of the various factors in plasma: those later in the cascade are present at higher concentrations than those which are active in the early stages.

Shortly after the cascade hypothesis was proposed, it became apparent that the model was an over-simplification of the physiological system. In particular, Hougie *et al* (1967) and Østerud and Rapaport (1970) suggested that the activation of factor X might be brought about by a complex of factors IX_a and VIII, rather than 'factor $VIII_a$' alone. Prothrombin is converted to thrombin via an analogous mechanism; in this case, the active principle is a complex of factors X_a and V (Papahadjopoulos and Hanahan, 1964; Esnouf and Jobin, 1965; Jobin and Esnouf, 1967).

The cascade scheme was further complicated by the discovery that fibrinogen is not the only protein in the coagulation system which is a substrate for thrombin. Lorand and Jacobsen (1958) suggested that thrombin acted enzymically on

factor XIII and it was subsequently shown (Lorand and Konishi, 1964) that this reaction produced factor $XIII_a$, an enzyme with transglutaminase activity. It was also demonstrated by Rapaport *et al* (1963) that trace amounts of thrombin enhanced the activity of factors V and VIII in a reaction which has recently been shown to depend on the proteolysis of factor V (Esmon, 1979; Nesheim and Mann, 1979) or factor VIII (Vehar and Davie, 1980).

The recognition of these reactions led to the proposal (Esnouf, 1977) of the modified cascade scheme shown in Figure 1.3. This model included both the intrinsic and extrinsic pathways for the activation of factor X, but the two pathways were rigidly separated.

Current Theories of Blood Coagulation

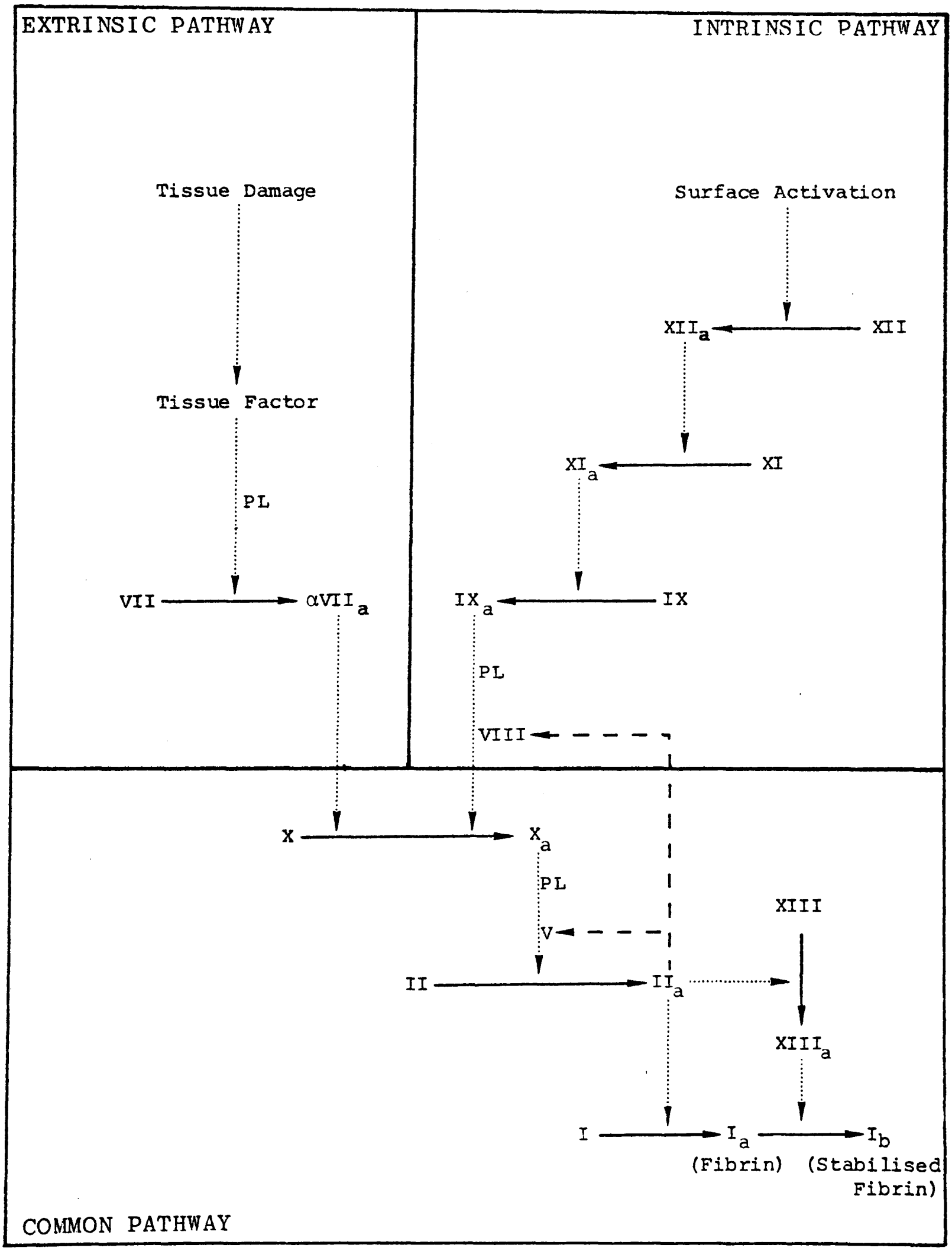
During the last ten years, it has become apparent that the arbitrary division of the early stages of coagulation into the intrinsic and extrinsic pathways has no physiological significance: the components of the two pathways interact at several points in the clotting process. Factor VII can be activated by either factor IX_a or factor XII_a (Seligsohn *et al*, 1979). Activated factor VII in combination with tissue factor will activate factor IX as well as factor X (Østerud and Rapaport, 1977, 1980). In plasma, the factor IX_a generated by this reaction will itself activate factor X in the presence of factor VIII (Østerud, 1980); the components of both the 'extrinsic' and 'intrinsic' pathways must be present in order for factor X to be activated at the fastest possible rate.

Recent observations have established that several stages

FIGURE 1.3

Modified Version of the Cascade Model of Blood Coagulation

(Esnouf, 1977)



of coagulation provide control points at which the activation of individual zymogens is either stimulated by positive feedback reactions or inhibited by negative feedback. The control of coagulation by a 'self damping' mechanism was first proposed by Nemerson *et al* (1974) who showed that thrombin could cleave prothrombin to yield prethrombin 1 and fragment 1. Prethrombin 1 is converted to thrombin only very slowly by the physiological activating system while fragment 1 competes with prothrombin for the binding site in the activator complex and thus further reduces the rate at which thrombin is generated. The most obvious example of a positive feedback system is provided by the reactions involved in the contact phase of coagulation during which there is a reciprocal activation of factor XII and prekallikrein (Cochrane and Griffin, 1979).

In view of the complexity of many of the reactions in coagulation, with interactions between proteins - and frequently phospholipids - being important for obtaining rapid rates of activation of the zymogens, the simple cascade model has now been superceded by a model in which there are discrete reaction complexes and reaction stages (Jackson and Nemerson, 1980). The coagulation system can be divided broadly into the contact phase, the activation of factor IX, the activation of factor X, the activation of prothrombin, and the formation of the fibrin clot.

The contact phase of coagulation involves factors XII and XI, together with prekallikrein and high molecular weight kininogen. *In vitro*, a sequence of reactions among these proteins is initiated by contact with a negatively-charged

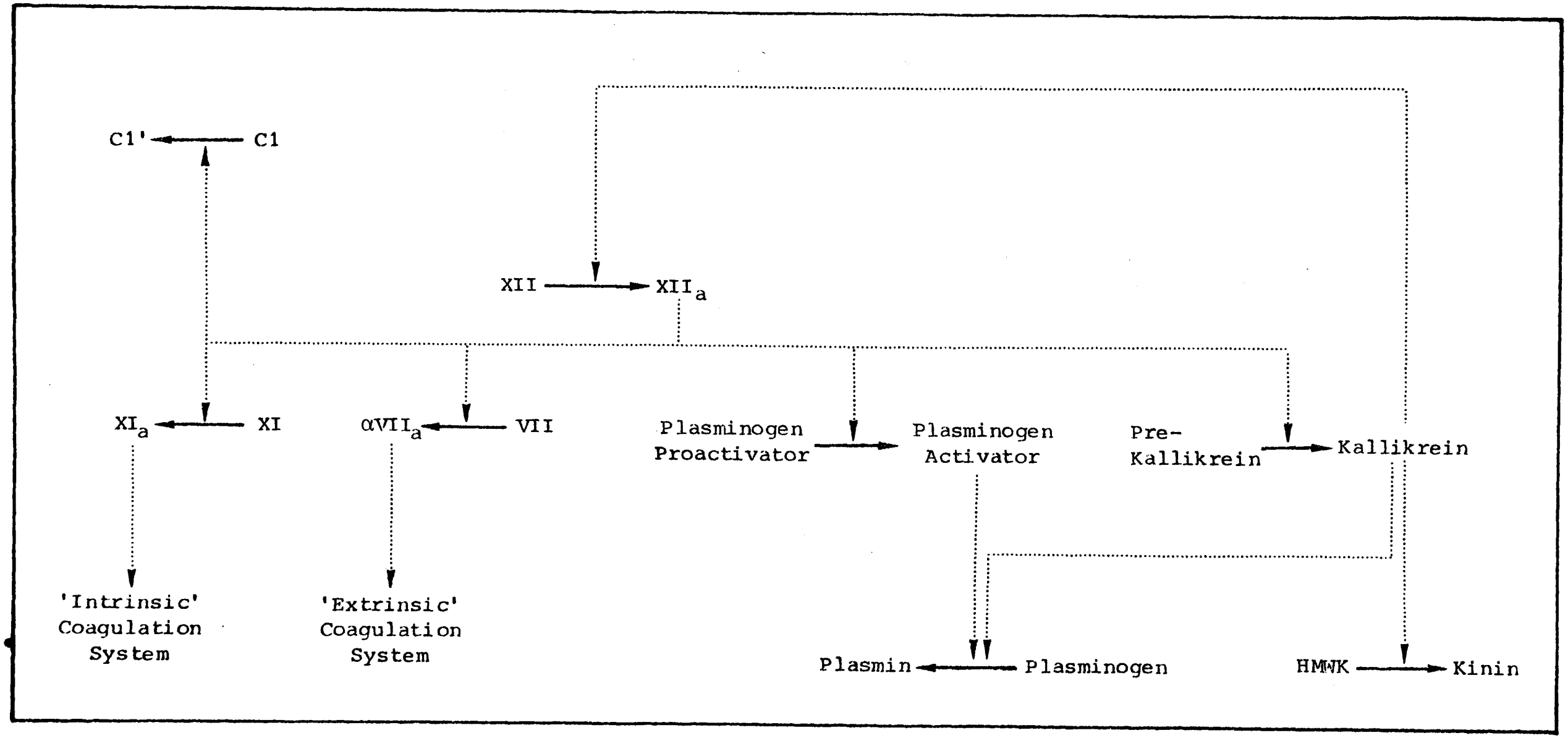
foreign surface such as glass or kaolin (Nossel, 1964), as a result of which factor XII is slowly activated. The physiological role of factor XII is, however, less well established: factor XII-deficient plasma has a prolonged clotting time, but patients who are deficient in factor XII have no haemorrhagic tendency (Ratnoff and Colopy, 1955). Activated factor XII converts prekallikrein to kallikrein, which will itself activate factor XII, suggesting (Cochrane and Griffin, 1979) that the interaction of prekallikrein and factor XII results in a reciprocal activation, thus amplifying the response in the early stages of coagulation. The activation of factor XII by kallikrein is greatly enhanced by the presence of high molecular weight kininogen which binds to the negatively-charged surface adjacent to the factor XII (Griffin and Cochrane, 1976). High molecular weight kininogen also has binding sites for both prekallikrein (Mandle *et al*, 1976) and factor XI (Thompson *et al*, 1977) and functions as a non-enzymatic surface co-factor assisting in the assembly of a complex of the other proteins of the contact phase. The final reaction of the contact system which is of importance in coagulation is the activation of factor XI by factor XII_a. Factor XII_a exists in two forms: a high molecular weight species (αXII_a) which remains bound to the surface, and a low (28 000) molecular weight fragment termed βXII_a which is released into solution. αXII_a preferentially activates factor XI (Cochrane and Griffin, 1979) and the factor XI_a which is formed also remains bound to the surface ensuring that the production of a clot is localised at the site of injury. βXII_a, however, activates prekallikrein

in solution and the kallikrein formed can initiate the generation of kinins and of plasmin throughout the bloodstream. In addition to the activation of coagulation, kinin-generation, and fibrinolysis via the production of kallikrein (Heimark *et al*, 1980), factor XII_a has also been implicated in the activation of the first component of the complement system (Donaldson, 1968) and of the vascular permeability factor, PF/dil (Ratnoff and Miles, 1964). The various consequences of the generation of activated factor XII are shown in Figure 1.4, which emphasises the potential controls available in haemostasis, with the initiation of coagulation at one site on a surface being accompanied by the initiation of the fibrinolytic system in solution.

The activation of the contact phase of coagulation eventually results in the production of activated factor VII and activated factor XI. Both factor XI_a and factor VII_a - in the presence of tissue factor - will activate factor IX (Fujikawa *et al*, 1974b; Østerud and Rapaport, 1977); these reactions require calcium. The activation of factor IX by either of these physiological systems involves the cleavage of two peptide bonds, resulting in the conversion of single-chain factor IX into two-chain factor $IX_{a\beta}$, with an activation peptide being released. In contrast, the activation of factor IX by an enzyme from Russell's viper venom involves only one cleavage and the 'activation peptide' remains attached to the light chain of the factor $IX_{a\alpha}$ molecule which is formed (Lindquist *et al*, 1978). Both forms of factor IX_a have coagulant activity but the specific activity of factor $IX_{a\alpha}$ is one half

FIGURE 1.4

Reactions of the Contact Phase of Coagulation



of that of factor $IX_{a\beta}$.

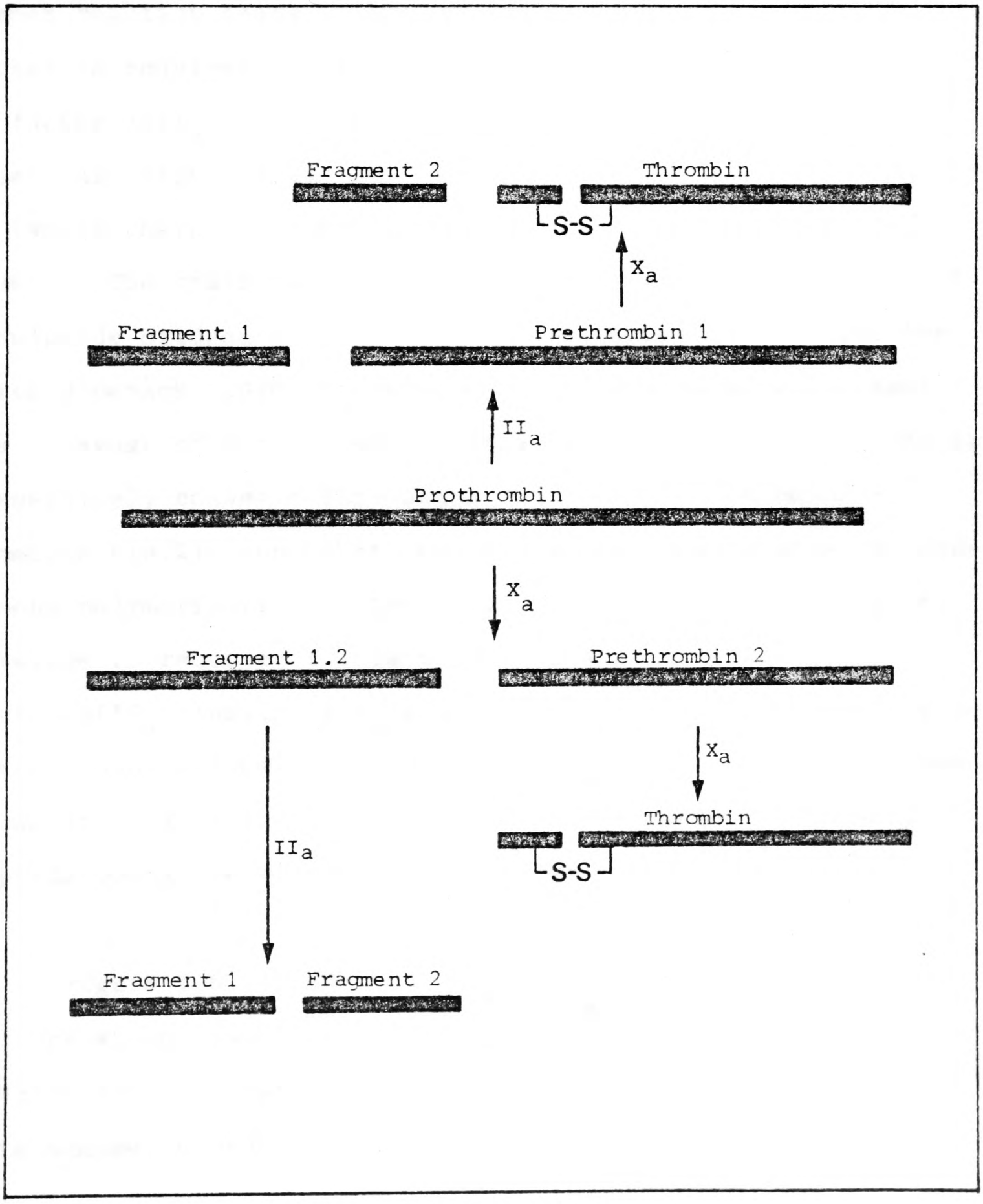
Factor X, like factor IX, can be activated by components of either the intrinsic or extrinsic pathways. In the presence of calcium ions, factor X is converted to factor $X_{a\alpha}$ by factor VII_a and tissue factor, or by factor IX_a, factor VIII and phospholipid (Radcliffe and Barton, 1973; Fujikawa *et al*, 1974a). Both of these systems cleave a peptide bond in the heavy chain of factor X, releasing an activation peptide. An identical form of factor $X_{a\alpha}$ is produced by the action of non-physiological activators such as Russell's viper venom or trypsin (Radcliffe and Barton, 1973). Factor X_a is a less specific proteinase than several of the enzymes of the coagulation system such as factor IX_a. In the presence of calcium and phospholipid, factor X_a will cleave a small peptide from the C-terminal of either factor X or factor X_a, giving rise to forms of the molecules termed factor X_β and factor $X_{a\beta}$ respectively (Fujikawa *et al*, 1974a; Jesty *et al*, 1974); this autolytic cleavage has no effect on the specific activity of the factor X. Factor X_a can activate factor VII (Radcliffe and Nemerson, 1975) in a reaction which appears to involve the formation of a ternary complex of factor X_a, tissue factor and factor VII (Morrison - Silverberg and Jesty, 1981). It has also been reported by Vehar and Davie (1980) that factor X_a can 'activate' factor VIII. Thus, factor X_a can accelerate the rate of its own production by both the intrinsic and extrinsic pathways.

The most important role of factor X_a in coagulation is,

however, its action on prothrombin. Factor X_a alone will activate prothrombin (Jobin and Esnouf, 1967), but only at a very slow rate; the efficient, rapid conversion of prothrombin to thrombin requires a complex of factor X_a, factor V, calcium ions and phospholipid (Papahadjopoulos and Hanahan, 1964; Jobin and Esnouf, 1967). Rosing *et al* (1980) have recently examined the kinetics of the activation of prothrombin and their data suggest that phospholipid principally reduces the K_m for prothrombin whereas the factor V increases the V_{max} of the reaction. Factor X_a is responsible for two cleavages in the prothrombin molecule (Figure 1.5). The first cleavage produces fragment 1.2 and prethrombin 2, and the second cleavage, which occurs within a disulphide loop, converts the latter into thrombin (Esmon *et al*, 1974). Thrombin itself will cleave fragment 1.2 into fragment 1 and fragment 2, and will also cleave the same bond in the intact prothrombin molecule, converting it to prethrombin 1 and fragment 1. The fragment 1 region of prothrombin is required for binding to phospholipids (Esmon and Jackson, 1974) and therefore prethrombin 1 has a reduced phospholipid-binding capacity. Its production thus lowers the rate of formation of thrombin since the activation of prethrombin 1 is much slower than that of prothrombin (Nemerson *et al*, 1974). This 'self-damping' mechanism provides a negative feedback control on the formation of thrombin; there is also a form of positive feedback control resulting from the increase in the activity of factors V and VIII when these factors are exposed to traces of thrombin (Rapaport *et al*, 1963).

FIGURE 1.5

Cleavage of Prothrombin by Factor X_a and Thrombin



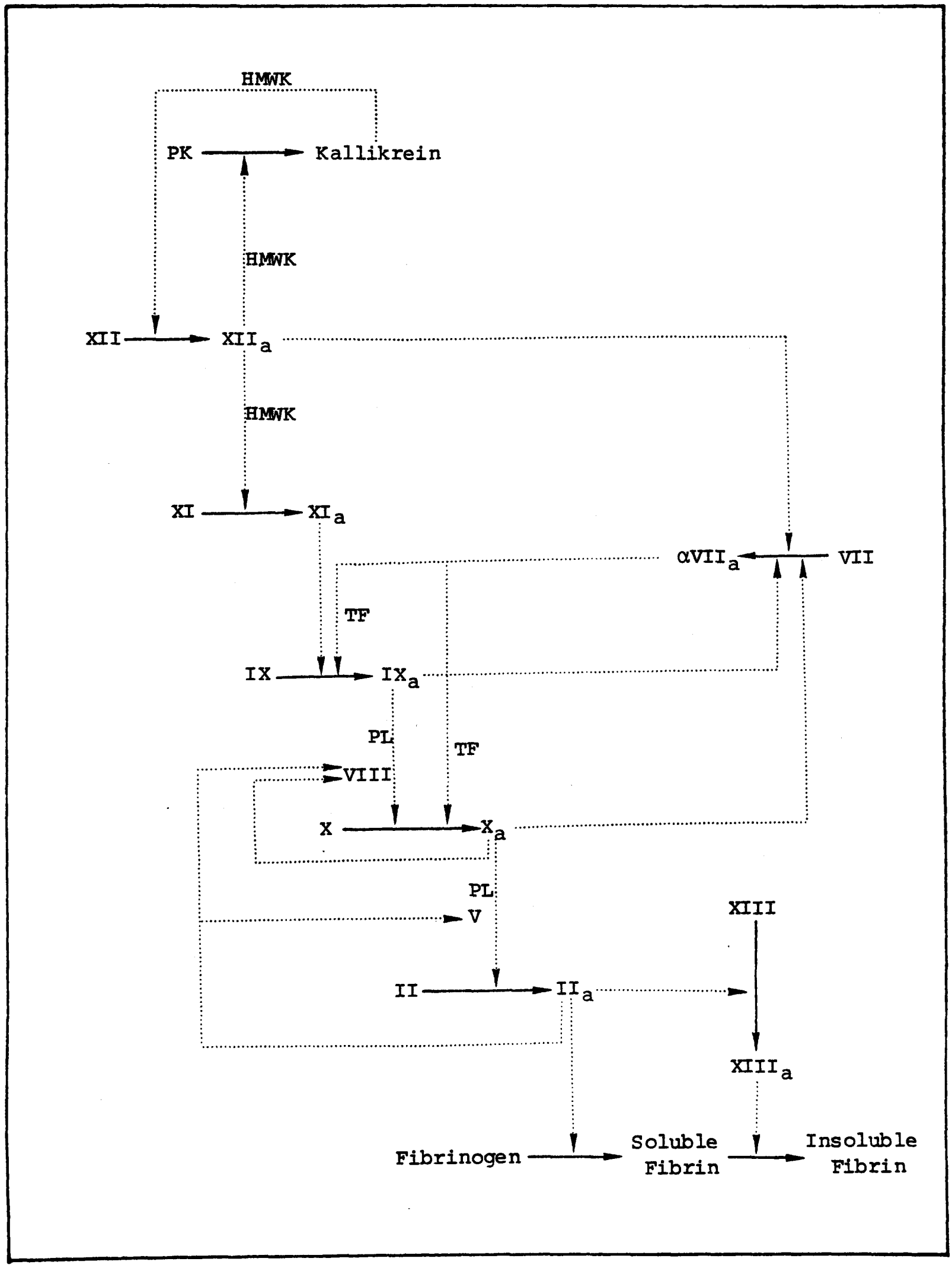
Physiologically, the formation of a fibrin clot requires thrombin to catalyse two reactions: the removal of the N-terminal fibrinopeptides from fibrinogen (Lorand, 1952) and the activation of factor XIII (Lorand and Konishi, 1964). The former reaction leads to the polymerisation of fibrin while the latter is required for the subsequent cross-linking of the clot by factor $XIII_a$. Fibrinogen is a large molecule, having a molecular weight of 340 000 (Schulman, 1953), and being composed of two Aα chains, two Bβ chains and two γ chains (McKee *et al*, 1966). The chains are linked together tightly by a series of disulphide bridges in the N-terminal regions, a structure for which Blombäck (1970) has proposed the term 'disulphide knot'. The cleavage of fibrinopeptides A and B from the α and β chains respectively converts fibrinogen into fibrin: Blombäck and Blombäck (1972) found that the former cleavage permits the end-to-end polymerisation of the fibrin monomers while the latter cleavage is required for lateral polymerisation to occur. Factor $XIII_a$ finally catalyses the formation of ε-(γ-glutamyl)-lysine bonds between the γ chains and subsequently between the α chains to give the cross-linked fibrin polymer. These iso-peptide bonds are not hydrolysed by any proteinase.

Figure 1.6 includes the interactions among the clotting factors which lead to the rapid formation of an insoluble fibrin clot. Negative feedback reactions are omitted from this scheme in order to avoid confusion.

Fibrin clots are eventually degraded enzymatically as a

FIGURE 1.6

Positive Feedback Reactions in Blood Coagulation



result of the action of plasmin, a process which is usually referred to as fibrinolysis. Plasmin is formed from plasminogen by a limited proteolytic cleavage catalysed by plasminogen activator. This activator is itself formed from an inactive precursor by the action of several proteinases, including factor XII_a (Revak and Cochrane, 1976).

Plasmin will degrade fibrinogen, soluble fibrin and insoluble fibrin (Gaffney, 1977), with the identical bonds in the subunit polypeptides being cleaved in each case. Figure 1.7 shows diagramatically the degradation of the fibrinogen molecule by plasmin. During fibrinolysis, fibrin is degraded into fragment E and fragment D dimers resulting from the presence of cross-links between the γ chains.

In addition to the lysis of clots, plasmin may play an important role in localising coagulation via its effects on fibrinogen. The activation of the fibrinolytic system in solution, following the initiation of the contact phase of coagulation on a surface (Cochrane and Griffin, 1979), leads to the degradation of fibrinogen in the bloodstream. There is, therefore, little chance of clots forming at sites around the body away from an injury, even if thrombin is produced and is not neutralised by the antithrombin III in the plasma, since the fibrinogen degradation products resulting from the action of plasmin are incoagulable.

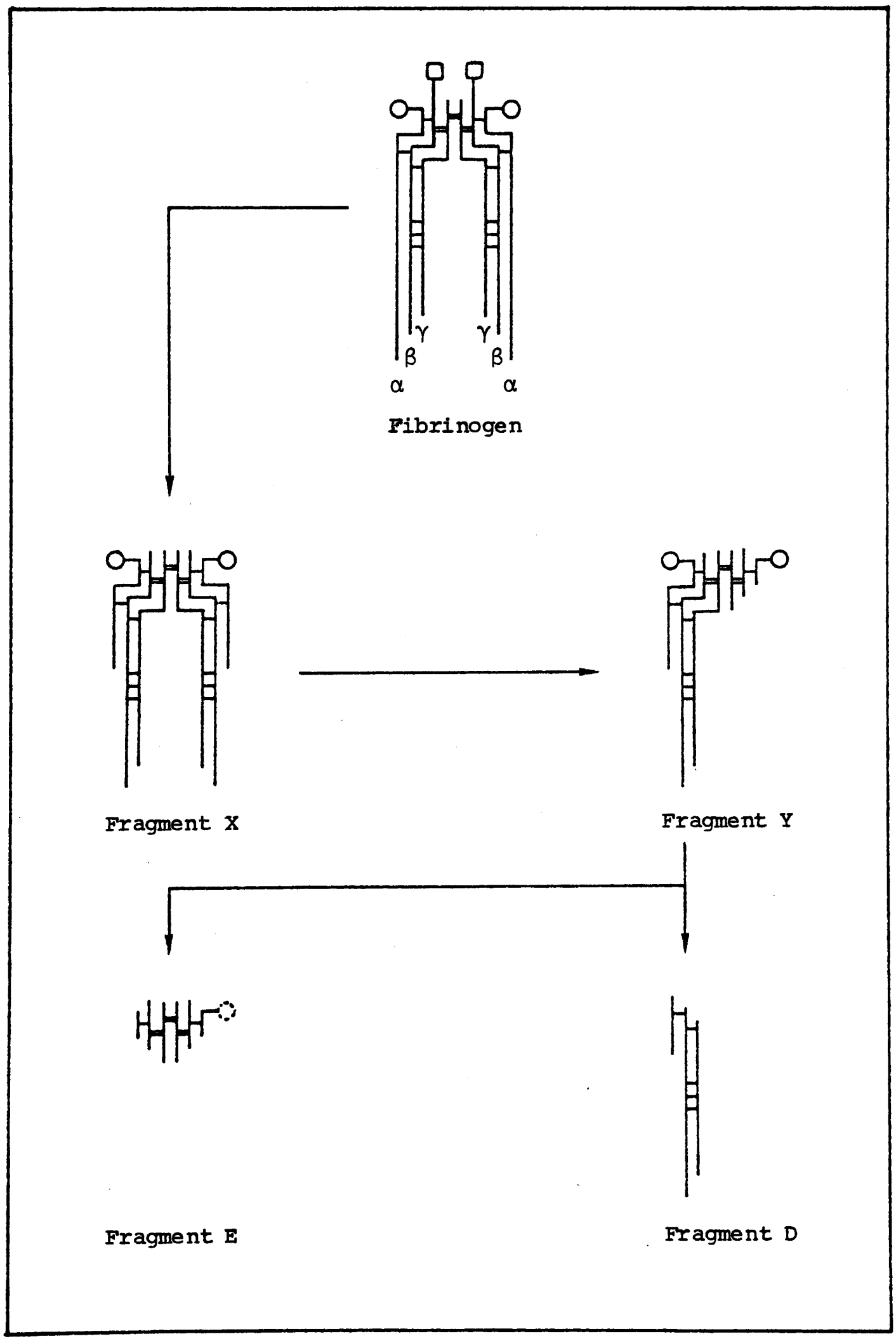
Natural Inhibitors of Coagulation

In addition to the various negative feedback reactions which operate during the normal coagulation process, and to

FIGURE 1.7

Plasmin Degradation of Fibrinogen

(Kopeć and Latallo, 1978)



the fibrinolytic action of plasmin, two other plasma proteins also regulate blood clotting: protein C and antithrombin III.

Several antithrombin activities have been described in blood but Lane and Biggs (1977) recently concluded that only the 'plasma cofactor of heparin' and the 'total progressive antithrombin' were physiologically important and that these terms represented two manifestations of the same protein - antithrombin III. This molecule inhibits thrombin (Lane and Biggs, 1977), factor X_a (Yin *et al*, 1971), factors VII_a, IX_a, XI_a and XII_a (Barrowcliffe *et al*, 1978; Rosenberg, 1978), plasmin (Crawford and Ogston, 1975) and kallikrein (Burrowes *et al*, 1975). In all cases, the *in vitro* activity of the antithrombin III is enhanced by heparin, but this effect cannot be significant *in vivo* as heparin is not found in the bloodstream.

Protein C (Stenflo, 1976) is the zymogen of a serine proteinase which is converted to its active form by trypsin (Esmon *et al*, 1976), Russell's viper venom (Kisiel *et al*, 1976) or thrombin (Kisiel *et al*, 1977). Activated protein C inhibits coagulation by inactivating factor V (Kisiel *et al*, 1977) and factor VIII (Vehar and Davie, 1980).

Factor VIII

Factor VIII is the only clotting factor which has not been isolated in a pure form and fully characterised. Several different activities are associated with 'factor VIII' in addition to the coagulant activity, which is denoted by factor VIII:C. The immunization of rabbits with a fraction of human

plasma enriched in factor VIII leads to the production of a specific antiserum which neutralises the factor VIII:C activity of plasma; the antiserum will also form a precipitin line with normal or haemophilic plasma (Zimmerman *et al*, 1971). The protein which is recognised by this antiserum is termed factor VIII-related antigen (factor VIIIR:Ag). It is present in the plasma of all haemophiliacs, even in cases where no factor VIII:C is detectable. A second form of hereditary deficiency of factor VIII coagulant activity was first described by von Willebrand (1926); this condition is normally referred to as von Willebrand's disease and is inherited as an autosomal characteristic. Patients with von Willebrand's disease are deficient in both factor VIII:C and factor VIIIR:Ag activities (Zimmerman *et al*, 1971).

Howard and Firkin (1971) found that the antibiotic ristocetin would cause platelet aggregation in platelet-rich plasma from normal persons and from haemophiliacs, but not from patients with von Willebrand's disease. This abnormality is corrected by plasma fractions enriched in factor VIII:C and factor VIIIR:Ag; the activity which is required is usually referred to as the von Willebrand factor (factor VIIIR:WF) or the ristocetin co-factor.

Factor VIII-rich fractions of porcine or bovine plasma will aggregate human platelets in the absence of ristocetin (Forbes and Prentice, 1973); they will also aggregate platelets from the same species in the presence of the antibiotic (Kalogjera and Owen, 1978). These properties of animal factor VIII concentrates are usually referred to as platelet aggregating

factor (PAF) and von Willebrand factor activities respectively, but Kalogjera and Owen (1978) showed that bovine PAF and factor VIIIR:WF are activities shared by a single molecule.

Haemophilic patients who have received large numbers of blood transfusions occasionally develop plasma 'inhibitors' which neutralise factor VIII:C activity (Biggs, 1970). The inhibitors are antibodies with determinants specific for factor VIII:C and can, in certain cases, be used to set up immunoradiometric assays for factor VIII:C (Peake and Bloom, 1978; Lazarchick and Hoyer, 1978). The activity which is measured in such an assay is termed factor VIII clotting antigen (factor VIII:CAg). The factor VIII:CAg appears to be more stable than the factor VIII coagulant activity - it is present in serum, for example - and the levels of the two activities in a sample containing factor VIII are, therefore, not necessarily identical (Peake *et al*, 1979).

At least four activities are associated with 'factor VIII': factor VIII:C, factor VIII:CAg, factor VIIIR:Ag and factor VIIIR:WF, which is probably identical with the platelet aggregating factor activity in bovine and porcine factor VIII. Preparations of human (Hershgold *et al*, 1971; Marchesi *et al*, 1972; Legaz *et al*, 1973; Shapiro *et al*, 1973) or bovine (Schmer *et al*, 1972; Legaz *et al*, 1975) factor VIII, which possessed all of the 'factor VIII-related' activities are now normally described by the term 'factor VIII-von Willebrand factor' (factor VIII/vWF). The nature of the relationships among the various activities of a factor VIII/vWF preparation is a source of debate. Bloom and Peake (1977) suggested three possible

models for the structure of factor VIII: it may be one molecule which has all of the associated activities; it may be two molecules, one carrying the factor VIII:C activity and one the factor VIIIR:Ag and factor VIIIR:WF activities; or it may be a molecular complex consisting of a low molecular weight factor VIII:C linked by non-covalent bonds to a high molecular weight carrier protein with factor VIIIR:Ag and factor VIIIR:WF activities. At present, the existence of a molecular complex, in which the factor VIII:C is only a minor component, is fairly widely accepted as a model for the factor VIII/vWF protein.

In order to establish the exact role of factor VIII:C in blood coagulation, it would be advantageous to prepare the molecule in a pure form, free from factor VIIIR:Ag and factor VIIIR:WF activities, but this is extremely difficult. Vehar and Davie (1980) have isolated bovine factor VIII:C which lacks platelet aggregating factor activity, but the method they devised involved the use of sulphydryl reducing agents. One of the major aims of the current project was, therefore, to prepare factor VIII:C by a different procedure from that employed for the isolation of factor VIII/vWF, and to use the material thus obtained to study the role of factor VIII in coagulation, and in particular, in the activation of factor X.

Chapter 2

THE PURIFICATION OF BOVINE FACTOR VIII

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THE PURIFICATION OF BOVINE FACTOR VIII

INTRODUCTION

The protein which is usually isolated from plasma as 'factor VIII' possesses factor VIIIR:Ag and platelet aggregating factor activities in addition to the factor VIII:C activity, and should therefore be referred to as factor VIII/vWF. The poorly-defined nature of the molecule involved in blood coagulation, and its relationship to the other activities has greatly hindered attempts to prepare pure factor VIII:C.

In 1911, Addis showed that the clotting defect in haemophilic plasma could be corrected by the addition of a fraction from normal plasma which contained prothrombin. Quick (1935), however, found that haemophilic plasma gave normal clotting times in his Prothrombin Time test when an excess of thromboplastin was present, suggesting that the level of prothrombin was normal. Patek and Taylor (1937) extended the work of Addis (1911) with the isolation, by precipitation at low ionic strength or acidic pH, of the 'plasma globulin fraction' containing the material capable of shortening the clotting time of haemophilic plasma. This substance, which precipitates from plasma optimally in the range pH5.9 to 6.4, was subsequently identified by Minot *et al* (1945) in Cohn fractions I and III-2, which are enriched in fibrinogen and β-globulins respectively.

Early attempts to isolate factor VIII from bovine plasma were made by Spaet and Kinsell (1953) and Bidwell (1955). The former used techniques previously employed with human plasma: precipitation at pH5.4 of a Cohn fraction I preparation from

which the fibrinogen had been removed by heating at 56°C. The latter, however, introduced a salt fractionation technique, which exploited the relative insolubility of bovine factor VIII in the presence of phosphate or citrate ions.

The production of factor VIII concentrates for clinical use in the treatment of haemophilic patients was greatly simplified by the discovery (Pool *et al*, 1964) that when frozen plasma was thawed slowly, much of the factor VIII:C activity remained in the 'cryoprecipitate'. This fibrinogen-rich material could be separated from the remainder of the plasma by centrifugation, and stored. Many purification procedures were later developed using the technique of cryoprecipitation, as a first stage, providing a convenient method of concentrating the plasma factor VIII:C into a small volume. A further refinement, following the Cohn fractionation procedure, employed ethanol as an aid to precipitation.

Various protein precipitants have been used in the preparation of factor VIII:C, including citrate and phosphate ions (Bidwell, 1955; Olson *et al*, 1977), ammonium sulphate (Hershgold *et al*, 1971; Olson *et al*, 1977), polyethylene glycol (Hershgold *et al*, 1971), β-alanine (Schmer *et al*, 1972), glycine (Legaz *et al*, 1975) and concanavalin A (Schmer *et al*, 1972; Legaz *et al*, 1973).

Several methods for the purification of factor VIII:C have included adsorption steps in which other clotting factors are removed either from plasma or at a later stage in the preparation. The vitamin K-dependent proteins, including prothrombin, can be adsorbed on to barium sulphate (Schmer *et*

al, 1972; Legaz *et al*, 1975), barium citrate (Olson *et al*, 1977) or alumina (Hershgold *et al*, 1971; Legaz *et al*, 1973). The factors involved in the contact phase are removed by adsorption with kaolin, and some of the plasma fibrinogen by adsorption with bentonite (Schmer *et al*, 1972).

Procedures for the purification of factor VIII/vWF invariably include gel filtration on 4% or 6% agarose (Johnson *et al*, 1967) at some stage. The principal advantage of this technique arises from the anomalous behaviour of factor VIII/vWF which elutes in the 'void volume' of the column, implying that it has a molecular weight in excess of 20 000 000. The majority of proteins are not excluded from the column and are therefore separated from the factor VIII/vWF.

Early attempts to utilize ion-exchange chromatography in the purification of factor VIII:C resulted in the preparation of a material with a high specific activity. Thus, Michael and Tunnah (1963, 1966) claimed to purify factor VIII:C by more than 100 000-fold over plasma by chromatography on Whatman ME-29, a specially-developed anion-exchange Cellulose. Veder (1966) purified factor VIII:C from human plasma by chromatography on DEAE-Sephadex or DEAE-Cellulose. This latter resin was also used by Shapiro *et al* (1973) and Olson *et al* (1977) in their purification procedures. Ion-exchange techniques have not, however, been widely used in the preparation of factor VIII:C, principally because of the very low recoveries of the activity (Hershgold, 1974).

The most detailed methods for the purification from bovine plasma of a material having factor VIII:C, factor VIIIR:Ag

and factor VIIIR:WF activities are those developed by E W Davie and his colleagues (Schmer *et al*, 1972; Legaz *et al*, 1973, 1975, 1976). They isolated an apparently homogenous, high molecular weight protein, consisting of several disulphide-linked sub-units of molecular weight 200 000, by a combination of procedures including cryo-ethanol precipitation, precipitation with polyethylene glycol, glycine or concanavalin A, adsorption with barium sulphate, kaolin and bentonite, agarose gel filtration and chromatography on tricalcium citrate-Cellulose.

The exact nature of these preparations of factor VIII/vWF, and others produced by simplified versions of the isolation procedure, is, however, in doubt. The various 'factor VIII-related' activities can be separated from one another by the use of several procedures described in Chapter 3, which are usually considered to 'dissociate' the components of the factor VIII/vWF complex.

The models proposed (Bloom and Peake, 1977) for the structure of factor VIII have included one in which a high molecular weight carrier protein with platelet aggregating factor activity circulates in the plasma in a complex with a lower molecular weight protein having factor VIII:C activity. It has, however, also been suggested (Rock *et al*, 1978) that factor VIII coagulant activity and the platelet aggregating activity are properties of two distinct molecules which become associated with one another only when chelators of calcium ions are used as anticoagulants during the collection of the blood. That is, the co-purification of these two activities might be simply an artefact arising from the procedure used. If this

is the case, then it is probable that the cryoprecipitation step will exacerbate the problem since the material produced is extremely glutinous even at a macroscopic level.

A novel procedure for preparing the factor VIII coagulant protein from bovine plasma has recently been reported by Vehar and Davie (1980). This method, however, results in the isolation of a form of factor VIII:C in which the disulphide bonds have been reduced with dithiothreitol and which is apparently composed of three polypeptides of molecular weight 93 000, 88 000 and 85 000. It is not certain that valid conclusions about the properties of native factor VIII:C in plasma can be drawn from the behaviour of this reduced material.

The development of a new procedure for the isolation of the native factor VIII coagulant protein from bovine plasma was therefore undertaken. A cryoprecipitation step was not included: firstly, in order to reduce any artefactual aggregation of the proteins and, secondly, because the recovery of factor VIII:C activity from this procedure is frequently low.

MATERIALS AND METHODS

Chromatography of Factor VIII

Commercially-available ion-exchange chromatography media were purchased from several suppliers, including Whatman Ltd., Maidstone, Kent, UK.; Pharmacia Fine Chemicals, London, UK.; and Serva Feinbiochemica, Heidelberg, German Federal Republic. They were equilibrated with the chosen buffer, usually 0.02M imidazole-HCl, pH7.0, in accordance with the manufacturer's instructions.

Other ion-exchange resins were prepared in the laboratory by Dr S I Chavin. Tricalcium citrate-Cellulose was prepared as described by Schmer *et al* (1972). The synthesis of hydroxylapatite was carried out according to the method of Bernardi (1971). Sulphate-Sepharose was produced by the treatment of Sepharose 4B with chlorosulphonic acid (Vehar and Davie, 1980).

Affinity resins for use in the isolation of factor VIII:C from plasma were synthesised by the technique of Cuatrecasas (1970). Sepharose 4B was activated with cyanogen bromide and the required ligand coupled via free amino groups to the activated gel. This method, which is described in detail in Appendix 9, was used to couple factors IX and X, and fibrin monomer to Sepharose. Factor VIII:C interacts with factors IX and X during coagulation, as described in Chapter 5; and Vehar and Davie (1980) used chromatography on factor X-Sepharose in their purification procedure.

'Hydrocarbon-Sepharoses' of three types were utilized; they were purchased commercially where possible, or prepared

in the laboratory if necessary. Type A were produced by coupling diamino-alkanes to Sepharose activated with cyanogen bromide; type B were prepared similarly, but using monoamino-alkanes; type C were synthesised by reacting Sepharose CL-4B with a glycidyl ether, to give a derivative in which the hydrocarbon chain was attached to the matrix via an uncharged, chemically stable ether linkage (Hjertén *et al*, 1974). The different types of gel are shown in Figure 2.1.

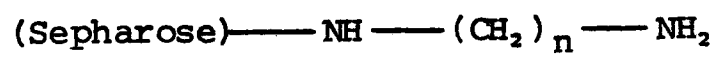
The chromatographic behaviour of factor VIII:C was studied with preparations of varying degrees of purity. In early experiments, bovine plasma was used, and - as progress in the purification of the activity was made - the most highly purified preparation of factor VIII:C available was subjected to chromatography on various resins to ascertain which might be of value as a subsequent step in the procedure.

In each case, a similar experimental design was adopted. A small column of the resin being tested was prepared and equilibrated with buffer. The factor VIII:C-containing mixture was diluted to an appropriate ionic strength and pumped on to the column. This was washed with starting buffer until the A_{280} of the eluate fell to zero. The adsorbed proteins were eluted by changing the ionic strength of the buffer washing the column, usually as a continuous linear gradient, but occasionally in discrete steps. The column eluate was collected in fractions of 2 or 5ml which were then assayed for factor VIII:C activity. Those fractions containing activity were pooled and the specific activity was determined, as a measure of the purification achieved.

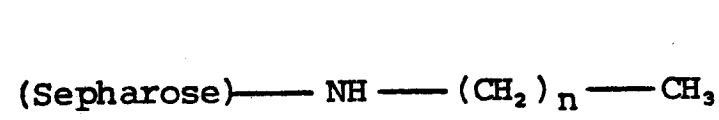
FIGURE 2.1

'Hydrophobic-Sepharoses'

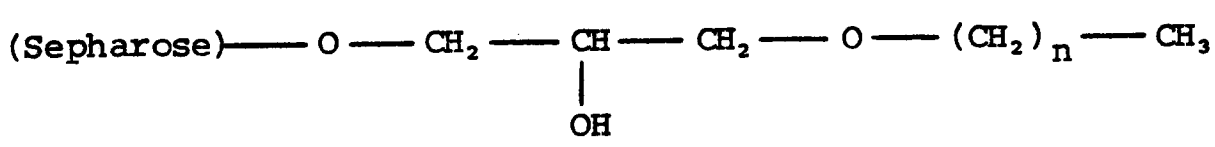
Type A



Type B



Type C



Precipitation of Factor VIII

It is possible to employ fractional precipitation of mixtures of proteins to isolate factor VIII:C which is a relatively insoluble protein. The suitability of various protein precipitants for use in the preparation of factor VIII:C was assessed by adding aliquots of the agent to a source of factor VIII:C, separating the precipitate from the supernatant by centrifugation and redissolving the former in buffer. Assays of the factor VIII:C activity in the redissolved precipitates obtained with different concentrations of each precipitant were used to determine the optimum range for removing the factor VIII:C from solution.

Dialysis and Ultrafiltration of Factor VIII

Several attempts were made to change the ionic strength or protein concentration of solutions containing factor VIII:C activity by means of dialysis or ultrafiltration.

Factor VIII:C solutions were dialysed against at least a ten-fold (v/v) excess of buffer of low ionic strength. The coagulant activity of the solution was measured in a two-stage assay before and after dialysis.

Solutions containing factor VIII:C were also concentrated by ultrafiltration using a PM10 or YM10 membrane in a stirred cell (Amicon Ltd., Woking, Surrey, UK). The ionic strength of the protein concentrate was then reduced by dilution with salt-free buffer. The factor VIII:C activity in the solution was determined before and after ultrafiltration.

The Preparation of 'Partially Purified Factor VIII:C'

The examination of the various techniques available for the purification of factor VIII:C resulted in the following procedure being developed for the isolation of the factor VIII:C activity from bovine plasma.

5000ml of bovine blood was collected, immediately after slaughter, into 500ml 0.1M sodium oxalate in a plastic bottle. The cells were removed by centrifugation at 5000g for 30 minutes in an MSE Coolspin Centrifuge (MSE Ltd, Crawley, Sussex, UK). The plasma was syphoned into a plastic beaker and stirred with 10mg/ml barium sulphate for 20 minutes. The barium sulphate was sedimented at 5000g for five minutes and the plasma was decanted and adsorbed with a second 10mg/ml aliquot of barium sulphate.

2000ml of adsorbed plasma was stirred with 500ml of gelatine-Sepharose, equilibrated with 0.02M imidazole-HCl, pH7.0, 0.50M NaCl, for 20 minutes. The mixture was centrifuged at 5000g for five minutes, and the supernatant was decanted. The gel was washed with two 800ml volumes of 0.02M imidazole-HCl, pH7.0, 0.50M NaCl and the washings were combined with the first supernatant.

Solid ammonium sulphate was then added to the supernatant pool, to give a final 25% saturation at 25^{o}C. The mixture was left to stand at 15^{o}C for 30 minutes, and centrifuged at 5000g for ten minutes. The supernatant was discarded and the precipitate was dissolved in approximately 1000ml 0.02M imidazole-HCl, pH7.0. The volume of the solution was adjusted to reduce the conductivity to less than 12mmho/cm.

The redissolved precipitate was applied to a column (10cm diameter x 20cm long) of ECTEOLA-Cellulose (Whatman Ltd., Maidstone, Kent, UK), equilibrated with 0.02M imidazole-HCl, pH7.0, 0.10M NaCl. The column was washed with the same buffer until the A_{280} of the eluate fell to less than 0.05. Adsorbed proteins were then eluted from the column by washing with 0.80M sodium chloride in 0.02M imidazole-HCl, pH7.0, and collected as a single pool in a total volume of approximately 500ml.

An equal volume of 2.0M phosphate-KOH, pH6.8, was added to the pool from the ECTEOLA-Cellulose chromatography. The mixture stood at 15^{o}C for 30 minutes before being centrifuged at 5000g for ten minutes. The supernatant was discarded and the precipitate was redissolved in 0.02M imidazole-HCl, pH7.0; care was taken to use a minimum quantity of buffer in order to keep the volume of the final solution to between 5 and 8ml.

The redissolved precipitate was applied to a column (2.5cm diameter x 90cm long) of Sepharose CL-4B at a flow rate of 50ml/hr. The proteins were eluted from the column at the same flow rate, using 0.02M imidazole-HCl, pH7.0, 0.30M NaCl. Fractions of 10ml were collected throughout, using an ISCO Model 328 Fraction Collector and accessories; they were assayed for factor VIII:C activity by means of the two-stage clotting assay. The fractions containing the highest levels of factor VIII:C, corresponding to the void volume, were pooled.

Solid ammonium sulphate was added to this pool, to give a final 35% saturated solution. The mixture was allowed to stand at 15^{o}C for 30 minutes and then centrifuged at 3300g for 15 minutes. The supernatant was discarded and the precipitate

was redissolved in 0.02M imidazole-HCl, pH7.0.

The solution containing the factor VIII:C activity was diluted to a total volume of 20ml with 0.11M potassium sulphate in 0.02M imidazole-HCl, pH7.0. It was then applied to a column (2.0cm diameter x 6.0cm long) of sulphate-Sepharose, equilibrated with 0.02M imidazole-HCl, pH7.0, 0.11M K_2SO_4. The column was washed with the same buffer at a flow rate of 75ml/hr, until the A_{280} of the eluate fell to less than 0.01. The adsorbed proteins were immediately eluted with 1.0M sodium chloride in 0.02M imidazole-HCl, pH7.0. During the elution, fractions of 5ml were collected and assayed for factor VIII:C activity. Those fractions containing the peak of the factor VIII:C were pooled.

Solid ammonium sulphate was added to this pool, to give a final 35% saturated solution. The mixture was left to stand at 15^{o}C for 30 minutes and centrifuged at 3300g for 15 minutes. The supernatant was discarded and the precipitate was redissolved in a mixture of 0.5ml 0.02M imidazole-HCl, pH7.0, and 0.5ml glycerol. The final product - 'partially purified bovine factor VIII:C' - was stored at -20^{o}C. Small aliquots were removed and used in other studies as required.

Preparation of Factor VIIIR:Ag

Factor VIIIR:Ag was prepared by Dr S I Chavin in this laboratory, by following the method described for 'factor VIII' by Legaz and Davie (1976).

The purified factor VIIIR:Ag was used to raise an anti-serum in a rabbit, as described in Appendix 7. The antiserum to factor VIIIR:Ag was prepared in the normal manner, and was

then rendered monospecific by adsorption with a pool of proteins from the included volume peak from the Sepharose 4B gel-filtration step in the factor VIIIR:Ag preparation.

RESULTS

Chromatography of Factor VIII

Factor VIII:C was adsorbed from solution on to all commercial anion-exchange resins which were tested. These included DEAE-Cellulose, DEAE-Sephadex A-50, DEAE-Sephacel, TEAE-Cellulose, QAE-Sephadex A-50, and ECTEOLA-Cellulose. In each case, the majority of the proteins in the solution also bound to these anion exchange resins in buffers at low ionic strength. The elution of the adsorbed proteins from the resin with a gradient of increasing ionic strength did not, in general, separate the factor VIII:C from significant amounts of the bulk protein. An example of the elution profile which was usually obtained in experiments of this type is given in Figure 2.2, which shows the chromatography of alumina-adsorbed bovine plasma on QAE-Sephadex.

Less protein was adsorbed on to the weak, low capacity resin, ECTEOLA-Cellulose, than on to any of the stronger anion-exchangers. The factor VIII:C activity was, however, completely removed from solution during chromatography on this material. Figure 2.3 shows the elution from ECTEOLA-Cellulose of a mixture of proteins prepared by chromatography of bovine plasma on DEAE-Sephadex A-50, followed by fractional precipitation with ammonium sulphate. Only a small proportion (approximately 20%) of the total protein applied to the column was recovered in the pool eluted at high ionic strength but this included the factor VIII:C. In addition to the increase in the specific activity of the factor VIII:C which was achieved by chromatography on ECTEOLA-Cellulose, an important qualitative change in the composition of

FIGURE 2.2

Chromatography of Plasma on QAE-Sephadex

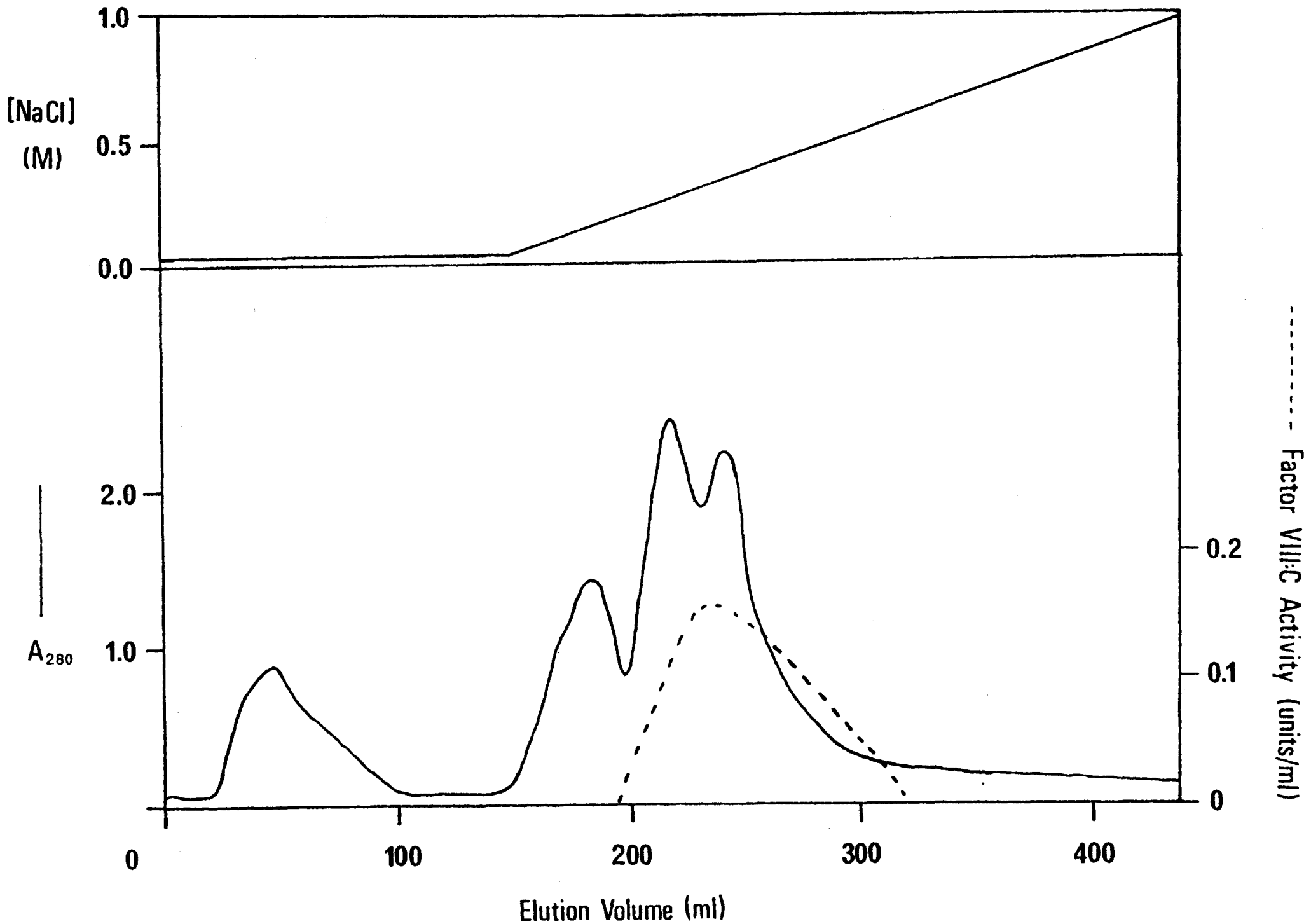
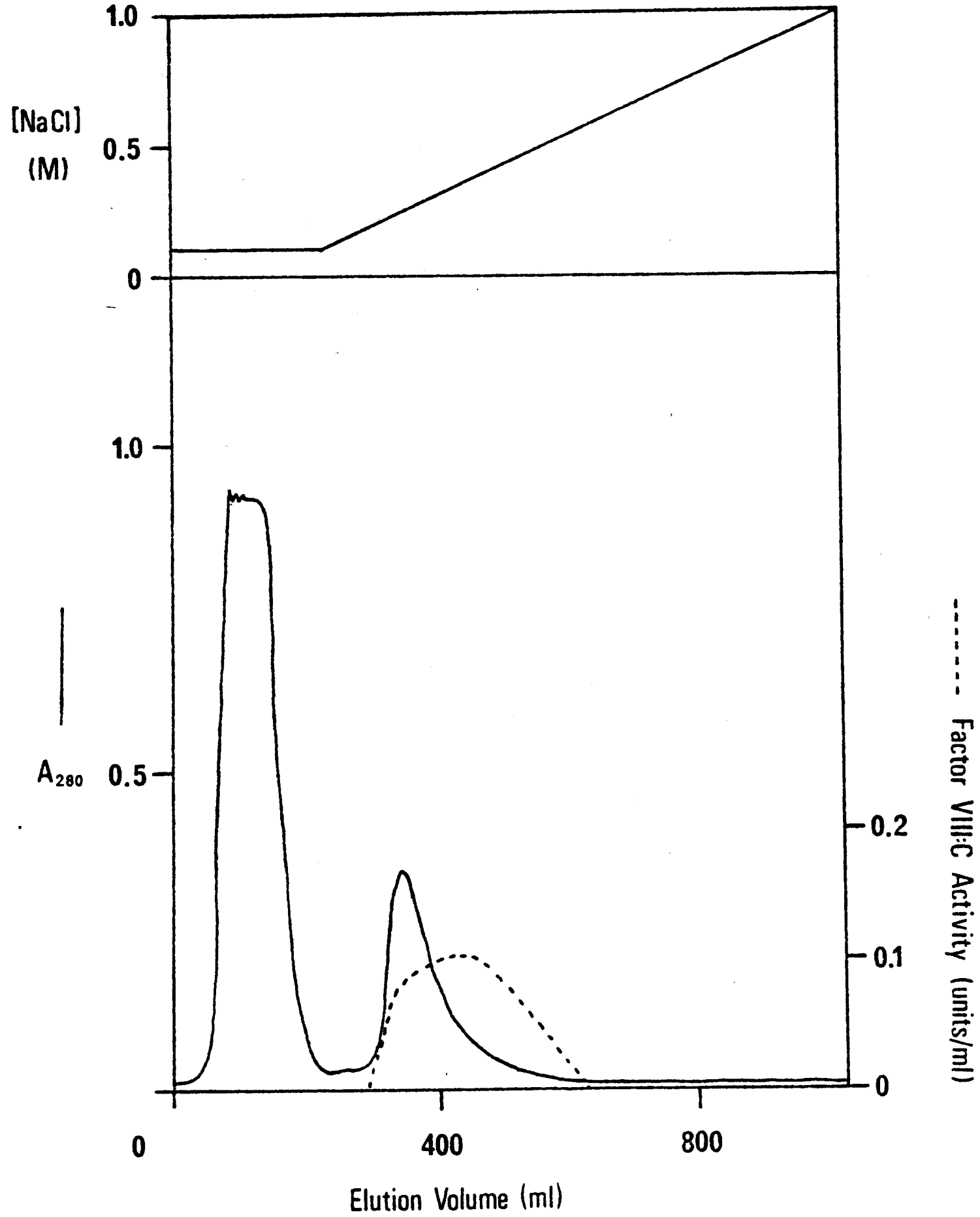


FIGURE 2.3

Chromatography of Factor VIII:C on ECTEOLA-Cellulose



the protein pool was observed on SDS-polyacrylamide gel electrophoresis. A major constituent of the mixture applied to the ECTEOLA-Cellulose column was fibrinogen, but little was detectable in the eluate pool containing the factor VIII:C. This observation was confirmed by immunoelectrophoresis which indicated that over 95% of the applied fibrinogen was separated from the factor VIII:C.

Factor VIII:C was also adsorbed on to several anion-exchange media which were synthesised in the laboratory. Hydroxylapatite adsorbed the factor VIII:C and all other components from a mixture of proteins which was applied. No protein was eluted from the resin by sodium chloride up to 3.0M. The proteins were, however, eluted from the column in a single peak by a gradient of increasing sodium phosphate concentration. Chromatography of a mixture of proteins including factor VIII:C on tricalcium citrate-Cellulose as described by Schmer *et al* (1972) resulted in the factor VIII:C activity being adsorbed on to the resin, but very little being eluted by EDTA.

Factor VIII:C was not adsorbed from solution on to such cation exchange resins as SP-Sephadex C-50 and CM-Sephadex C-25. These gels adsorbed very little protein from plasma or from mixtures of proteins obtained at various stages in the purification of factor VIII:C.

Factor VIII:C, together with most other proteins in the mixtures which were tested, was bound by Cellulose phosphate. The use of a phosphate buffer gradient to elute the adsorbed

proteins from the column did not bring about a significant purification of the factor VIII:C from the other proteins.

True hydrophobic interaction chromatography on 'type C' hydrophobic Sepharoses (Figure 2.1), with the hydrocarbon chain attached to the gel matrix via an uncharged, chemically stable ether linkage, resulted in severe losses of factor VIII:C activity. At sodium chloride concentrations of 0.50M or greater, factor VIII:C was adsorbed from solution on to phenyl-Sepharose, as was the majority of the protein in the mixture applied to the gel. The use of a gradient of decreasing sodium chloride concentration to elute the adsorbed proteins failed to separate the factor VIII:C from the other proteins.

Neither factor VIII:C nor the other proteins in the mixtures tested was adsorbed on to octyl-Sepharose or butyl-Sepharose at a sodium chloride concentration of 1.0M.

The Shaltiel Hydrophobic Chromatography Kit: Agarose-C_n Series (Miles Laboratories Ltd., Slough, Bucks, UK) was utilised for the investigation of 'type B' hydrophobic agaroses (Figure 2.1). Table 2.1 summarises the effects of the length of the hydrocarbon side chain on the adsorption and elution of factor VIII:C. The pool of protein containing factor VIII:C was applied to the gels in 0.02M imidazole-HCl, pH7.0, and the adsorbed proteins were eluted with 1.0M sodium chloride in the same buffer. The other proteins in the pool applied to the hydrophobic agaroses behaved in a very similar manner to the factor VIII:C: the majority of the protein was adsorbed by the gels with a side chain of two or more carbon atoms, and only

TABLE 2.1

Chromatography of Factor VIII:C on Alkyl-Agaroses

No. of Carbon Atoms per Hydrocarbon Chain	Recovery of Factor VIII:C	
	Unadsorbed Pool	Eluate Pool
0	69%	5%
2	0%	48%
4	0%	43%
6	0%	10%
8	0%	0%
10	0%	0%

a single peak was eluted by a gradient of increasing sodium chloride concentration.

The adsorption of factor VIII:C and protein by amino-alkyl-Sepharose (type A hydrophobic-Sepharoses in Figure 2.1) is summarised in Table 2.2. A solution containing 6 units of factor VIII:C and 75mg total protein was mixed with 10ml of gel equilibrated with 0.1M sodium chloride in 0.02M imidazole-HCl, pH7.0. The gel was then washed successively with 0.3M and 0.5M sodium chloride in the same buffer. In later experiments, chromatography of 'partially purified factor VIII:C' on aminobutyl-Sepharose or aminohexyl-Sepharose columns resulted in a complete overlap of the factor VIII:C activity with the main protein peak eluted from the column.

The chromatographic behaviour of factor VIII:C on heparin-Sepharose was examined using a mixture of proteins prepared by chromatography of plasma on TEAE-Cellulose and ECTEOLA-Cellulose, accompanied by fractional ammonium sulphate precipitation of the eluate pools. Characteristically, all of the protein in this pool was adsorbed on to heparin-Sepharose in the presence of 0.1M sodium chloride. The elution of the column with a linear gradient from 0.10M to 1.0M sodium chloride separated the bound proteins into two peaks as shown in Figure 2.4. The factor VIII:C activity did not coincide with either protein peak and its exact location could be changed by adjusting the pH of the elution buffer: the more alkaline the solution, the earlier the factor VIII:C was eluted from heparin-Sepharose. Despite this, it was not possible to adjust the conditions in

TABLE 2.2

Chromatography of Factor VIII:C on Amino-Alkyl-Sepharoses

a) Recovery of Factor VIII:C

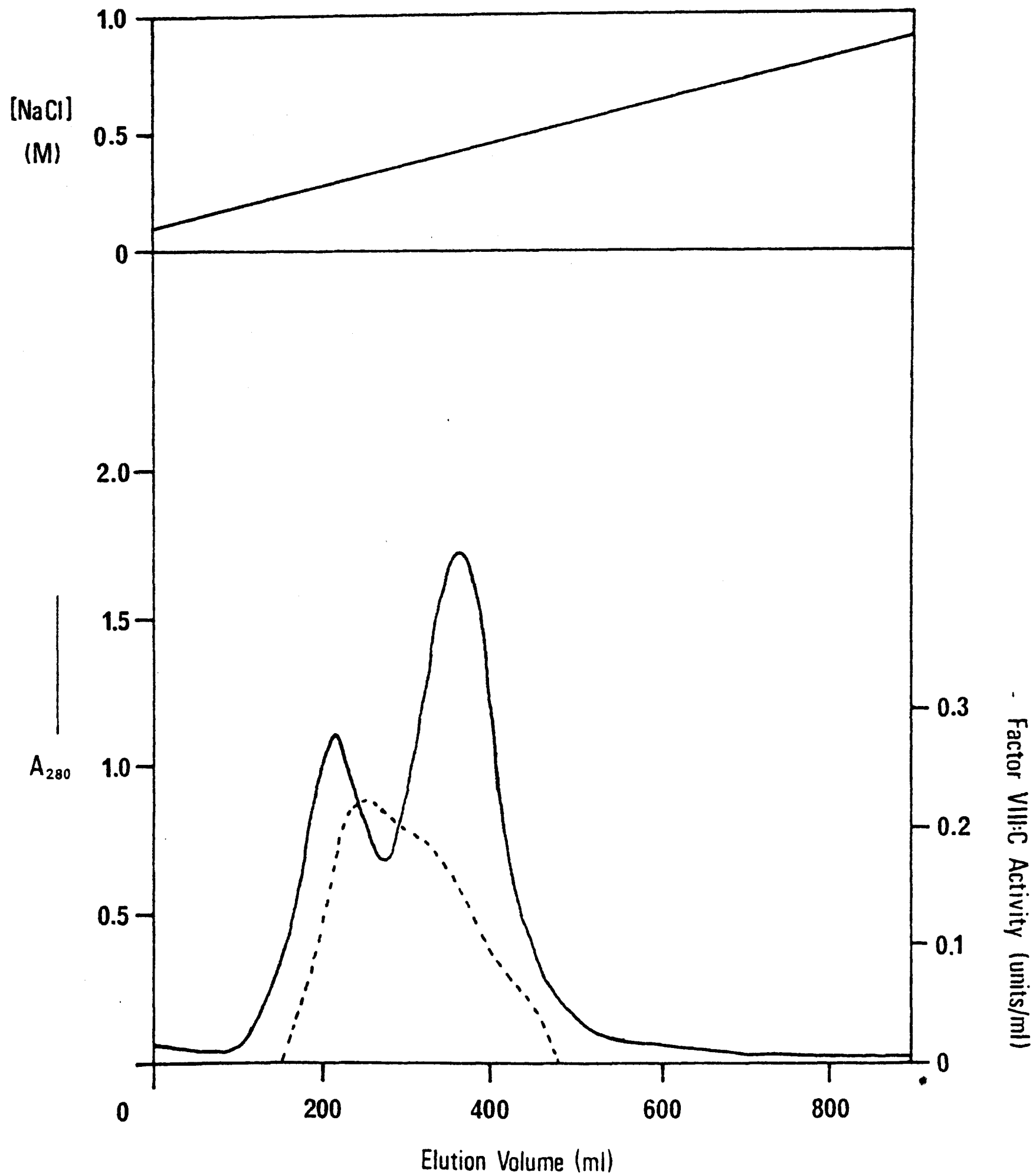
No. of Carbon Atoms per Hydrocarbon Chain	0.1M NaCl Pool	0.3M NaCl Pool	0.5M NaCl Pool
2	100%		
4	0%	100%	
5	0%	83%	5%
6	0%	70%	18%

b) Recovery of Bulk Protein

No. of Carbon Atoms per Hydrocarbon Chain	0.1M NaCl Pool	0.3M NaCl Pool	0.5M NaCl Pool
2	97%	3%	
4	70%	25%	10%
5	70%	35%	
6	57%	41%	8%

FIGURE 2.4

Chromatography of Factor VIII:C on Heparin-Sepharose



such a manner as to give a significant separation of the factor VIII:C activity from the remainder of the protein.

The use of dextran sulphate-Sepharose for the chromatography of a similar mixture of proteins to that applied to heparin-Sepharose gave very similar results. The bulk protein was eluted by a sodium chloride gradient in two peaks, but the factor VIII:C activity was spread across both.

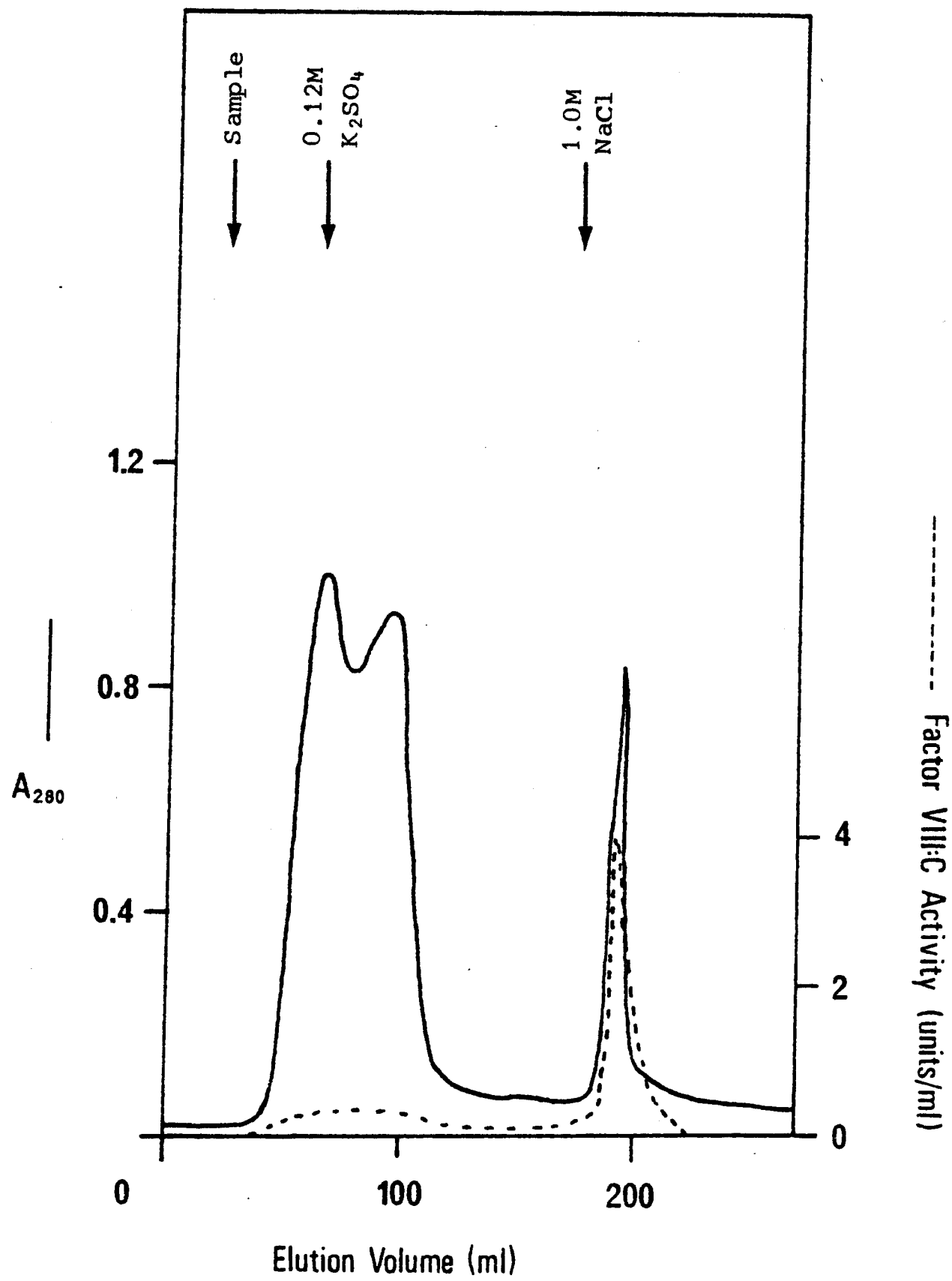
Factor VIII:C was adsorbed from solution on to sulphate-Sepharose, prepared by treating Sepharose CL-4B with chlorosulphonic acid. Very little protein was adsorbed by this gel in the presence of 0.11M potassium sulphate and those proteins which were bound - including the factor VIII:C - could be eluted by washing the column with 1.0M sodium chloride. Figure 2.5 shows a typical separation of factor VIII:C from contaminating proteins on sulphate-Sepharose.

A severe difficulty which was encountered during experiments involving 'affinity' resins was the inactivation of the factor VIII:C by traces of proteolytic enzymes - especially activated coagulation factors - in the proteins coupled to the Sepharose.

Factor VIII:C was not adsorbed from solution on to factor X-Sepharose. Several batches of gel were synthesised, using both factor X_1 and factor X_2, but no binding of factor VIII:C was detectable in any case. The addition of calcium chloride, at concentrations up to 0.08M, or of 0.5mM dithiothreitol, to the buffer in which the factor X-Sepharose was

FIGURE 2.5

Chromatography of Factor VIII:C on Sulphate-Sepharose



equilibrated failed to induce the adsorption of factor VIII:C.

Factor IX-Sepharose did not adsorb factor VIII:C from solutions in 0.02M imidazole-HCl, pH7.0, containing only sodium chloride. In the presence of calcium ions and phospholipid, some factor VIII:C activity was removed from solution by this resin, but no activity could be eluted by washing the gel with 1.0M sodium chloride. Some, but not all, of the factor VIII:C activity in protein solutions was adsorbed by factor IX-Sepharose in the presence of soluble factor X; some of the bound activity was subsequently eluted when the gel was washed with 1.0M sodium chloride. The results were, however, extremely variable: between 25 and 90% of the applied factor VIII:C was removed from solution while only 10 to 40% of the applied activity was recovered in the material eluted from the column. Factor IX-Sepharose adsorbed approximately 25% of the total protein present in the mixture containing factor VIII:C.

Fibrin-monomer-Sepharose did not adsorb factor VIII:C from solution. It did, however, remove approximately 50% of the protein from the mixture which was applied in 0.02M imidazole-HCl, pH7.0, 0.1M sodium chloride, at a temperature of $4^{o}C$. Factor VIII:C activity was either lost or irreversibly adsorbed to the column at lower ionic strength, while less protein was bound at high ionic strength or at higher temperature. SDS-polyacrylamide gel electrophoretic analysis of the proteins in the pools applied to the column and not adsorbed by the gel indicated that the principal component which was bound to the gel was fibrinogen.

Factor VIII:C was eluted in the 'void volume' during gel filtration on Sepharose 4B or Sepharose CL-4B as shown in Figure 2.6. There was a certain amount of 'trailing' of the activity into the included volume of the column, but the majority of the factor VIII:C was associated with the very turbid, apparently lipid-rich, peak eluted by a volume of buffer equivalent to approximately 30% of the total volume of gel in the column.

Precipitation of Factor VIII:C

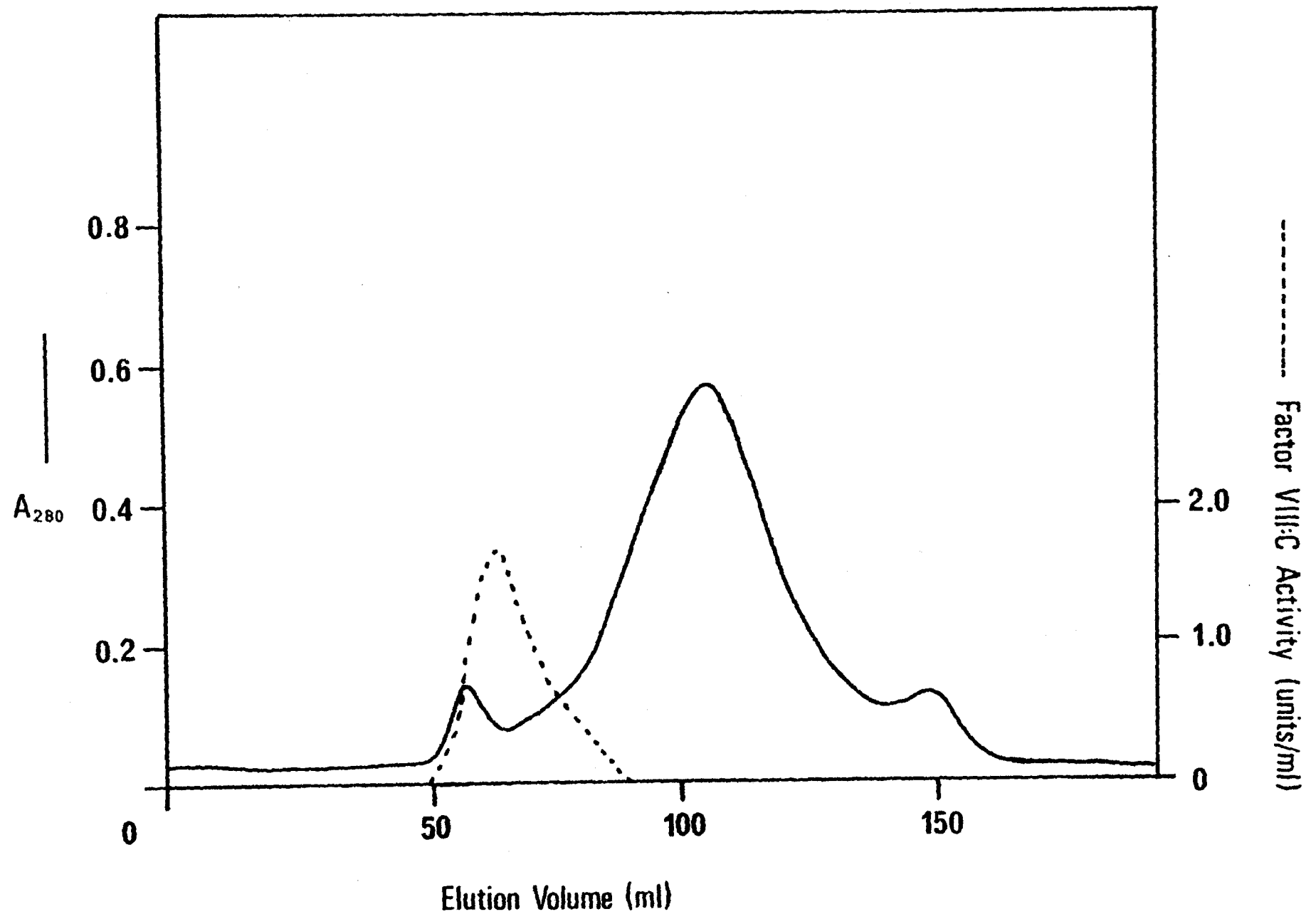
Factor VIII:C was precipitated from plasma by approximately 1.0M ammonium sulphate, equivalent to 25% saturation at 25°C. After the precipitate had been separated from the supernatant by centrifugation, it dissolved readily in salt-free buffer. The redissolved precipitate contained all of the factor VIII:C activity together with only approximately 10% of the plasma protein.

More dilute protein solutions were less readily precipitated by ammonium sulphate. In general, it was found that complete precipitation of factor VIII:C was obtained only if the salt concentration was increased to 35% saturation, or 1.4M ammonium sulphate.

Polyethylene glycol (PEG) 6000, at a final concentration of 5% (w/v), precipitated factor VIII:C from solution, provided that there was a sufficiently high concentration of protein in the original material. The precipitate was, however, very difficult to redissolve after it had been

FIGURE 2.6

Gel-filtration of Factor VIII:C on Sepharose CL-4B



separated from the supernatant, and the recovery of factor VIII:C was not quantitative.

Factor VIII:C was precipitated from a pool of protein prepared by chromatography on TEAE-Cellulose and ECTEOLA-Cellulose, and fractional precipitation with ammonium sulphate, by the addition to the solution of an equal volume of 2.0M phosphate-KOH, pH6.9, giving a final mixture containing 1.0M phosphate ions. The redissolved precipitate contained all of the factor VIII:C activity together with approximately 20% of the bulk protein in the starting material.

Dialysis and Ultrafiltration of Factor VIII:C

Dialysis of factor VIII:C was always accompanied by a marked fall in activity; in many cases the coagulant activity was lost completely. Very low recoveries were also obtained when solutions containing factor VIII:C were subjected to ultrafiltration. It is probable that in both cases the factor VIII:C activity was lost as a result of adsorption to the surface of the dialysis sac or ultrafiltration cell membrane.

The ionic strength and protein concentration in solutions could be changed most conveniently by precipitating the protein and redissolving it in a buffer of low ionic strength. The solution containing factor VIII:C activity was taken to 35% saturation with ammonium sulphate and left to stand at 15^{o}C for 30 minutes until precipitation was complete. It was then centrifuged at 5000g for 15 minutes,

the supernatant was discarded and the precipitate was dissolved in 0.02M imidazole-HCl, pH7.0. The conductivity of the solution was measured and the volume was adjusted with buffer to give the required ionic strength.

The Preparation of 'Partially Purified Factor VIII:C'

The results from eight preparations of 'partially purified factor VIII:C' are summarised in Table 2.3. The chromatographic separations of the factor VIII:C from the other proteins on ECTEOLA-Cellulose, Sepharose 4B and sulphate-Sepharose are shown in Figures 2.7, 2.6 and 2.5 respectively. The polypeptide compositions of the precipitated protein pools were examined by SDS-polyacrylamide slab gel electrophoresis under reducing conditions. Figure 2.8 shows a typical slab gel from which it is apparent that the 'partially purified factor VIII:C' obtained at the completion of the procedure has a heterogeneous polypeptide composition. The gel also shows some of the changes in the protein composition of the pools containing factor VIII:C as the purification proceeds. The general trend appears to be an increase in the relative concentration of peptides with molecular weights in the range 200 000 to 500 000. It is also noticeable that very little of the plasma albumin is present in the first ammonium sulphate precipitate, while the fibrinogen concentration is greatly decreased following chromatography on ECTEOLA-Cellulose.

The final 'partially purified factor VIII:C' was dissolved in a total volume of 1.0ml in a solution containing

TABLE 2.3

Preparation of 'Partially Purified Factor VIII:C' from Bovine Plasma

	Total Factor VIII:C (units)	Recovery of Factor VIII:C	Total Protein (A_{280} units)	Specific Activity (units VIII:C/A_{280} unit)
Plasma	2000	100%	115950	0.017
Precipitated gelatine-Sepharose adsorbed plasma	1930	97%	17290	0.11
Precipitated eluate from ECTEOLA-Cellulose Chromatography	1160	58%	150	7.73
Precipitated void volume peak from Sepharose 4B	440	22%	13	34
Precipitated eluate from Sulphate-Sepharose Chromatography	150	8%	1.6	94

FIGURE 2.7

Chromatography of Factor VIII:C on ECTEOLA-Cellulose

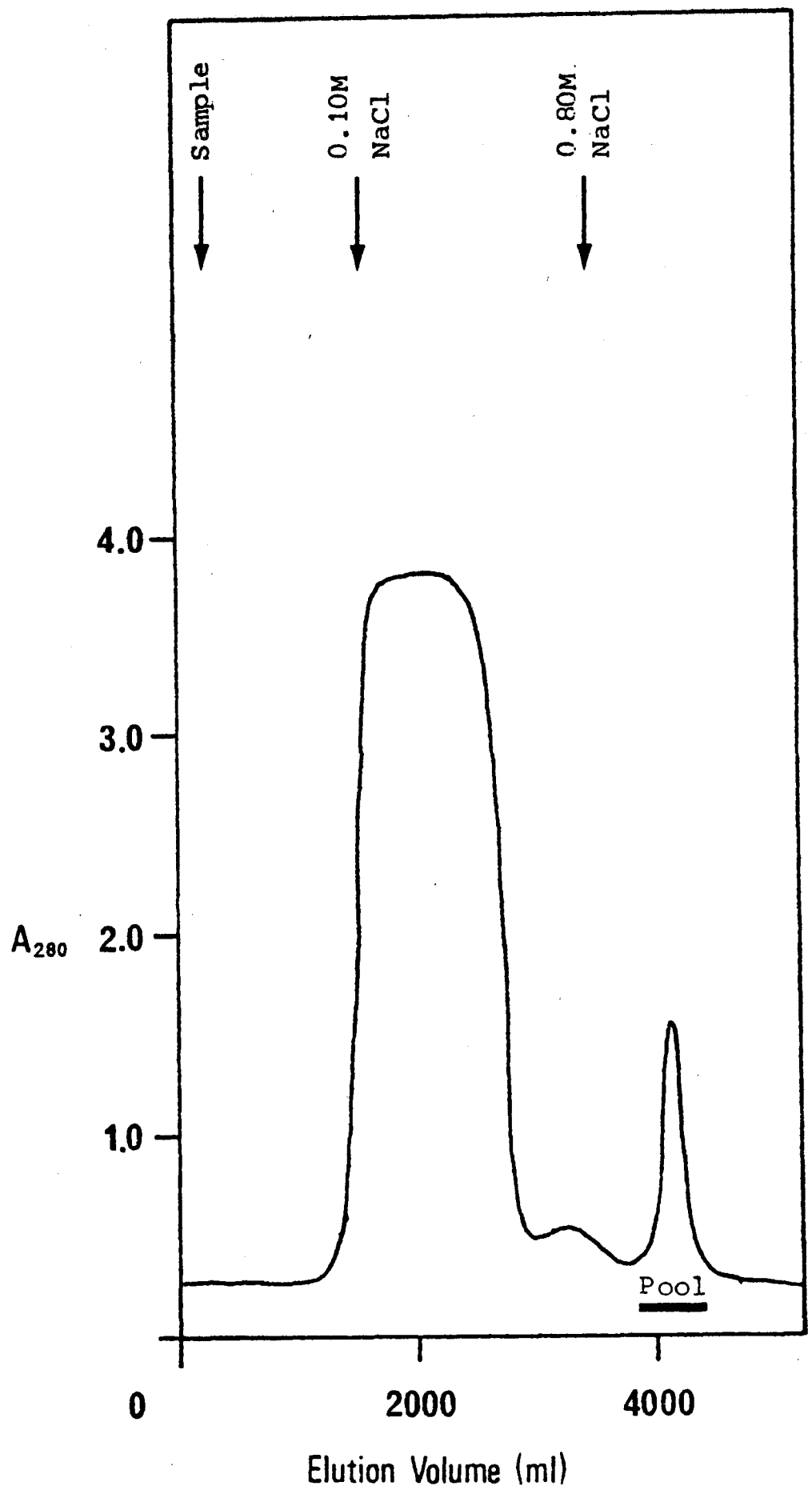
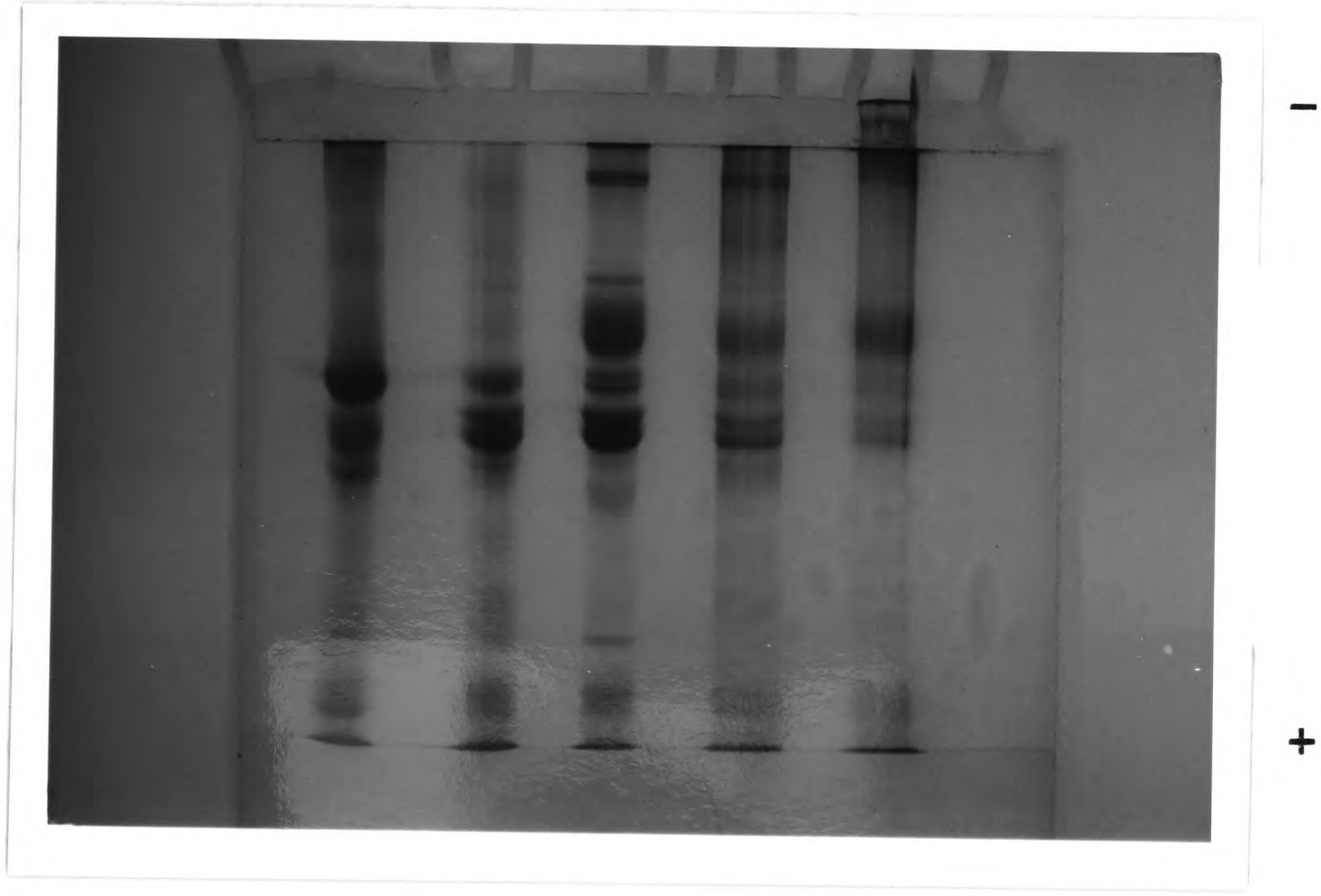


FIGURE 2.8

SDS-PAGE of Samples Taken During the Preparation of 'Partially Purified Factor VIII:C'



1. Plasma (70µg)
2. Precipitated gelatine-Sepharose adsorbed plasma (75µg)
3. Precipitated eluate from ECTEOLA-Cellulose chromatography (60µg)
4. Precipitated void volume peak from Sepharose CL-4B (70µg)
5. 'Partially purified factor VIII:C' (70µg)

50% glycerol. It contained a mean of 150 units of factor VIII:C, as measured by a two-stage assay, and 1.6mg of protein. Typically, the preparation contained 0.35µg of organic-extractable phosphorus; if all of this phosphorus were present in the form of phospholipid, this would correspond to 8.8µg of phospholipid in the 'partially purified factor VIII:C'. In addition to the coagulant activity, the factor VIII:C preparation also had platelet aggregating factor activity, causing the aggregation of suspensions of fresh human platelets, and factor VIIIR:Ag activity, forming precipitin lines with an antiserum raised to conventionally purified factor VIIIR:Ag. In Ouchterlony double immunodiffusion studies, a reaction of identity between the 'partially purified factor VIII:C' and conventionally purified factor VIIIR:Ag was obtained.

The Preparation of Factor VIIIR:Ag

The factor VIIIR:Ag isolated from bovine plasma by Dr Chavin migrated as a single major band of high molecular weight on SDS-polyacrylamide gel electrophoresis in the presence of reducing agents. It was used to raise an antiserum in a rabbit, which - in turn - was used to assess the factor VIIIR:Ag content of other preparations of factor VIII. The antiserum was made monospecific by adsorption with the protein eluting in the included volume of a Sepharose 4B gel-filtration column.

DISCUSSION

The isolation of pure factor VIII:C from bovine plasma poses many problems. The behaviour of the factor VIII:C during ion-exchange chromatography is very similar to that of other plasma proteins and little purification of factor VIII:C can be achieved by the use of such resins as DEAE-Sephadex, QAE-Sephadex or TEAE-Cellulose. The only anion-exchanger which proved to be of value for purifying factor VIII:C was ECTEOLA-Cellulose (van Creveld *et al*, 1961), which appeared to have a higher affinity for factor VIII:C than for the majority of the plasma proteins. The failure of over 95% of the plasma fibrinogen to bind to ECTEOLA-Cellulose was particularly valuable since fibrinogen and factor VIII:C frequently co-purify (Michael and Tunnah, 1966). The low levels of fibrinogen which remained associated with the factor VIII:C could be removed by affinity chromatography on fibrin-monomer-Sepharose, but this was found to be unnecessary when chromatography on sulphate-Sepharose was employed at a subsequent stage of the preparation as this procedure separated the factor VIII:C from the residual fibrinogen.

In general, cation-exchange resins adsorbed too little protein to be of value in the purification of factor VIII:C. Neither the factor VIII:C activity nor the other proteins in the mixtures tested was adsorbed by SP-Sephadex or CM-Sephadex.

Many of the chromatography media examined did adsorb factor VIII:C, but they also bound many of the other proteins

in the mixtures which were applied. Little resolution of the factor VIII:C from the contaminants was obtained even when a salt gradient was used to elute the column. This lack of resolution, together with the frequently low yields of factor VIII:C, prohibited the use of these media in the purification of factor VIII:C. The possibility of employing any of the hydrocarbon-Sepharoses, heparin-Sepharose, or dextran-sulphate-Sepharose was eliminated by criticisms of this type.

A frequent contaminant of factor VIII preparations is fibronectin (Legaz *et al*, 1975), but this can be removed completely by chromatography on gelatine-Sepharose (Engvall and Ruoslahti, 1978). The most convenient method of removing the fibronectin during the purification of factor VIII:C was to carry out a batch adsorption of the starting plasma with gelatine-Sepharose before attempting to fractionate the proteins by ion-exchange chromatography. In this way, it was possible to avoid the presence of fibronectin, which is adhesive and very poorly soluble, in the factor VIII:C-containing protein pools at later stages in the purification.

The anomalous behaviour of the factor VIII:C activity during gel filtration on agarose columns can be utilized during a purification procedure. The apparent exclusion of factor VIII:C from the gel matrix is probably due either to aggregation of the molecules or to the factor VIII:C molecule having a long, rod-like shape. Less than 10% of the total protein applied to the Sepharose CL-4B column was eluted in the

'void volume' whereas approximately 80% of the recovered factor VIII:C was in this pool. The inclusion of a gel-filtration step in the purification of factor VIII:C does, however, result in the isolation of a material which is relatively enriched in phospholipid as this is also excluded from the column.

Fractional precipitation of factor VIII:C is most useful in purification procedures because the activity is associated with a relatively insoluble protein. It is, therefore, possible to precipitate factor VIII:C with low concentrations of, for example, ammonium sulphate. During the preparation of factor VIII:C, the precipitation of the gelatine-Sepharose-adsorbed plasma with 25% saturated ammonium sulphate separated the factor VIII:C from approximately 90% of the plasma proteins, including albumin. A subsequent precipitation of the factor VIII:C with 1.0M phosphate ions left the less insoluble proteins in solution and gave a further increase in the specific activity of the preparation.

The complete purification scheme devised for the preparation of factor VIII:C involved the adsorption of specific contaminants from bovine plasma on to barium sulphate and gelatine-Sepharose, followed by chromatography on ECTEOLA-Cellulose, Sepharose CL-4B and sulphate-Sepharose, and fractional precipitation with ammonium sulphate and phosphate ions. Two further precipitations with 35% saturated ammonium sulphate were used during the purification as the most convenient

method of concentrating the proteins in chromatography column eluates and simultaneously reducing the ionic strength of the solutions.

Using 2000ml batches of plasma as the starting material, the purification procedure described in this Chapter gave a final product with a mean factor VIII:C content of 150 units, together with 1.6mg of protein, as estimated from adsorbance measurements. The specific activity was, therefore, 94 units of factor VIII:C per mg protein, representing a 5000-fold purification of the coagulant activity relative to plasma.

The 'bovine factor VIII' prepared by the method of Legaz and Davie (1976) was originally reported to have a specific activity of 95 units/mg, but a subsequent publication from the same laboratory (Vehar and Davie, 1980) quotes a specific activity of 20 units/mg for preparations of this type. These preparations are now generally referred to as factor VIII/vWF: they possess factor VIII:C, factor VIIIR:Ag and platelet aggregating factor activities. SDS-polyacryl-amide gel electrophoresis of factor VIII/vWF under reducing conditions normally reveals a single polypeptide chain of molecular weight approximately 200 000 (Schmer *et al*, 1972; Shapiro *et al*, 1973) and it was originally thought that this subunit possessed all of the 'factor VIII-related' activities. It is, however, most unlikely that the factor VIII/vWF, or the subunit of molecular weight 200 000, is pure factor VIII:C. Firstly, the 'partially purified factor VIII:C' prepared in the present study is heterogeneous but has a specific coagulant activity similar to, or higher than, that of the factor VIII/

vWF. Secondly, Vehar and Davie (1980) have isolated a reduced form of the factor VIII coagulant molecule from bovine plasma which has a specific activity of 4500 units/mg. Hence, there is little doubt that the principal component of factor VIII/vWF is that responsible for the factor VIIIR:Ag and platelet aggregating factor activities. The factor VIII:C component is probably a minor constituent of the preparation, which is not detectable by SDS-polyacrylamide gel electrophoresis.

It was not possible, during the present work, to devise a method which was capable of purifying bovine factor VIII:C to homogeneity. Unlike the factor VIII/vWF preparations, however, in which there is one major polypeptide species with an apparently high affinity for the coagulant activity, the 'partially purified factor VIII:C' contains numerous components. This should provide greater scope for further purification of the factor VIII:C in the future. It appears from the data of Vehar and Davie (1980) that it will be necessary to purify the activity by an additional factor of approximately fifty in order to obtain pure factor VIII:C. In this context, however, it is essential that the condition of the factor VIII coagulant activity is taken into account. As is described in Chapter 3, the treatment of factor VIII:C with thrombin results in a transient increase in activity, which is detectable only if a one-stage clotting assay is used. Vehar and Davie (1980) used this type of assay, whereas two-stage clotting assays, which are insensitive to thrombin activated factor VIII:C, were used to determine the activity of the 'partially purified

factor VIII:C' prepared in this laboratory. A comparison of the specific activities of the two preparations might, therefore, not be valid.

It may be possible to use recently developed chromatography media such as the solid phase ethylene-maleic anhydride 'polyelectrolytes' described by Johnson *et al* (1978), or techniques such as isoelectric focusing, in the purification of the factor VIII:C to homogeneity. The 'partially purified factor VIII:C' contains both platelet aggregating factor and factor VIIIR:Ag activities. Theoretically, this suggests that the use of gel-filtration in buffers at high ionic strength or other methods designed to dissociate the 'factor VIII complex' might produce an increased purification of the coagulant activity. As described in Chapter 3, however, the factor VIII:C obtained by gel-filtration in the presence of 0.25M calcium chloride appears to be very unstable.

The failure of the factor VIII:C preparation to bind to factor X-Sepharose or - in a consistent manner - to factor IX-Sepharose is rather surprising at first sight. The data presented in Chapter 5, and the results of other workers (Hougie *et al*, 1967; Østerud and Rapaport, 1970; Chuang *et al*, 1972) suggest that the activation of factor X involves the formation of a complex with factors IX_a and VIII:C. It has also been reported (Vehar and Davie, 1980) that chromatography on factor X-Sepharose can be used as the final step in the purification of factor VIII:C. In analogous experiments with factor V, however, which forms a complex with •

factor X_a and prothrombin during the activation of the latter (Paphadjopoulos and Hanahan, 1964; Jobin and Esnouf, 1967; Nesheim *et al*, 1979), Esmon *et al* (1973) found that native bovine factor V would not bind to prothrombin-Sepharose whereas activated factor V was adsorbed by the gel. Thus, it is possible that factor VIII:C might bind to factor X-Sepharose only after 'activation' by, for example, exposure to traces of thrombin or factor X_a. In addition, Vehar and Davie (1980) used factor X-Sepharose under reducing conditions to separate factor VIII:C from a relatively small quantity of contaminating protein - 30% of the material applied to the column was factor VIII:C. The failure of the 'partially purified factor VIII:C' described in this thesis to bind to either factor IX-Sepharose or factor X-Sepharose could, therefore, be the result of using inappropriate conditions for the chromatography. Alternatively, it may reflect the interactions between the proteins being of an inherently weak nature, making it impossible to isolate one protein through the formation of a complex with another component of the factor X-activating system.

Chapter 3

CHEMICAL AND PHYSICAL MODIFICATIONS OF FACTOR VIII

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CHEMICAL AND PHYSICAL MODIFICATIONS OF FACTOR VIII

INTRODUCTION

Many groups have attempted, by the use of several different techniques, to separate the various 'factor VIII-related' activities which are present in normal preparations of bovine and human factor VIII/vWF. The coagulant and platelet aggregating factor activities can be 'dissociated' from one another by centrifugation or gel-filtration in the presence of 1M NaCl (Thelin and Wagner, 1961; Weiss *et al*, 1972), 0.25M $CaCl_2$ (Owen and Wagner, 1972; Rick and Hoyer, 1973) or 1M $MgCl_2$ (Marcus *et al*, 1978). Chromatography on anion-exchange resins (Baugh *et al*, 1974; Brown *et al*, 1974; Olson *et al*, 1977), on solid phase ethylene-maleic anhydride 'polyelectrolytes' (Johnson *et al*, 1978), or on aminohexyl-Sepharose (Austen, 1979) can also be used to separate the coagulant and platelet aggregating activities. Affinity chromatography using Sepharose-bound antibodies raised against either the coagulant moiety or the portion of the molecule responsible for platelet aggregation (Holmberg and Ljung, 1978; Barrow *et al*, 1979; Tuddenham *et al*, 1979; Cooper *et al*, 1980) will selectively adsorb the appropriate fraction from a mixture.

Several workers (Austen *et al*, 1975; Blombäck *et al*, 1978; Savidge *et al*, 1979) have reported that a dissociation of the 'factor VIII-related' activities of factor VIII/vWF can be brought about by the mild reduction of the protein with dithiothreitol. Austen *et al* (1975) reported that the

coagulant activity of factor VIII was easily destroyed by reduction whereas the factor VIIIR:Ag and platelet aggregating activity were more resistant. Others, however, have found that human factor VIIIR:WF activity is more sensitive to reduction than the coagulant activity (Blombäck *et al*, 1978; Counts *et al*, 1978; Savidge *et al*, 1979).

The action of proteolytic enzymes on factor VIII has generally been studied using factor VIII/vWF preparations (Pasquini and Hershgold, 1973; Rick and Hoyer, 1977; Switzer and McKee, 1977, 1979; Atichartakarn *et al*, 1978). No correlation between the changes in the factor VIII:C activity and the polypeptide pattern on SDS-polyacrylamide gel electrophoresis was observed in these experiments, but this was probably a reflection of the composition of the protein.

Most proteinases will destroy the coagulant activity of factor VIII preparations. In the case of thrombin (Rapaport *et al*, 1963) or factor X_a (Davie *et al*, 1975) a transient increase in the factor VIII:C activity precedes its destruction.

Changes in activity similar to those observed with factor VIII/vWF preparations were seen when reduced highly purified factor VIII:C (Vehar and Davie, 1980) was treated with proteinases. In this case, however, it was possible to correlate the changes in coagulant activity with alterations in the SDS-polyacrylamide gel electrophoresis patterns.

METHODS

Dissociation of Factor VIII

Two techniques were used in attempts to separate the coagulant activity of the 'partially purified factor VIII:C' (Chapter 2) from the factor VIIIR:Ag and platelet aggregating factor activities in the preparation: gel-filtration on Sepharose 4B in the presence of a high ionic strength buffer, and chromatography on aminohexyl-Sepharose.

'Partially purified factor VIII:C' was pumped on to a column (0.9cm diameter x 15cm long) of Sepharose 4B, equilibrated with 0.25M calcium chloride in 0.02M imidazole-HCl, pH7.0, at a flow rate of 7ml/hr. The proteins were eluted from the column with the same buffer and fractions of 0.8ml were collected. The factor VIII:C activity of the eluate fractions was measured by a two-stage clotting assay.

The 'partially purified factor VIII:C' was also subjected to gel-filtration under the same conditions but using buffer containing 1.0M sodium chloride and no calcium chloride.

Chromatography on aminohexyl-Sepharose was carried out using a column (1.5cm diameter x 3cm long) of gel prepared by coupling 1,6-diaminohexane to Sepharose 4B activated with cyanogen bromide (Cuatrecasas, 1970). Originally, the column was equilibrated with 0.10M sodium acetate-acetic acid, pH5.5, containing 0.10M lysine (Austen, 1979). In subsequent experiments, however, 0.02M imidazole-HCl, pH7.0, was used as the equilibrating buffer.

'Partially purified factor VIII:C' was applied to the column of aminohexyl-Sepharose at a flow-rate of 50ml/hr, and the column was then washed with 0.10M sodium chloride in buffer until the A_{280} of the eluate fell to zero. The adsorbed proteins were eluted from the column with a gradient from 0.10M to 1.5M sodium chloride in the appropriate buffer. Fractions of approximately 2ml were collected throughout; they were assayed for factor VIII:C, factor VIIIR:Ag and platelet aggregating factor activities using the techniques described in the Appendices.

Reduction and Carboxymethylation of Factor VIII

'Partially purified factor VIII:C' was reduced by incubating it at 4°C for 16 hours with 2-mercaptoethanol at a final concentration of 0.03M in a solution containing 0.02M calcium chloride.

The reduced factor VIII:C was carboxymethylated by adding iodoacetamide to the mixture of 'partially purified factor VIII:C' and 2-mercaptoethanol, to a final concentration of 0.04M. After incubation in the dark at room temperature for 60 minutes, a one-tenth volume of 0.30M 2-mercaptoethanol was added to the mixture to react with excess iodoacetamide.

The activity of the factor VIII in the mixture was measured by a two-stage clotting assay before and after reduction and carboxymethylation.

Interactions of Factor VIII with Proteolytic Enzymes

(i) Thrombin

The effect of thrombin on factor VIII:C was studied using bovine thrombin (Diagnostic Reagents Ltd., Thame, Oxon, UK) reconstituted in water to a concentration of 50 international units per ml, as directed by the manufacturer, and then diluted to 0.5iu/ml with 0.02M imidazole-HCl, pH7.0, for use. The diluted thrombin was mixed with 'partially purified factor VIII:C' in various proportions, and the mixtures were incubated at 37°C. At intervals, aliquots were removed from the incubation mixtures and assayed for factor VIII:C activity by a one-stage or two-stage clotting assay.

The reaction between factor VIII:C and thrombin was investigated further by using hirudin, prepared as described in Appendix 10 by the method of Markwardt (1970). 0.35 units of 'partially purified factor VIII:C' was incubated at 37°C for 120 seconds with 0.01iu thrombin; 0.04 atu hirudin was added either immediately before the thrombin, or at the completion of the incubation. Control samples of factor VIII:C were incubated either alone or with thrombin. After the incubation, the mixtures were diluted, and the factor VIII activity was measured by a one-stage clotting assay.

(ii) Activated Protein C

Activated protein C, prepared as described in Chapter 4, was diluted to a final concentration of 0.02mg/ml in 0.025M tris-HCl, pH7.5. Aliquots of this solution were then

incubated at 37°C with 'partially purified factor VIII:C' in the presence and absence of phospholipid. The changes in the factor VIII:C activity were followed as described above for thrombin.

(iii) Trypsin

Bovine pancreatic trypsin was dissolved in 0.025M tris-HCl, pH7.5, at concentrations of 0.02mg/ml and 0.56mg/ml. 2.7 units of 'partially purified factor VIII:C' was incubated at 37°C with aliquots of trypsin solution; samples were taken immediately after mixing and after 30 minutes at 37°C, and the factor VIII:C activity was measured by a two-stage clotting assay.

RESULTS

Dissociation of Factor VIII

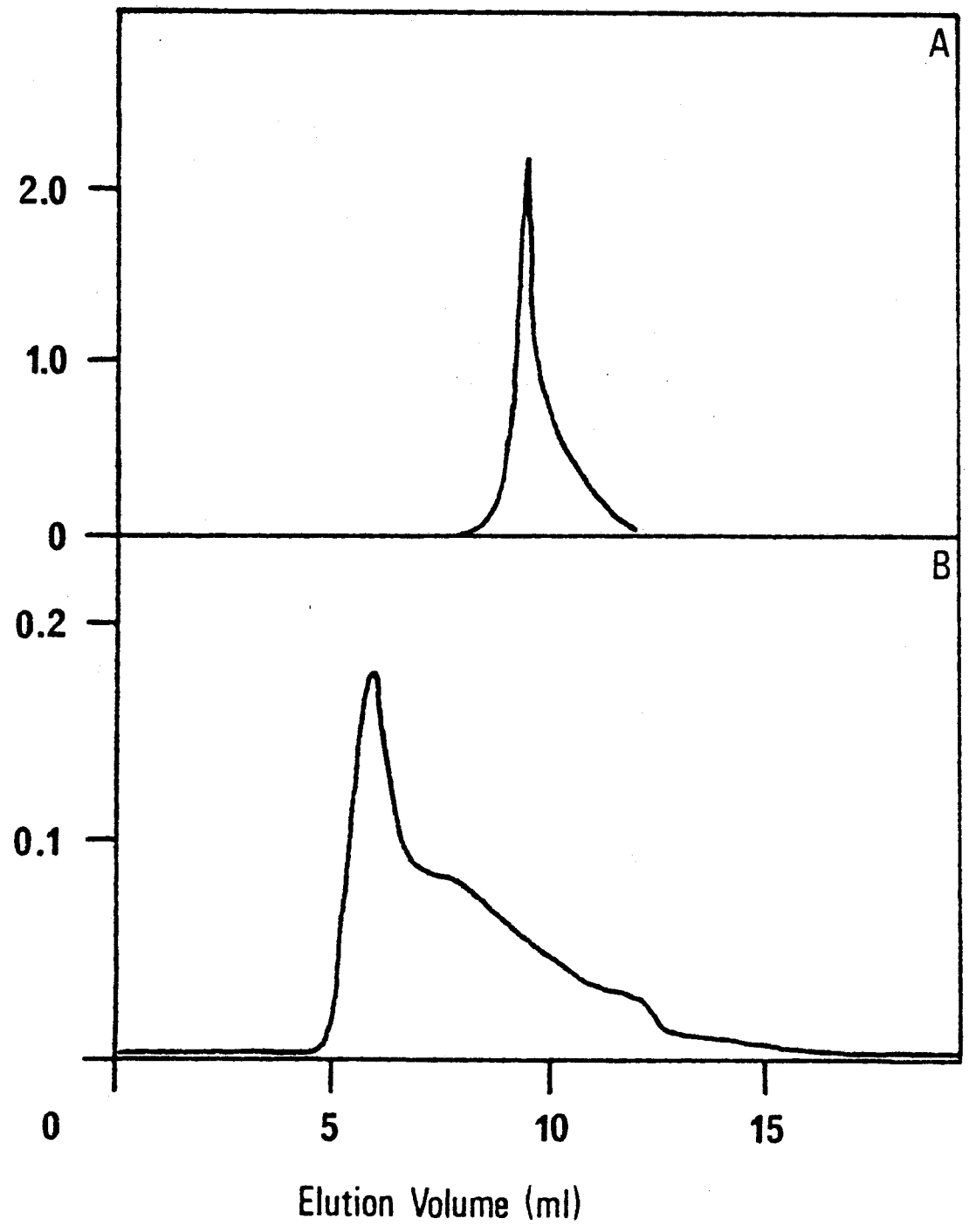
Gel-filtration of 'partially purified factor VIII:C' on Sepharose 4B in the presence of 0.25M calcium chloride resulted in the elution of a single major protein peak from the column as shown in Figure 3.1. The factor VIII:C activity, however, was not associated with the main peak of protein in the excluded volume of the column: it was displaced into the included volume and eluted at the trailing edge of the protein. A very high proportion of the factor VIII:C activity was not recovered in the column eluate: 15 units of factor VIII:C were mixed with calcium chloride and applied to the column, but the pooled eluate fractions contained only 1 unit. The protein concentration in this pool was approximately 0.05mg/ml which was too low to permit the examination of the polypeptide composition by SDS-polyacrylamide gel electrophoresis.

The elution of the 'partially purified factor VIII:C' from the Sepharose 4B column in the presence of 1M sodium chloride was indistinguishable from that seen in buffers containing low concentrations of salt. The protein was eluted as a single major peak in the excluded volume of the column, identical to the pattern shown in Figure 3.1B. In this case, however, the factor VIII:C activity was also eluted in the 'void volume'.

The chromatography of 'partially purified factor VIII:C' on aminohexyl-Sepharose columns equilibrated with 0.10M

FIGURE 3.1

Gel-filtration of 'Partially Purified Factor VIII:C' on Sepharose 4B in 0.25M Calcium Chloride



Panel A: Factor VIII:C (units/ml)

Panel B: Protein (A_{280})

sodium acetate-acetic acid, pH5.5, was unsuccessful. No factor VIII coagulant activity was detectable in any of the fractions eluted from the column.

The use of 0.02M imidazole-HCl, pH7.0, as the buffer for chromatography on aminohexyl-Sepharose produced very variable results. On occasion, elution patterns similar to that shown in Figure 3.2 were obtained. That is, the factor VIIIR:Ag and platelet aggregating factor activities were not adsorbed by the gel whereas the factor VIII:C activity was bound and subsequently eluted by the sodium chloride gradient. On other occasions, however, the separation of the 'factor VIII-related' activities was much less well defined, despite the use of apparently identical conditions. The factor VIII:C was frequently divided into two fractions, with activity being present in the material which did not adsorb to the gel as well as in the proteins eluted by the salt gradient. In addition, part or all of the platelet aggregating factor applied to the column was adsorbed by the gel in some experiments; when this occured, the PAF activity was eluted in the same fractions as the coagulant activity.

Reduction and Carboxymethylation of Factor VIII

The treatment of 5.0 units of factor VIII:C with 0.03M 2-mercaptoethanol at 4^{o}C for 16 hours resulted in a fall in the activity, measured by a two-stage clotting assay, to a mean value of 2.75 units. That is, a loss of approximately 45% of the coagulant activity was associated with the reduction

FIGURE 3.2

Chromatography of 'Partially Purified Factor VIII:C' on Amino-hexyl-Sepharose

Panel A: Sodium chloride gradient (Molarity of NaCl)

Panel B: PAF Activity

Panel C: Factor VIIIR:Ag; height of immunoelectrophoresis peak (mm)

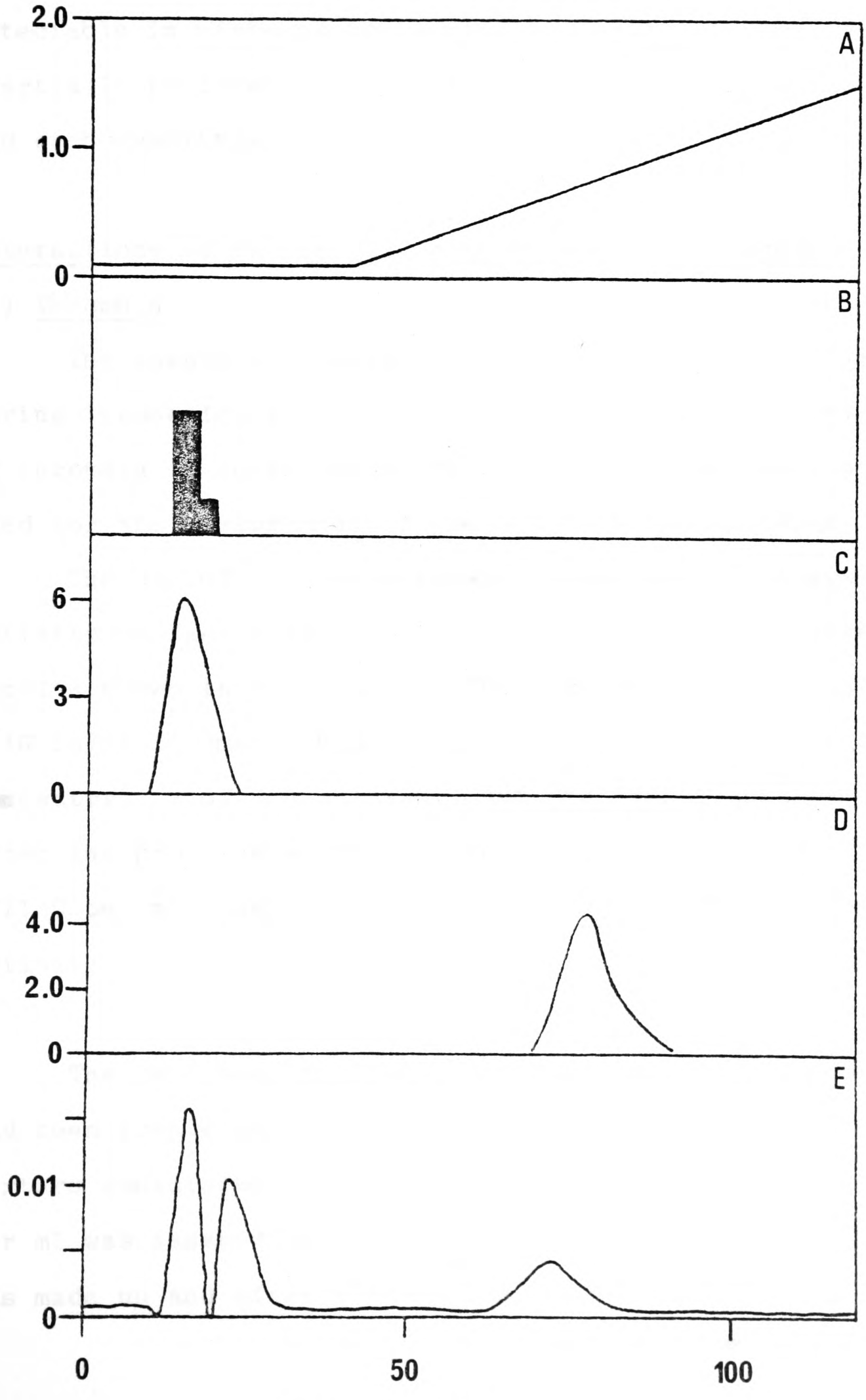
Panel D: Factor VIII:C (units/ml)

Panel E: Protein (A_{280})

FIGURE 3.2

Chromatography of 'Partially Purified Factor VIII:C'

on Amino-hexyl-Sepharose



process. No further loss of factor VIII:C activity was observed when the reduced protein was stored at 4°C in the presence of 2-mercaptoethanol for an additional 72 hours.

Less than 0.2 units of factor VIII:C activity was detectable in mixtures containing 5.0 units of unmodified 'partially purified factor VIII:C' following the reduction and carboxymethylation of the proteins.

Interactions of Factor VIII with Proteolytic Enzymes

(i) Thrombin

The change in the factor VIII:C activity with time during incubation at 37°C with three different concentrations of thrombin is shown in Figure 3.3. One-stage assays were used for the measurement of the activity in all cases.

The use of two-stage assays to measure the coagulant activity of factor VIII in a mixture with thrombin gave the results shown in Table 3.1. The mixture, which contained 0.16 iu/ml thrombin, had a nominal original factor VIII:C concentration of 3.0 units/ml, but the sample assayed immediately after the proteins were mixed showed only 1.5 units of factor VIII:C per ml, suggesting that the thrombin was affecting the activity.

The one- and two-stage assays of factor VIII:C which had been treated with thrombin were compared directly. A mixture containing 4.5 units factor VIII:C and 0.17 iu thrombin per ml was assayed by both techniques immediately after it was made up and after six minutes incubation at 37°C. The

FIGURE 3.3

'Activation' of 'Partially Purified Factor VIII:C'

by Thrombin

Solutions containing approximately 8 units factor VIII per ml were incubated with (A) 4 iu/ml, (B) 0.33 iu/ml or (C) 0.15 iu/ml bovine thrombin. At intervals, aliquots were diluted in citrate-saline and the factor VIII:C concentration was determined using a one-stage assay.

FIGURE 3.3

'Activation' of 'Partially Purified Factor VIII:C'

by Thrombin

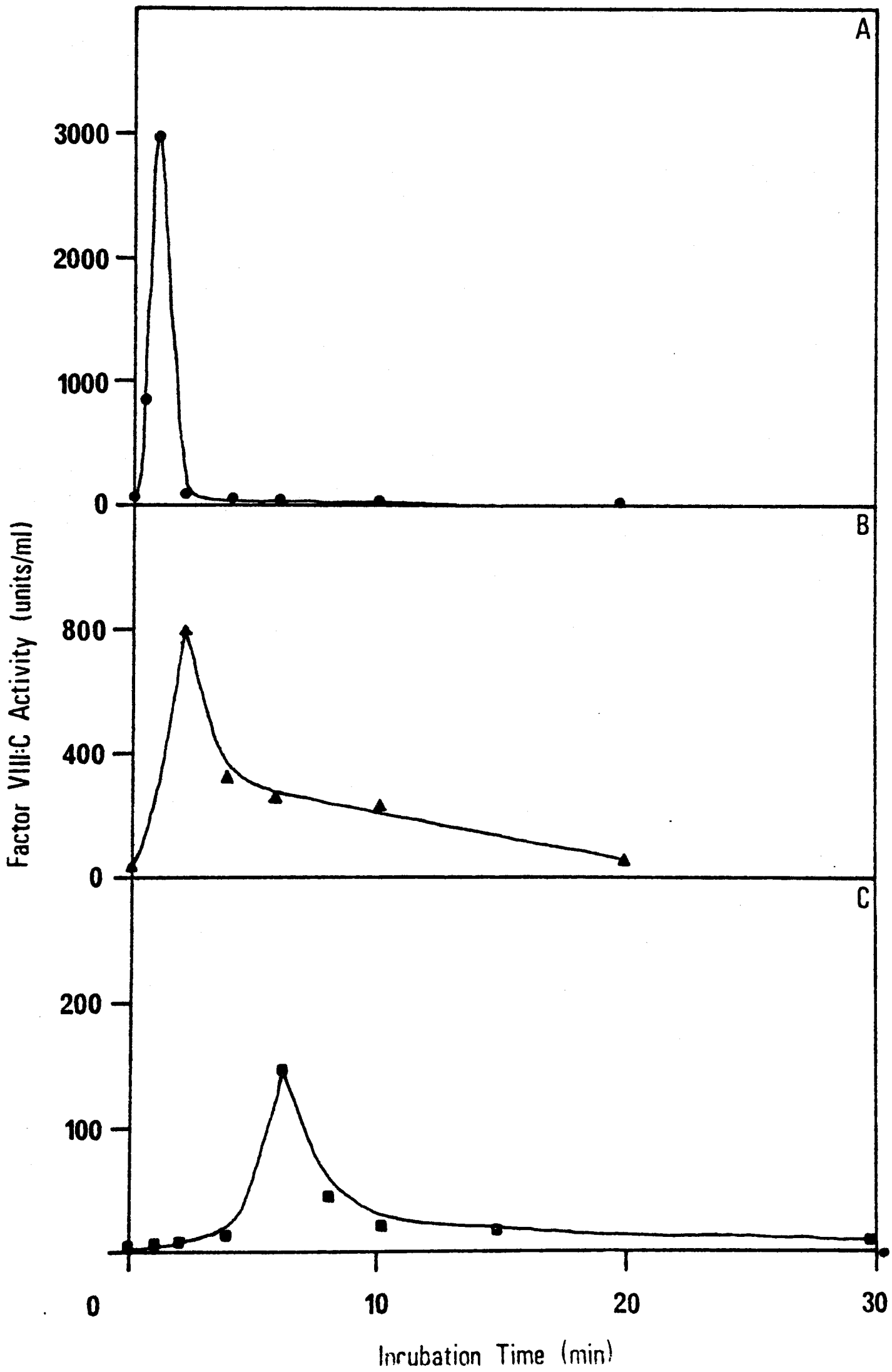


TABLE 3.1

Interaction of Factor VIII with Thrombin
(Two-Stage Clotting Assays of Factor VIII)

Incubation Period (Minutes)	Factor VIII:C Activity (units/ml)
0	1.5
1	1.1
2	1.0
3	0.7
4	0.7
6	0.5
8	<0.4
10	<0.4

'Partially purified factor VIII:C', at a nominal concentration of 3.0 units/ml, was incubated at 37°C with 0.16 iu/ml thrombin. At intervals, the factor VIII:C activity was measured by two-stage clotting assays.

one-stage assay indicated that the factor VIII:C concentration in this mixture was 4.6 units/ml before incubation and 20.6 units/ml afterwards. The two-stage assay indicated that immediately after adding the thrombin, the mixture contained 4.4 units/ml of factor VIII:C, but no activity was detectable after incubation.

Two-stage assays of thrombin-treated factor VIII:C were investigated further by incubating a mixture containing 4.5 units of factor VIII:C and 0.17 iu of thrombin per ml at 37°C for six minutes, diluting into citrate-saline, and setting up a series of identical mixtures for the first stage of the assay. These mixtures were incubated at 37°C for periods ranging from 10 seconds to the 10 minutes normally used for the assay. No factor VIII coagulant activity was detected in any of the samples.

The influence of hirudin on the interaction between thrombin and factor VIII:C is summarised in Table 3.2. In all cases, the coagulant activity was determined by means of a one-stage assay.

(ii) Activated Protein C

The change in the detectable factor VIII:C concentration when 1.9 units/ml of 'partially purified factor VIII:C' was incubated at 37°C with 0.004mg/ml activated protein C in the presence of 0.01M calcium chloride is shown in Figure 3.4. The results were not affected by the addition of 0.10mg/ml

TABLE 3.2

The Effect of Hirudin of the 'Activation' of Factor VIII:C by Thrombin

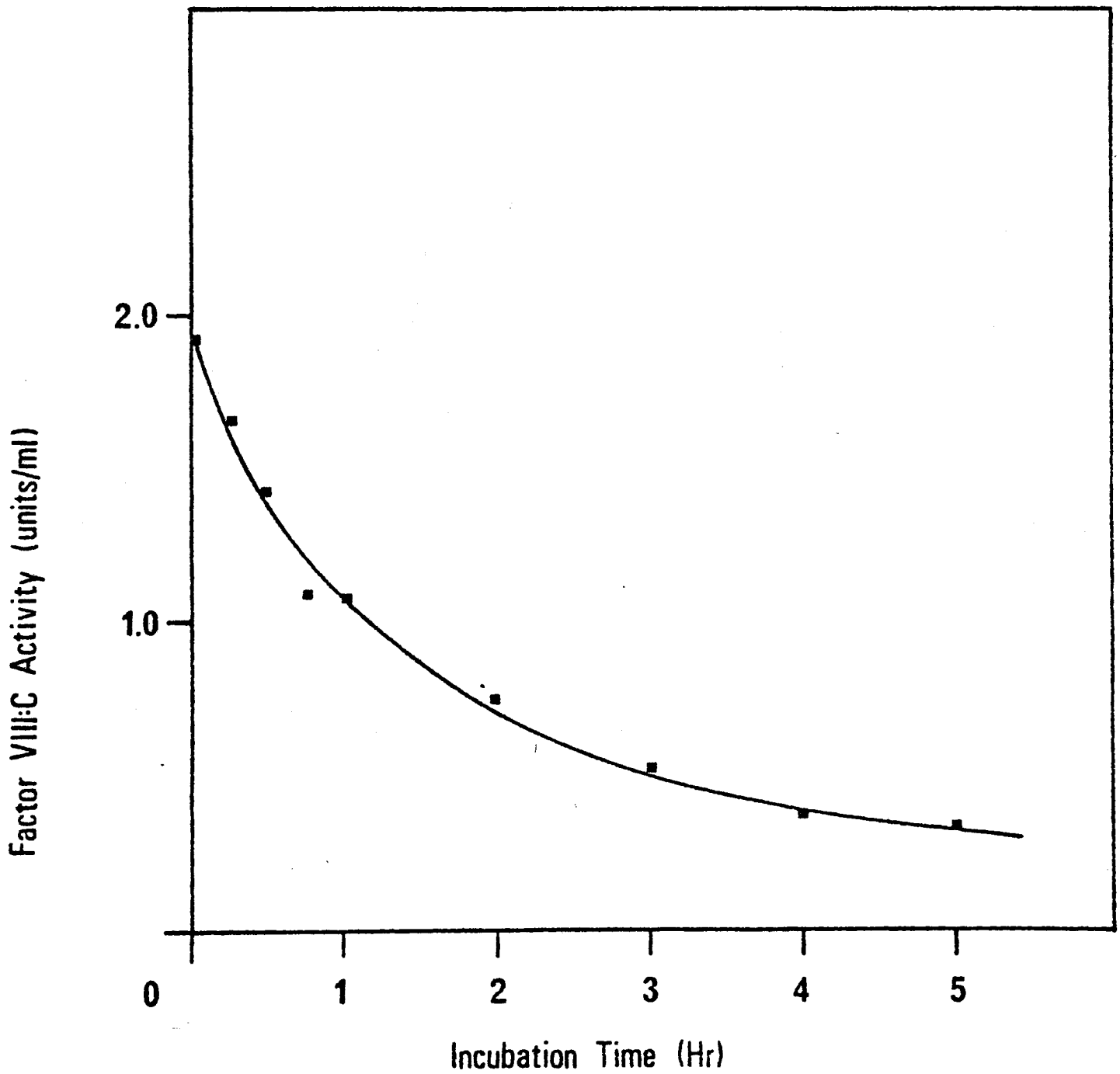
	Total Factor VIII:C
Factor VIII incubated alone	0.36 units
Factor VIII incubated with thrombin	3.12 units
Factor VIII incubated with thrombin and hirudin	0.37 units
Factor VIII incubated with thrombin; hirudin added after incubation	3.34 units

0.35 units of 'partially purified factor VIII:C' was incubated at 37°C for 120 seconds with 0.01iu thrombin and 0.04atu hirudin was added either immediately before the thrombin or at the completion of the incubation. The factor VIII:C activity was determined in a one-stage assay immediately the incubation was complete.

FIGURE 3.4

Inactivation of 'Partially Purified Factor VIII:C'

by Activated Protein C



1.9 units/ml 'partially purified factor VIII:C' was incubated at 37°C with 0.004mg/ml activated protein C and 10mM $CaCl_2$. At intervals, the coagulant activity was measured with a two-stage factor VIII:C assay.

Centrolex P lecithin to the incubation mixtures. In the absence of activated protein C, the incubation of 1.9 units/ml 'partially purified factor VIII:C' at 37°C in a solution containing 0.01M calcium chloride resulted in a slight increase in the measurable activity over a period of five hours.

(iii) Trypsin

Table 3.3 summarises the changes in coagulant activity seen when 2.7 units of 'partially purified factor VIII:C' was incubated at 37°C with varying concentrations of trypsin in a total volume of 0.06ml. In the case of the factor VIII:C sample incubated with 0.09mg/ml trypsin, no activity was detectable when it was assayed within ten seconds of mixing.

TABLE 3.3

The Effect of Trypsin on Factor VIII:C

Final Concentration of Trypsin (mg/ml)	Factor VIII:C Activity (units/ml)	
	Immediately After Mixing	After 30 Minutes Incubation
0.000	48	42
0.004	53	0
0.090	0	0

'Partially purified factor VIII:C' (45 units/ml) was mixed with various concentrations of trypsin and the coagulant activity was determined immediately after mixing and following 30 minutes incubation at 37°C.

DISCUSSION

The late elution of factor VIII:C activity which was observed during gel-filtration in the presence of 0.25M calcium chloride is in agreement with the results of other workers (Owen and Wagner, 1972; Rick and Hoyer, 1973). The separation of the coagulant activity from the main protein and from the factor VIIIR:Ag, which is reported to elute in the column 'void volume' irrespective of the ionic strength of the buffer, is an interesting phenomenon, particularly with regard to models of the structure of the factor VIII protein or complex in plasma. This 'dissociation' is not, however, a viable means for the preparation of factor VIII:C uncontaminated with platelet aggregating factor. Less than 10% of the coagulant activity applied to the column was recovered in the eluted material, and even this appeared to be relatively unstable. It has been suggested (Switzer and McKee, 1977) that the 'dissociation' of factor VIII/vWF into coagulant and platelet aggregating fractions depends on the limited proteolytic cleavage by thrombin of a single entity having both activities. If this is the case, it would account for the loss and destabilization of the coagulant activity.

The failure of the factor VIII:C activity to 'dissociate' during gel-filtration in the presence of 1M sodium chloride is in contrast to the results of Weiss *et al* (1972). This may simply reflect the different types of preparations used since it appears that more highly purified factor VIII 'dissociates' less readily than cruder material.

A successful separation of the factor VIII:C activity

from the factor VIIIR:Ag and factor VIIIR:WF activities by chromatography on aminohexyl-Sepharose has been reported by only one group (Austen, 1979), who used relatively impure sources of human and porcine factor VIII. It is possible that the inconsistent results obtained with different preparations of 'partially purified bovine factor VIII:C' reflect slight variations in their composition: as with gel-filtration in high ionic strength buffers, 'dissociation' of factor VIII on aminohexyl-Sepharose may occur more readily when a less highly purified starting material, such as cryoprecipitate is used.

The reduction of factor VIII/vWF with dithiothreitol has been claimed (Austen, 1974) to 'dissociate' the coagulant and factor VIIIR:Ag activities. Blombäck *et al* (1978) also found that treating plasma with dithiothreitol and iodoacetic acid resulted in the factor VIII:C activity being eluted in the included volume during gel-filtration on Sepharose 2B, whereas the factor VIIIR:Ag remained excluded from the column. These groups found that 10^{-4}M or 10^{-3}M dithiothreitol had very little effect on the level of factor VIII:C, but that higher concentrations of the reducing agent destroyed the activity. In the present work, a loss of approximately 45% of the coagulant activity of the 'partially purified factor VIII:C' was observed after reduction with 0.03M 2-mercaptoethanol. In contrast with the observations of Blombäck *et al* (1978), however, alkylation of the reduced 'partially purified factor VIII:C' with iodoacetamide destroyed the coagulant activity. These losses of factor VIII:C

activity following reduction strongly suggest that intact disulphide bridges are important for the expression of the coagulant activity of factor VIII.

The reduced 'partially purified factor VIII:C' was not subjected to gel-filtration. It was prepared principally for chromatography on factor X-Sepharose following a report (Vehar and Davie, 1980) that factor VIII:C could be purified by chromatography on this resin under reducing conditions. As is described in Chapter 2, however, chromatography of reduced 'partially purified factor VIII:C' on factor X-Sepharose was no more successful than when the unreduced material was used.

The destruction of the factor VIII:C activity of 'partially purified factor VIII:C' by proteinases accords with reports (Pasquini and Hershgold, 1973; Atichartakarn *et al*, 1978; Triantaphyllopoulos, 1979; Varadi *et al*, 1980; Andersen *et al*, 1980; Cockburn *et al*, 1981) that the coagulant activity of factor VIII/vWF is destroyed by plasmin, trypsin, chymotrypsin, granulocyte proteinases and factor X_a. Several of these authors observed changes in the SDS-polyacrylamide gel electrophoresis pattern of the protein after proteolytic digestion, but, in most cases, there was no correlation between these changes and the loss of the factor VIII:C activity. It is likely that this was due to the factor VIII:C being only a minor component of the factor VIII/vWF preparations. Cockburn *et al* (1981), using a modified gel electrophoresis technique, detected a polypeptide of molecular weight 85 000, comprising approximately 2% of their

human factor VIII/vWF, which was degraded by plasmin at a rate corresponding to the loss of the factor VIII:C activity from the preparation. Vehar and Davie (1980) were able to correlate the degradation of one polypeptide in their highly purified factor VIII:C preparation with the loss of the coagulant activity during treatment with activated protein C. In general, the destruction of factor VIII coagulant activity by proteinases is probably a simple result of proteolysis of the molecule. The breakdown of factor VIII:C by activated protein C described in this Chapter and by Vehar and Davie (1980) may - together with the action of this enzyme on factor V (Kisiel *et al*, 1977; Walker *et al*, 1979) - provide a mechanism whereby *in vivo* coagulation is controlled.

The effect of thrombin on factor VIII:C has been a source of controversy for several years. The 'activation' of factor VIII:C in plasma by thrombin was first reported by Rapaport *et al* (1963). It has since been described in greater detail, and with purified systems, by Shapiro *et al* (1973), Legaz *et al* (1975), Rick and Hoyer (1977, 1978), Switzer *et al* (1979, 1980) and Hultin and Jesty (1981). The increase in factor VIII:C activity following the addition of thrombin was measured by all of these authors by means of a one-stage assay; the extent of the 'activation' varied from fourfold (Shapiro *et al*, 1973) to more than 100-fold (Legaz *et al*, 1975). Switzer and McKee (1980) have shown that the level of factor VIII:C which is seen depends on the quantity of thrombin used; this was confirmed during the present work, and may account for the variability among the other reports.

In contrast, Vukovich *et al* (1978, 1980) concluded on the basis of experiments carried out with thrombin coupled to Sepharose that the enzyme is responsible solely for the inactivation of factor VIII:C. These workers saw only a decline in the coagulant activity of thrombin-treated factor VIII/vWF when a two-stage assay was employed. In addition, they found that there was no increase in the factor VIII:C activity determined in a one-stage assay if the thrombin-Sepharose was removed from the test sample. They therefore concluded that the 'activation' of factor VIII:C is an artefact resulting from an effect of thrombin on the one-stage assay system.

The failure of Vukovich *et al* (1978) to show activation of factor VIII:C in samples from which thrombin-Sepharose had been removed prior to the assay may be accounted for by the suggestion (Hultin and Jesty, 1981) that 'activated factor VIII:C' is actually a factor VIII:C-thrombin complex. According to this model, both components would be required in the assay in order for an effect to be detectable. The failure of hirudin to reduce the activity of activated 'partially purified factor VIII:C' when it was added after the incubation of the factor VIII:C with thrombin, in contrast to the inhibition of factor VIII:C activation observed when the hirudin was added prior to the incubation with thrombin, would suggest that the components of a putative thrombin-factor VIII:C complex must have a high affinity for one another and that the active site of the thrombin molecule is likely to be involved in the binding. Hultin and Jesty

(1981) found that inhibitors of thrombin, including hirudin, DAPA (Nesheim *et al*, 1979), and diisopropyl fluorophosphate, would inhibit the activation of factor VIII:C; they also reported that the addition of such inhibitors to mixtures of thrombin and factor VIII:C when the coagulant activity reached a peak enhanced the rate at which the activity was subsequently lost.

The treatment of 'partially purified factor VIII:C' with thrombin did not increase its activity in a two-stage clotting assay, confirming the results obtained with factor VIII/vWF by Vukovich *et al* (1978) and Cockburn *et al* (1981). This may be a result of the ten minute incubation period in the first stage of the assay, which is presumably sufficiently long for factor X_a and thrombin to be generated and to react with the factor VIII:C. If the factor VIII:C is 'activated' prior to its addition to a two-stage assay, the first stage incubation provides time for the factor VIII:C to be inactivated and hence only a fall in activity can be seen. Tran *et al* (1979) have suggested that the activation of factor VIII:C can be seen in the form of a shortening of the time required for the first incubation in the two-stage assay, but no activity was detectable in thrombin-treated 'partially purified factor VIII:C' when shorter incubation periods were used.

The results obtained with the 'partially purified factor VIII:C', together with those reported by other groups investigating the interaction of factor VIII/vWF with thrombin, indicate that the enzyme causes a transient increase in the

factor VIII:C activity, followed by a decline. This effect can be detected in a one-stage assay, but not in a two-stage assay, and its magnitude depends on the relative concentrations of factor VIII:C and thrombin. Both Vehar and Davie (1980) and Cockburn *et al* (1981) have reported changes in the polypeptide composition of factor VIII preparations which can be correlated with the thrombin 'activation' of the coagulant activity. The former group found that the 'activation' of their highly purified bovine factor VIII:C by thrombin or factor X_a was accompanied by a conversion of the polypeptide triplet to a doublet of molecular weight 69 000 and 73 000, a third major component of molecular weight 38 000 and a minor component of molecular weight 55 000. The latter group observed a decrease in the molecular weight of a minor component in their human factor VIII/vWF from 85 000 to 77 000 during 'activation' by thrombin. Neither of these reports, which suggest that there may be a difference between species, has yet been confirmed.

The 'partially purified factor VIII:C' used in these studies has a different polypeptide composition from that of factor VIII/vWF. The biological activity of the factor VIII:C in the two types of preparation appears, however, to be the same. It can be separated from the factor VIIIR:Ag and platelet aggregating factor activities under appropriate conditions, but is rendered unstable by this procedure. The factor VIII:C activity is decreased by disulphide bond reduction but the stability is not affected. Thrombin 'activates''partially purified factor VIII:C' and it, and

other proteinases, will destroy the coagulant activity.

Chapter 4

THE PURIFICATION OF THE VITAMIN K-DEPENDENT PROTEINS FROM BOVINE PLASMA

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THE PURIFICATION OF THE VITAMIN K-DEPENDENT PROTEINS FROM BOVINE PLASMA

INTRODUCTION

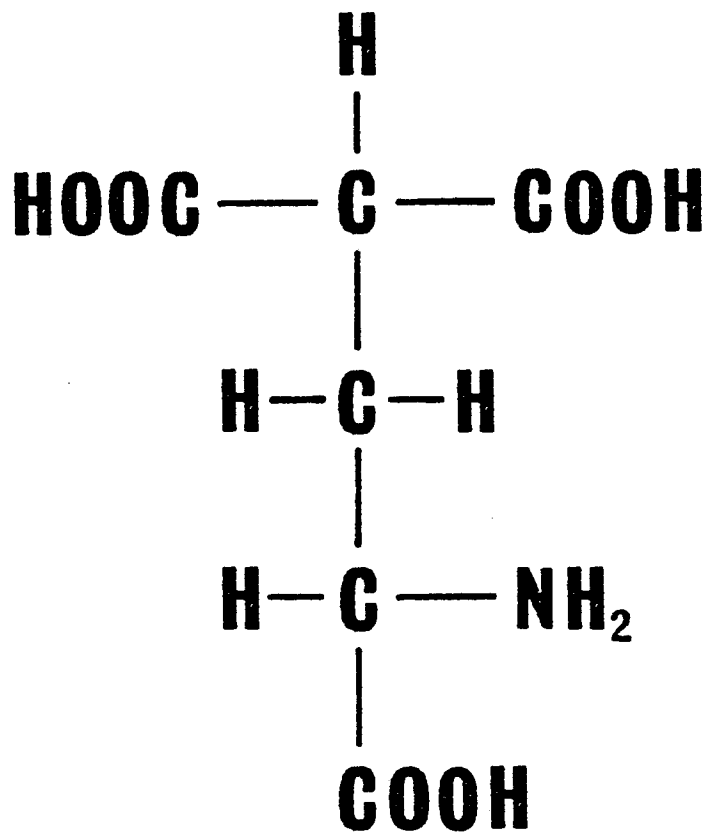
Seven proteins present in bovine plasma are dependent upon vitamin K for their biosynthesis (Esnouf, 1982). The vitamin K-dependent proteins, prothrombin, factor VII, factor IX, factor X, protein C, protein S and protein Z, all contain glutamic acid residues in the amino-terminal region of the molecule which have undergone a post-translational modification converting them to γ-carboxyglutamic acid (Figure 4.1).

Prothrombin, and factors VII, IX and X are well characterised components of the blood coagulation cascade (Davie *et al*, 1979; Jackson and Nemerson, 1980; Esnouf, 1982). Factor X from bovine plasma has two unusual properties: it is found only as a two-chain molecule; that is, a single chain form of factor X has never been isolated; and it can be resolved into two forms (Jackson and Hanahan, 1968) by chromatography on DEAE-Sephadex. The two forms of the molecule are usually designated X_1 and X_2, and the difference between them has been variously ascribed to a difference in their carbohydrate content (Jackson, 1972), to the presence of a sulphated tyrosine residue at position 18 of the heavy chain of factor X_2 but not factor X_1 (Morita and Jackson, 1979), and to a difference in the number of γ-carboxyglutamic acid residues in the light chains of the proteins (Neal *et al*, 1976).

Protein C (Stenflo, 1976) is the zymogen of a serine proteinase which has a marked anticoagulant activity (Kisiel

FIGURE 4.1

γ-Carboxyglutamic Acid (Gla)



et al, 1977), due - at least in part - to the inactivation of factor V. It has also been reported that factor VIII is inactivated by activated protein C (Vehar and Davie, 1980). Recently, it has been proposed (Walker, 1980, 1981) that protein S (Di Scipio and Davie, 1979) might function as a high molecular weight cofactor for activated protein C.

Protein Z (Prowse and Esnouf, 1977) was originally believed to be a single chain form of factor X (Mattock and Esnouf, 1973). The amino acid composition (Petersen *et al*, 1979) and the sequence of the amino-terminal region (Petersen *et al*, 1980) of protein Z are, however, distinct from those of factor X. The function of protein Z is yet to be determined.

The amino acid sequences of the amino-terminal regions of the vitamin K-dependent plasma proteins are homologous, with a characteristic gla-gla pair at residues 7 and 8. The sequence homology among these proteins has led to the suggestion (Katayama *et al*, 1979) that they may have evolved from a common ancestral protein.

The vitamin K-dependent proteins are all adsorbed from plasma on to insoluble barium salts (Magnusson, 1971). This property, which depends on the presence of γ-carboxyglutamic acid residues in the molecules, provides the means of separating the vitamin K-dependent proteins from the remaining components of the plasma. Prothrombin - the most abundant member of the group - can be purified one hundred-fold in a single step by adsorption on to barium sulphate (Magnusson, 1971).

Although barium sulphate was used as the adsorbent in the

first stage of several procedures for the purification of the vitamin K-dependent proteins (Jackson *et al*, 1968; Fujikawa *et al*, 1972a, 1973; Esnouf *et al*, 1973) it is not the most suitable barium salt for this purpose because the barium sulphate pellets are difficult to handle and less than 50% of the plasma prothrombin can be recovered from them. These problems can largely be overcome by the use of barium citrate in place of barium sulphate (Prowse, 1976). The recovery of prothrombin in high ionic strength eluates from barium citrate is almost complete, and the citrate forms a softer, more tractable pellet in the centrifuge.

The physical properties of the vitamin K-dependent proteins are very similar and it is, therefore, difficult to obtain a complete fractionation of the various components which are adsorbed from plasma on to barium citrate and subsequently eluted. Chromatography on anion-exchange resins such as DEAE-Sephadex will separate the vitamin K-dependent proteins from any contaminating material and also provide an initial fractionation into 'prothrombin' and 'factor X' (Esnouf *et al*, 1973). The prothrombin pool also contains factors VII and IX and proteins C and S, while the factor X pool also includes protein Z.

Several groups of workers have devised procedures for tne isolation of the components of the protein pools obtained from DEAE-Sephadex. The methods employed have been described by Fujikawa *et al* (1972a, 1973); Kisiel and Hanahan (1973), Stenflo (1976); Prowse and Esnouf (1977), Di Scipio *et al*.

(1977, 1979) and Østerud *et al* (1978). In some cases, the purification of a single protein has been attempted, whereas other groups have been able to purify several of the vitamin K-dependent proteins simultaneously.

The procedures used for the purification of the vitamin K-dependent proteins in this laboratory and described in this thesis include modifications of the methods originally employed by various workers. Minor changes in the procedure have been made from time to time in an attempt to improve the yield and purity of the products. The detailed methods described are, therefore, those used in the most recent preparations.

MATERIALS

Anion-exchange resins for the chromatographic separation of the vitamin K-dependent proteins, including DEAE-Sephadex A-50, DEAE-Sepharose CL-6B, and QAE-Sephadex A-50, were purchased from Pharmacia Fine Chemicals, London, U.K. Heparin-Sepharose was prepared by coupling porcine mucosal heparin to Sepharose 4B (Pharmacia Fine Chemicals) using the cyanogen bromide activation technique of Cuatrecasas (1970) which is described in detail in Appendix 9.

The factor X activating enzyme (RVV-X) was isolated from Russell's viper (*Vipera russellii russellii*) venom by chromatography on TEAE-Cellulose (Serva Feinbiochemica, Heidelberg, German Federal Republic) as described by Williams and Esnouf (1962). It was further purified by gel-filtration on Ultrogel AcA 44 (LKB Ltd., Croydon, Surrey, U.K.) using 0.05M phosphate-NaOH, pH7.5, as the eluant. The purified protein was stored in small aliquots at a concentration of 2mg/ml at -20°C.

'Contact Product', which is a crude preparation of factor XI_a (Nossel, 1964) was isolated from bovine plasma by mixing the plasma with 40mg/ml Celite 512 for ten minutes, and then filtering. The Celite 512 was washed with 0.17M NaCl in 0.05M tris-HCl, pH7.5, until no protein was detectable in the eluate. The adsorbed proteins were then eluted by washing the Celite 512 with 1.7M NaCl in 0.05M tris-HCl, pH7.5. This fraction (contact product) was dialysed into 0.05M tris-HCl, pH7.5, and stored in small aliquots at a concentration of 1mg/ml at -20°C.

ASSAY METHODS

The activity of the vitamin K-dependent proteins was measured by means of clotting assays, or immunoelectrophoretically, using the techniques described in Appendices 3 and 7. The purity of the proteins was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Both rod- and slab-gels were employed, using the buffer systems indicated in Appendix 8.

EXPERIMENTAL METHODS AND RESULTS

Isolation of Proteins from Bovine Blood

200 litres of blood was collected, immediately after the death of the cattle at the slaughterhouse, into 20 litres of 4% trisodium citrate, in plastic buckets, and the froth was removed. The blood was transported to the laboratory in plastic bottles as rapidly as possible. Within 60 minutes of collection, the plasma was separated from the cells in a Westfalia continuous flow centrifuge (Model SAOOH 205 from Westfalia Separator Ltd., Wolverton, Bucks, U.K.) which was operated in the separator mode with the following parameters:

Blank vanes at the top

Centripetal pump: 38mm

Regulating ring dam: 36mm

Back pressure: 20-22 psi

Speed of rotation of bowl: 10 000rpm

Inlet flow rate: 180 litres/hour

The plasma (100 litres) was stirred for 30 minutes with a cold (4^{o}C) suspension of 3000g barium chloride in 6 litres of distilled water. The precipitate of barium citrate produced at this step was separated from the supernatant using the Westfalia centrifuge in the clarifier mode with the following parameters:

Blank vanes at the bottom

Centripetal pump: 38mm

Regulating ring dam: 27mm

Back pressure: 18 psi

Inlet flow rate: 70 litres/hour

A few drops of octan-2-ol were added to the barium citrate suspension to disperse the surface foam, and the material was recycled through the Westfalia centrifuge and washed with cold 5mM barium chloride. The precipitate was then collected, mixed with cold 5mM barium chloride and sedimented at 5000g in an MSE Coolspin centrifuge (MSE Ltd., Crawley, Sussex, U.K.), using a 6 x 1000ml swing-out rotor. The supernatant was discarded and the precipitate was again washed with 5mM barium chloride and spun down. The pellet was resuspended in 8 litres 0.2M citric acid-NaOH, pH6.9, and solid ammonium sulphate was added to give a final 30% saturation at 20°C. The mixture was stirred for 30 seconds with a Silverson mixer emulsifier, using octan-2-ol to prevent foaming, and left to stand at 4°C for 30 minutes. The precipitate was spun down at 5000g in the Coolspin centrifuge for 20 minutes and the supernatant was decanted. Solid ammonium sulphate was added to the supernatant until 65% saturation was reached, and the mixture was left to stand at 4°C for 60 minutes. It was then spun at 5000g for 30 minutes in the Coolspin centrifuge; the pellet was collected and respun at 50 000g for 15 minutes in a Beckman Model L2-65B centrifuge (Beckman Instruments Inc., Palo Alto, California, USA). The pellet was redissolved in 300ml 0.02M citric acid-NaOH, pH6.9, and dialysed at 4°C for five hours against 50 litres of the same buffer.

The dialysed solution was then loaded on to a column (8cm diameter x 20cm long) of DEAE-Sephadex A-50, equilibrated

with 0.05M citric acid-NaOH, pH6.9. The column was washed with 500ml of the same buffer, and elution was carried out with a linear gradient (2 x 3500ml) from 0.05M to 0.50M citric acid-NaOH, pH6.9, at a flow rate of 360ml/hr; 25ml fractions were collected. When the A_{280} of the main - prothrombin - peak fell below 1.0, the gradient was stopped and the remainder of the proteins were eluted by washing the column with 0.60M citric acid-NaOH, pH6.9.

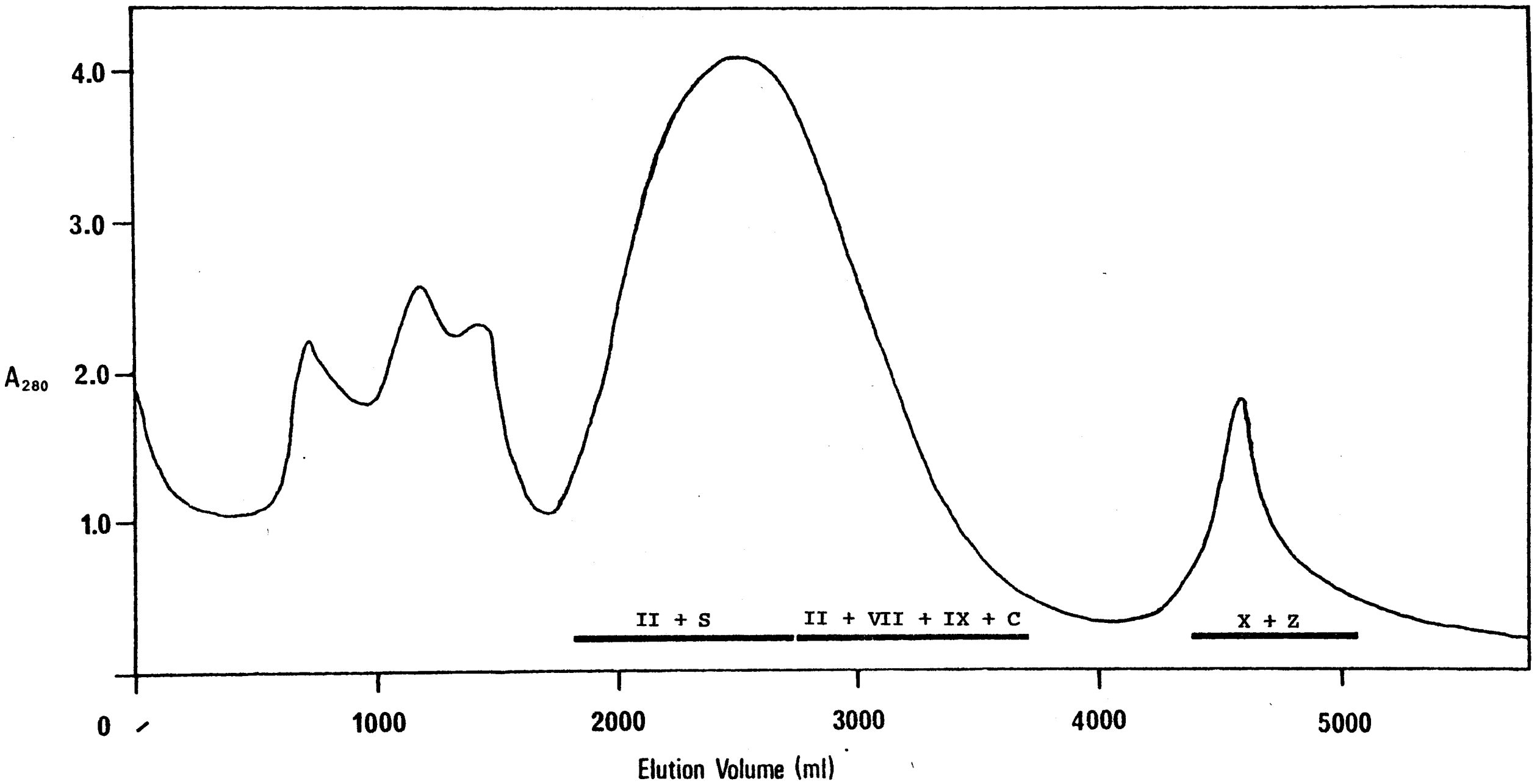
A typical elution profile is shown in Figure 4.2. The fractions were divided into three pools. The leading side of the prothrombin peak, which is enriched in Protein S, was used by other workers in the laboratory as a source of the latter protein. The remainder of the prothrombin peak, which includes most of the factor VII, factor IX and Protein C, was pooled separately and used in the isolation of the various components. The material eluted from the column by 0.60M citric acid-NaOH, pH6.9, was collected as a single pool.

The Isolation of Prothrombin

The pool containing prothrombin, protein C and factors VII and IX was concentrated in an ultrafiltration cell (Amicon Ltd., Woking, Surrey, U.K.) fitted with a PM10 membrane, to a volume of approximately 300ml, and dialysed at 4^{o}C for 24 hours against 20 litres of 0.05M imidazole-HCl, pH6.0. The dialysed pool was then pumped on to a column (4.5cm diameter x 9cm long) of heparin-Sepharose equilibrated in the same buffer, at a flow rate of 350ml/hr. The column was washed with 0.05M imidazole-HCl, pH6.0, until the A_{280} of the eluate fell to

FIGURE 4.2

Chromatography of Barium Eluate on DEAE-Sephadex



less than 0.01. The adsorbed proteins were then eluted by washing the column with 0.5M NaCl in 0.05M imidazole-HCl, pH 6.0 and collected as a single pool. A typical elution profile is shown in Figure 4.3.

The unadsorbed protein was collected as a single pool, the total volume of which was usually between 1200 and 2000ml. Solid ammonium sulphate was added to this pool to a final 70% saturation; the mixture was left at 4°C for 60 minutes, then spun at 5000g in the Coolspin centrifuge for 20 minutes. The pellet was redissolved in 500ml 0.05M citric acid-NaOH, pH6.9, and dialysed overnight at 4°C against 10 litres of the same buffer.

The dialysed pool was then pumped on to a column (6cm diameter x 20cm long) of DEAE-Sephadex, A-50, equilibrated with 0.05M citric acid-NaOH, pH6.9. The proteins were eluted from the column with a linear gradient (2 x 2000ml) from 0.05M to 0.50M citric acid-NaOH, pH6.9. 25ml fractions were collected, and those fractions at the centre of the protein peak (Figure 4.4) were pooled. Examination of the pool by SDS-polyacrylamide gel electrophoresis showed only a single polypeptide chain corresponding to prothrombin.

The Isolation of Factor IX

The pool containing the proteins adsorbed to heparin-Sepharose, and subsequently eluted with sodium chloride (Figure 4.3) was dialysed at 4°C for 18 hours against 10 litres of 0.05M imidazole-HCl, pH6.0. 1.0M calcium chloride was then added to the dialysed pool to give a final calcium chloride concentration of 2.5mM, and the solution was pumped

FIGURE 4.3

Chromatography of Prothrombin Pool on Heparin-Sepharose

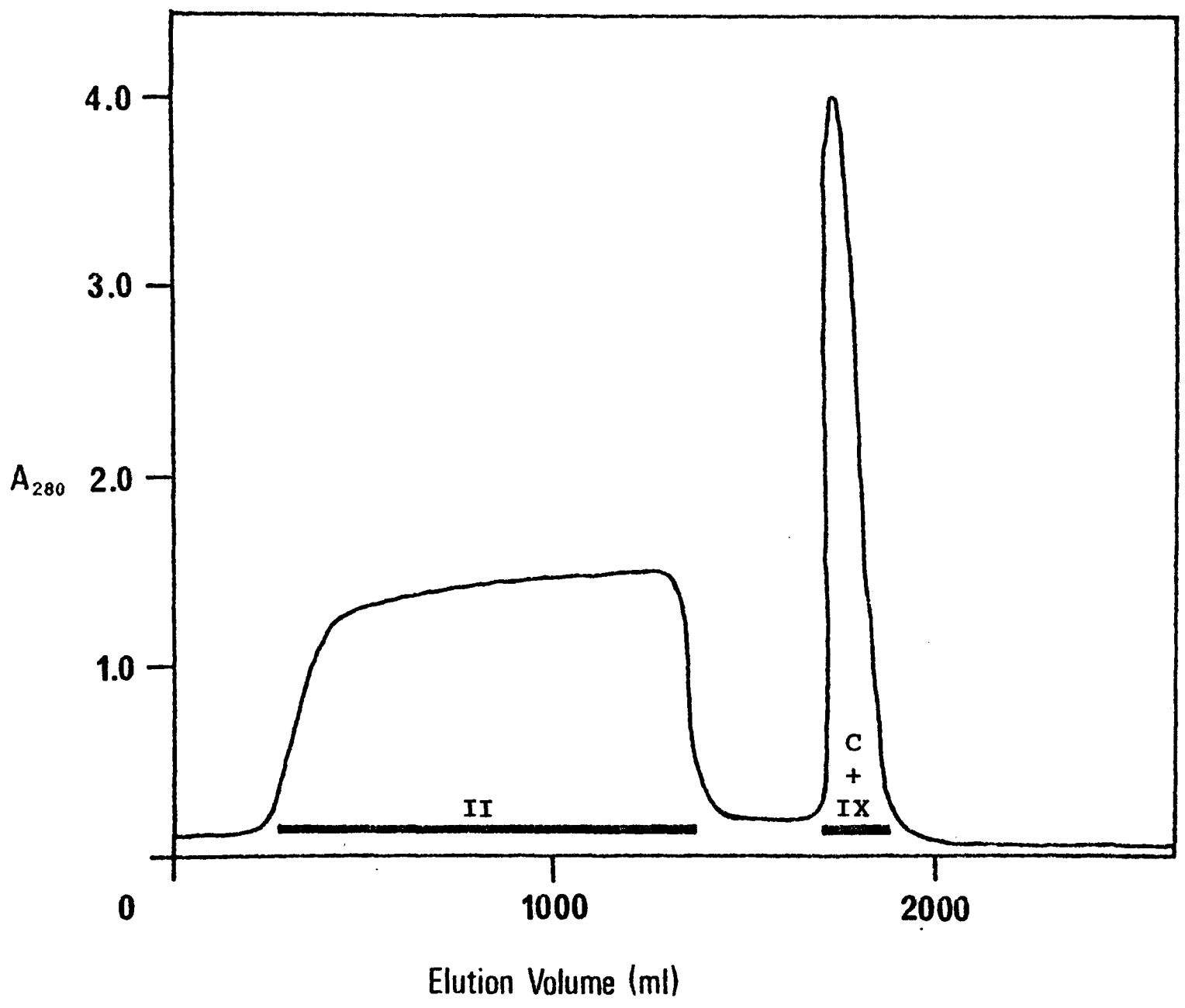
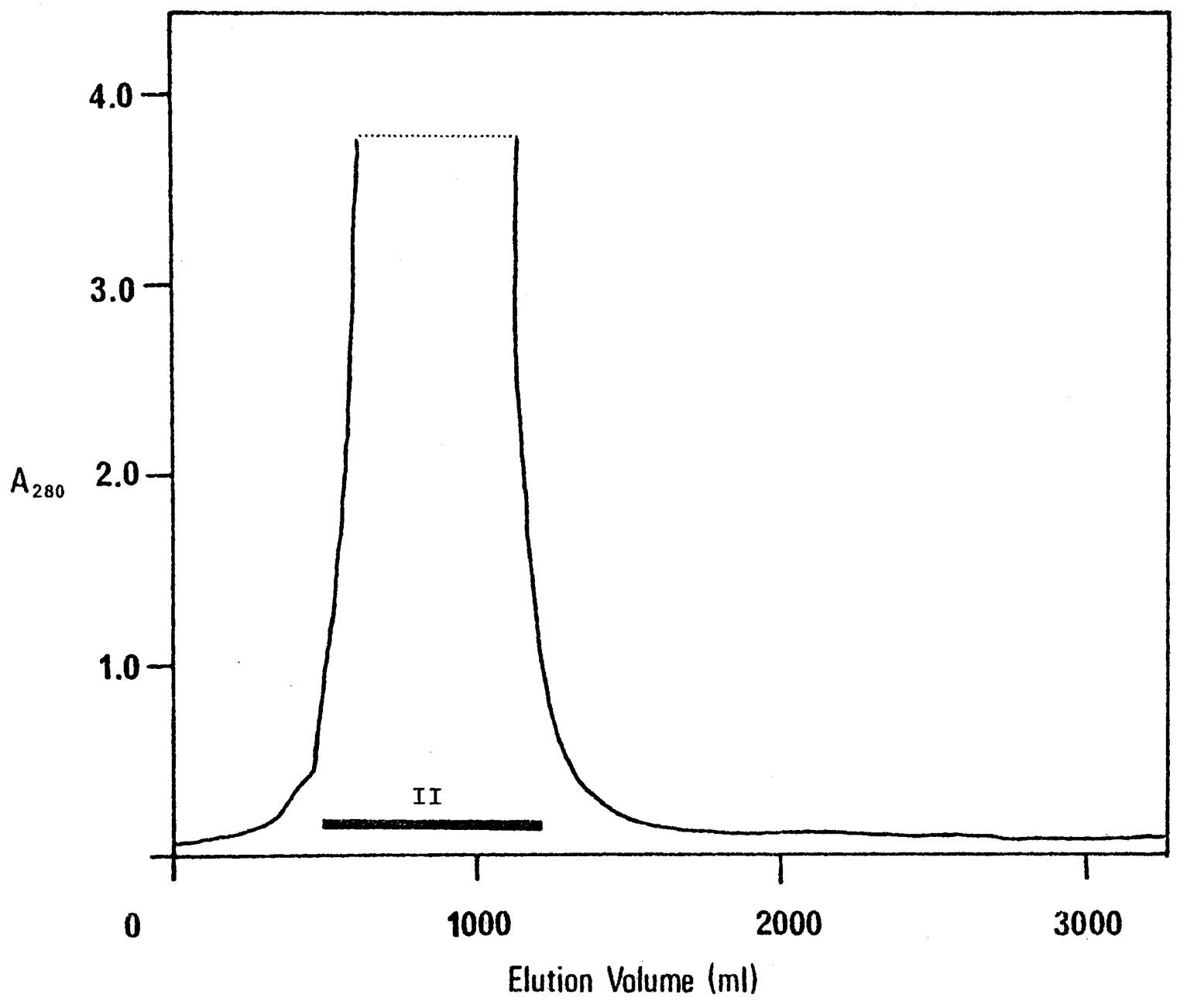


FIGURE 4.4

Rechromatography of Prothrombin on DEAE-Sephadex



on to a column (4.5cm diameter x 9cm long) of heparin-Sepharose equilibrated with 0.05M imidazole-HCl, pH6.0, 2.5mM $CaCl_2$. After washing the column with the same buffer at a flow rate of 120ml/hr, the adsorbed proteins were eluted with a linear gradient (2 x 750ml) from 0 to 0.9M NaCl in 0.05M imidazole-HCl, pH6.0, 2.5mM $CaCl_2$. Fractions of 15ml were collected and assayed for factor IX by Laurell rocket immunoelectrophoresis. As shown in Figure 4.5, the factor IX was the last protein to elute from the column and was present as a single, discrete peak. The fractions containing factor IX were pooled and examined by SDS-polyacrylamide gel electrophoresis: a single polypeptide chain, corresponding to factor IX, was visible in the stained gel.

The Isolation of Protein C

The fractions eluted from the heparin-Sepharose column during the isolation of factor IX were also assayed by Laurell rocket immunoelectrophoresis for protein C. Those fractions containing protein C were pooled (Figure 4.5) and dialysed at $4^{o}C$ for 18 hours against 5000ml 0.05M tris-HCl, pH7.5.

The dialysed pool was pumped on to a column (4cm diameter x 30cm long) of QAE-Sephadex A-50, equilibrated with 0.05M tris-HCl, pH7.5. The column was washed with the same buffer at a flow rate of 120ml/hr and the adsorbed proteins were eluted with a linear gradient (2 x 1000ml) from 0 to 0.60M sodium chloride in 0.05M tris-HCl, pH7.5. During the elution, 20ml fractions were collected: they were assayed for protein C by Laurell rocket immunoelectrophoresis. As Figure 4.6 shows, the

FIGURE 4.5

Chromatography of Factor IX and Protein C on Heparin-Sepharose

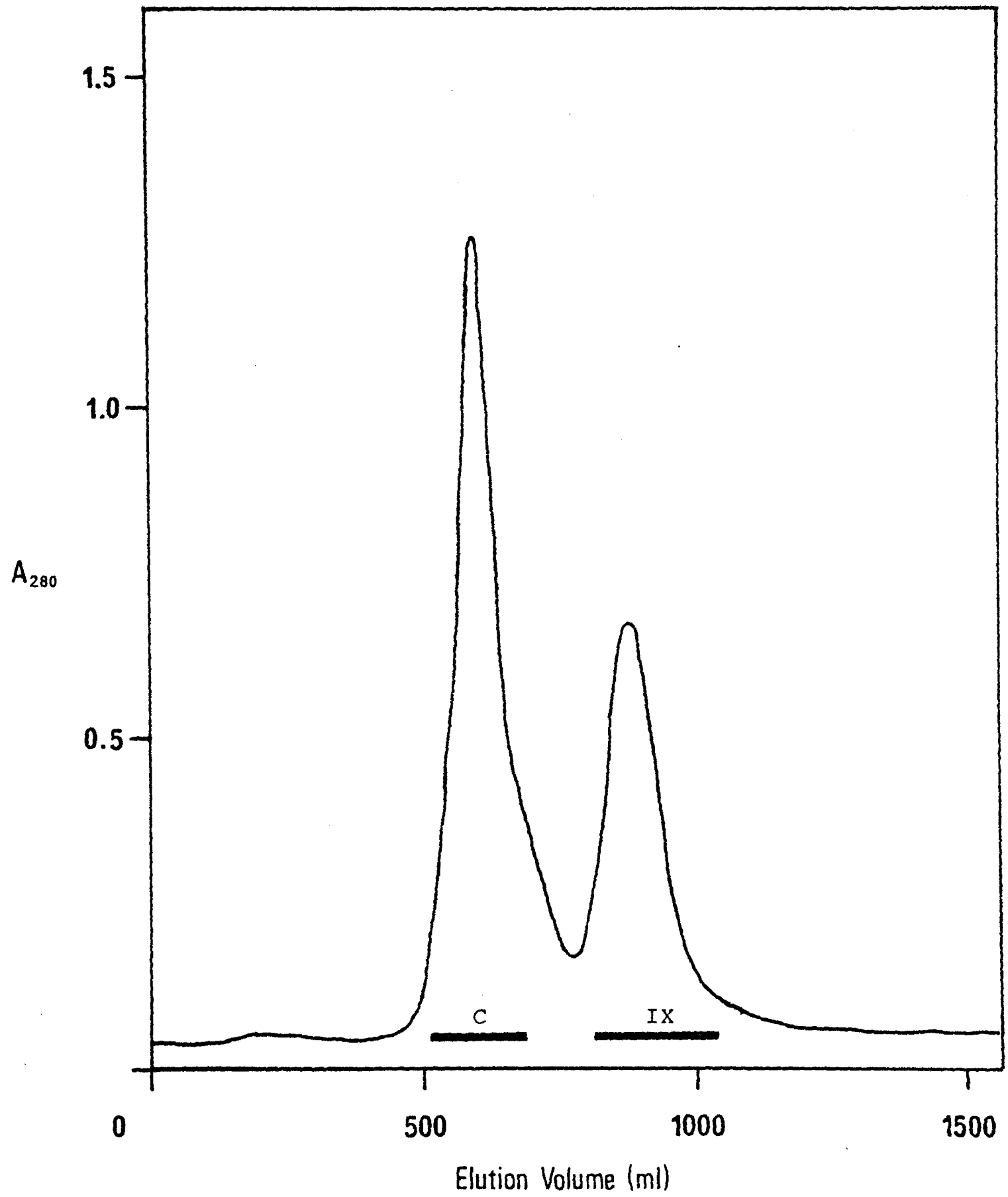
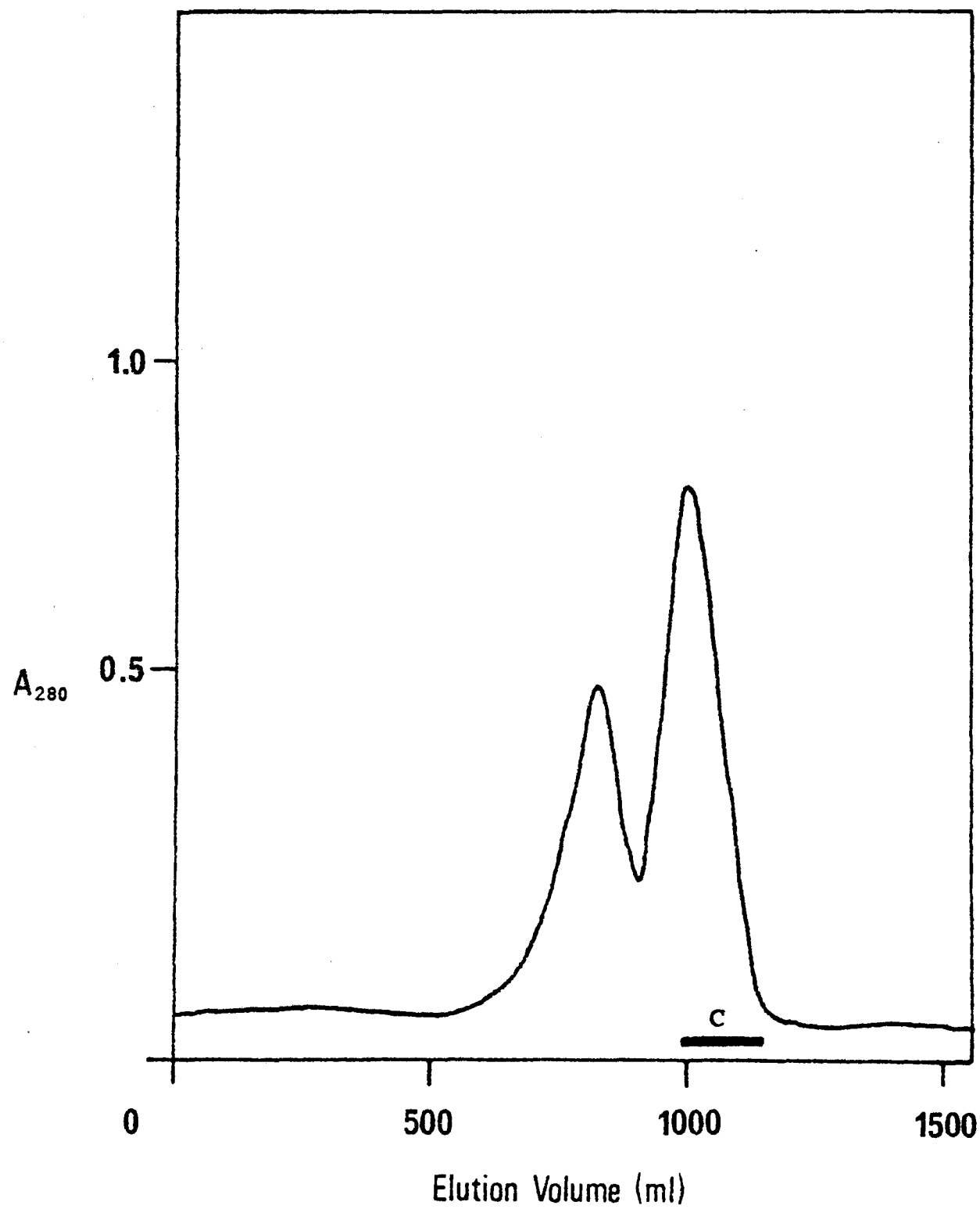


FIGURE 4.6

Chromatography of Protein C Pool on QAE-Sephadex



protein C was present in the second major protein peak. The fractions containing protein C were pooled and dialysed at 4°C for 18 hours against 5000ml 0.05M tris-HCl, pH7.5, containing 0.10M NaCl.

The dialysed pool was pumped on to a column (2.6cm diameter x 30cm long) of QAE-Sephadex equilibrated with 0.05M tris-HCl, pH7.5, 0.10M NaCl. The column was washed with 0.20M NaCl in 0.05M tris-HCl, pH7.5, and then the adsorbed proteins were eluted, at a flow rate of 120ml/hr, with a linear gradient (2 x 750ml) from 0.25M to 0.60M NaCl in 0.05M tris-HCl, pH7.5. During the elution, fractions of approximately 20ml were collected and assayed for protein C by Laurell rocket immunoelectrophoresis. Those fractions containing protein C (Figure 4.7) were pooled, and dialysed at 4°C for 18 hours against 5000ml 0.05M citric acid-NaOH, pH6.9.

The dialysed pool containing protein C was pumped on to a column (2.6cm diameter x 30cm long) of DEAE-Sepharose CL-6B, equilibrated with 0.05M citric acid-NaOH, pH6.9. After washing the column with the same buffer at a flow rate of 120 ml/hr, the adsorbed proteins were eluted with a linear gradient (2 x 750ml) from 0.10M to 0.50M citric acid-NaOH, pH6.9. Fractions of 15ml were collected and assayed for protein C by Laurell rocket immunoelectrophoresis. As is shown in Figure 4.8, the final peak of protein eluted from the column contained the protein C. The fractions were pooled as indicated and examined by SDS-polyacrylamide gel electrophoresis. The major band visible in the stained gel corresponded to the heavy chain of the protein C, but several minor contaminants were also

FIGURE 4.7

Rechromatography of Protein C Pool on QAE-Sephadex

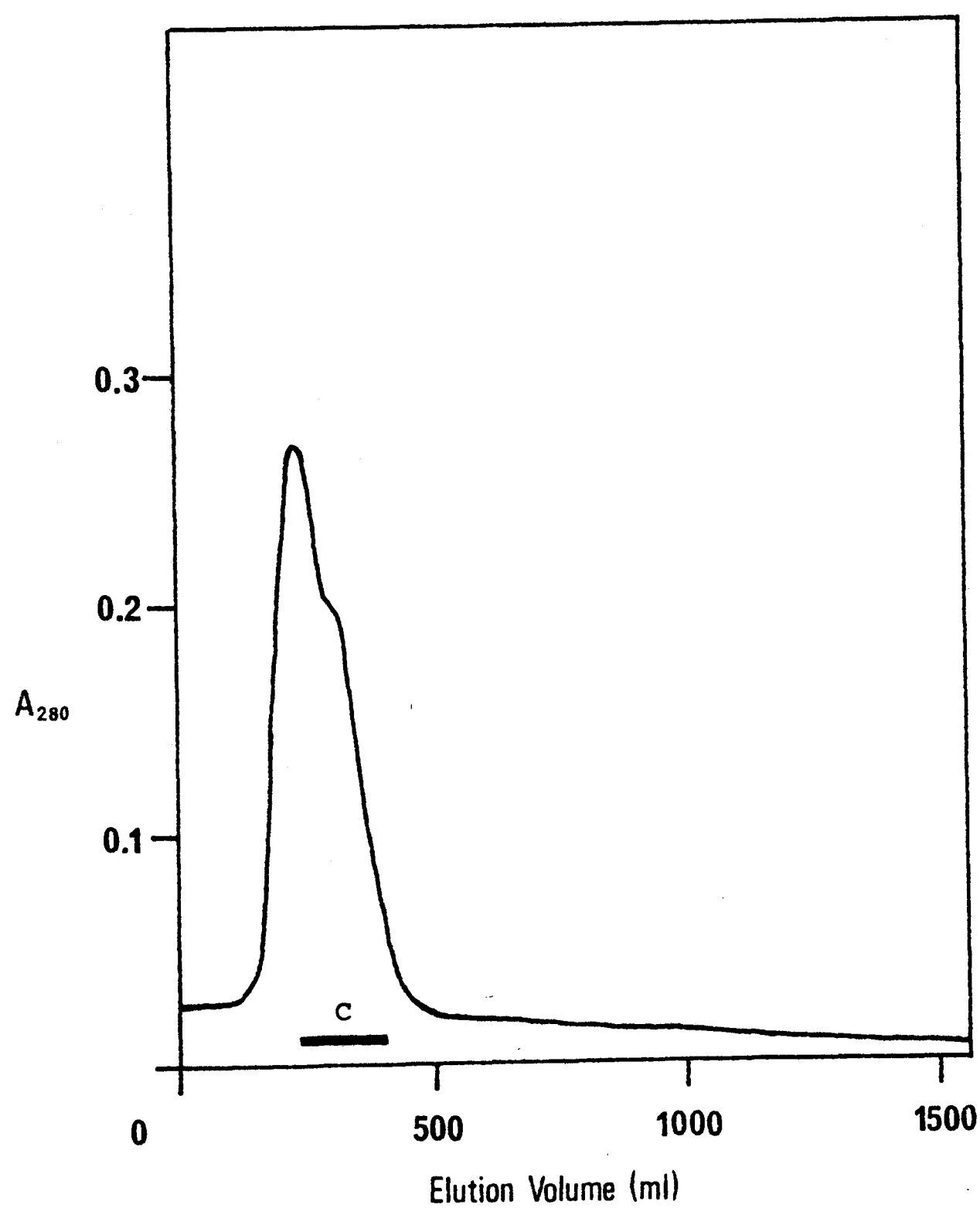
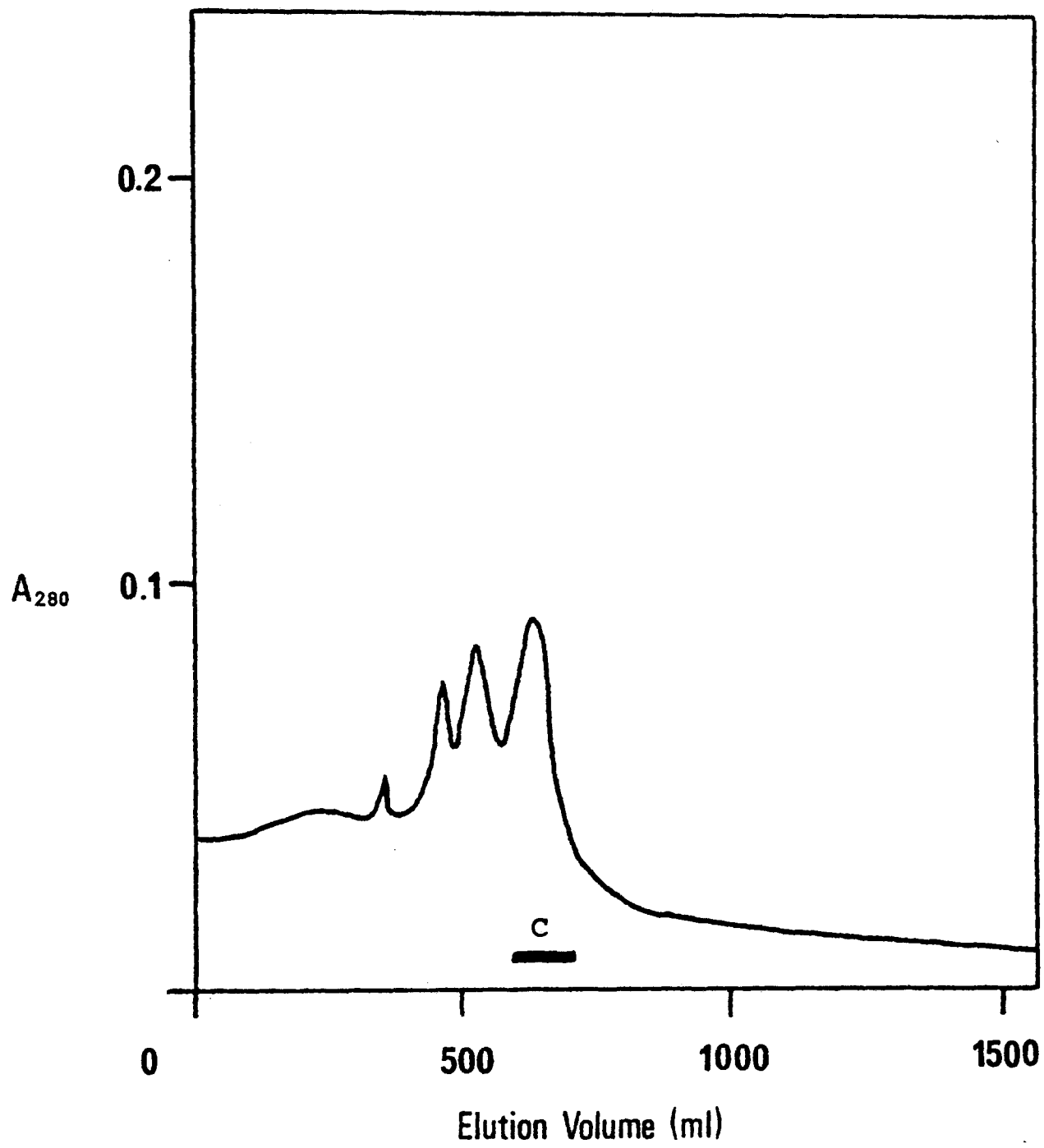


FIGURE 4.8

Chromatography of Protein C on DEAE-Sepharose



visible. This preparation of protein C lengthened the activated partial thromboplastin time of normal bovine plasma. The effect was not increased by a pretreatment of the protein C with thrombin, suggesting that the protein C had been activated during its preparation. This is probably the result of the protein C being exposed to traces of thrombin generated from prothrombin.

The Isolation of Factor X and Protein Z

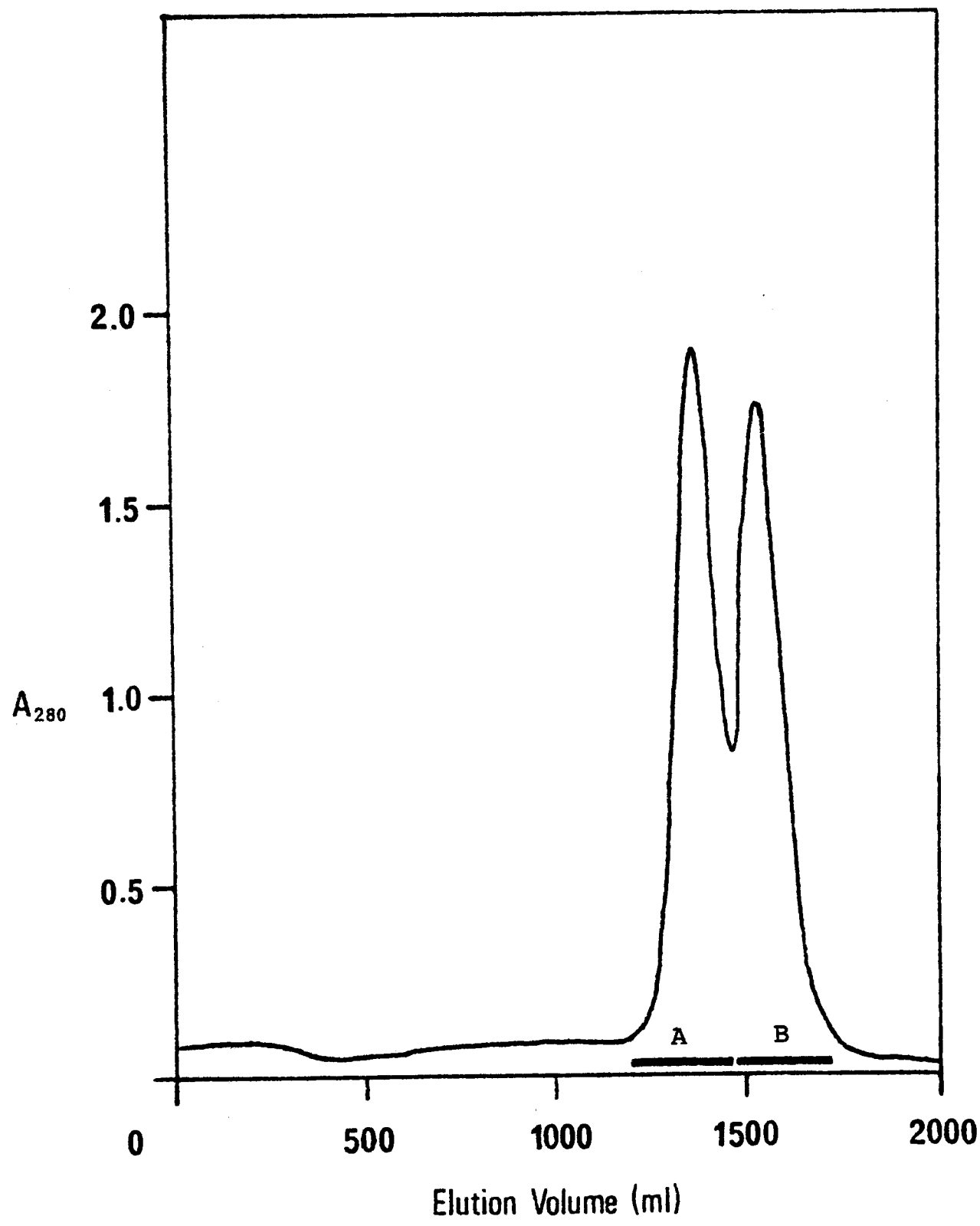
The pool of protein eluted from the first DEAE-Sephadex A-50 column with 0.60M citric acid-NaOH, pH6.9, (Figure 4.2) was concentrated in an ultrafiltration cell (Amicon Ltd., Woking, Surrey, U.K.) fitted with a YM10 membrane, to a volume of 150 ml. It was then dialysed at $4^{o}C$ for 18 hours against 10 litres of 0.025M citric acid-NaOH, pH6.9.

The dialysed pool was pumped on to a column (3.5cm diameter x 30cm long) of DEAE-Sephadex A-50 equilibrated with 0.05M citric acid-NaOH, pH6.9. The column was washed with the same buffer at a flow rate of 60ml/hr and then the proteins were eluted with a linear gradient (2 x 1000ml) from 0.05M to 0.60M citric acid-NaOH, pH6.9; 20ml fractions were collected throughout. The fractions were tested for factor X by means of a clotting assay using Russell's viper venom as the activator, and those fractions containing factor X were pooled as shown in Figure 4.9.

Pool A, which contains principally factor X_1, was concentrated in an Amicon ultrafiltration cell to a volume of 30ml. It was then dialysed at $4^{o}C$ for 18 hours against

FIGURE 4.9

Chromatography of Factor X Pool on DEAE-Sephadex



10 litres of 0.05M tris-HCl, pH7.5.

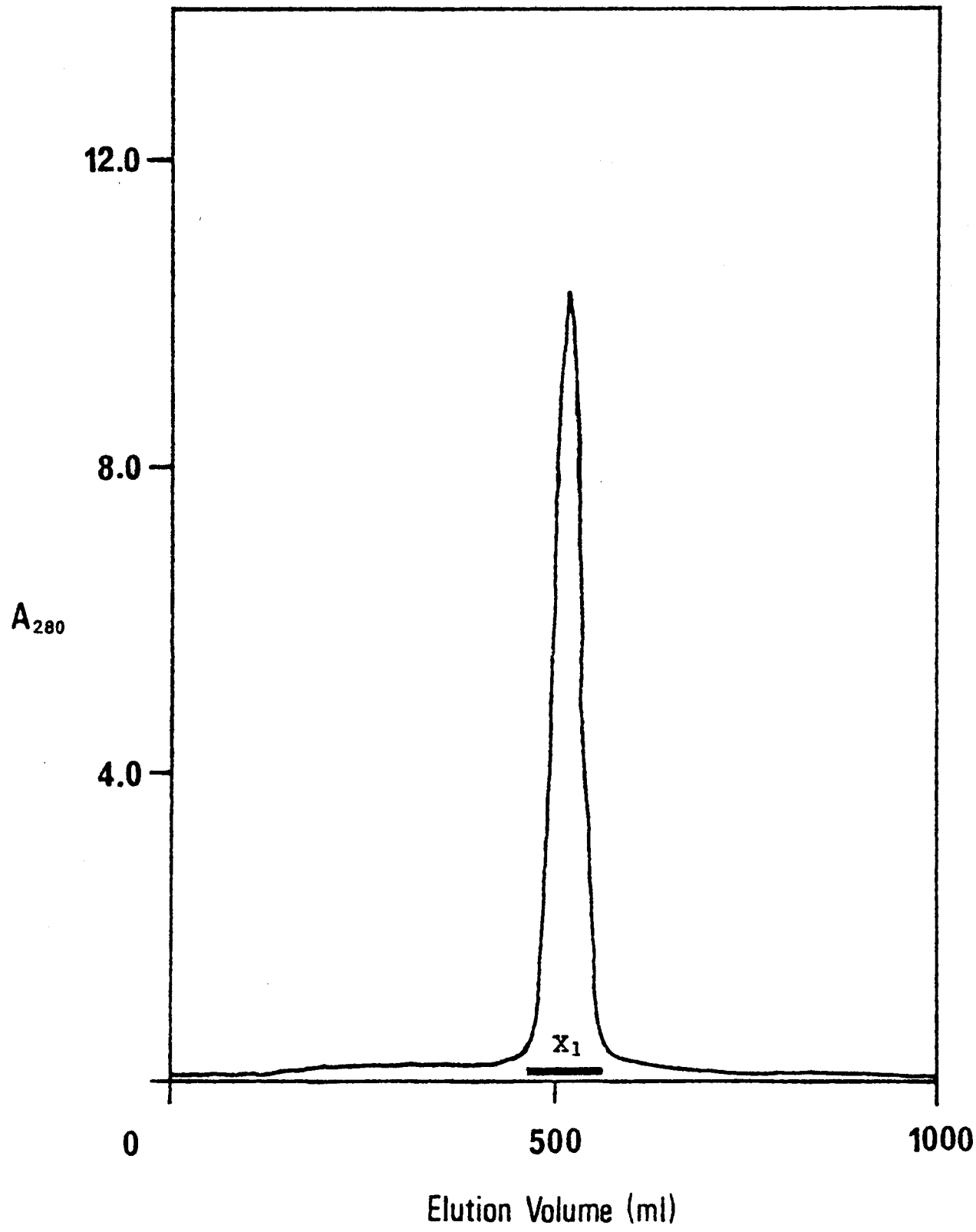
The dialysed pool was pumped on to a column (2.2cm diameter x 35cm long) of DEAE-Sephadex A-50, equilibrated with 0.05M tris-HCl, pH7.5. The column was washed with the same buffer at a flow rate of 60ml/hr and the proteins were eluted with a linear gradient (2 x 500ml) from 0.10M to 1.0M NaCl in 0.05M tris-HCl, pH7.5. 10ml fractions were collected during the gradient elution. An aliquot from the top of the main protein peak (Figure 4.10) was reserved for the studies on the activation of factor X described in Chapters 5 and 6. The remaining fractions containing factor X_1 were pooled and the activity was measured in a clotting assay. When this pool was examined by SDS-polyacrylamide gel electrophoresis under reducing conditions, only two polypeptides were visible: the heavy and light chains of factor X.

Pool B from the first DEAE-Sephadex A-50 column (Figure 4.9), which contains factor X_2 and protein Z, was concentrated in an Amicon ultrafiltration cell to a volume of 40ml. It was then dialysed at $4^{o}C$ for 18 hours against 10 litres of 0.05M citric acid-NaOH, pH6.9.

The dialysed pool was pumped on to a column (2.2cm diameter x 40cm long) of DEAE-Sepharose CL-6B, equilibrated with 0.05M citric acid-NaOH, pH6.9. The column was washed with the same buffer at a flow rate of 60ml/hr and the proteins were eluted with a linear gradient (2 x 500ml) from 0.05M to 0.60M citric acid-NaOH, pH6.9. 10ml fractions were collected and assayed for factor X and protein Z by Laurell

FIGURE 4.10

Rechromatography of Factor X_1 on DEAE-Sephadex



rocket immunoelectrophoresis. As shown in Figure 4.11, the first protein peak eluted from the column contains factor X and the second peak contains protein Z. The fractions were pooled as indicated in the figure, with those fractions containing both proteins being discarded. The activity of the factor X_2 pool was measured in a clotting assay, and both pools were examined by SDS-polyacrylamide gel electrophoresis. Under reducing conditions, two polypeptides were detected in the factor X_2 pool, corresponding to the heavy and light chains of the protein, whereas the protein Z migrated as a doublet of higher molecular weight.

Recovery of the Vitamin K-Dependent Proteins

The yields of the various vitamin K-dependent proteins, and the degree of purification achieved at the different stages in the procedures are summarised in Tables 4.1, 4.2 and 4.3. The values quoted are typical of those obtained in several preparations of these proteins. The recoveries of prothrombin, factor IX and protein C are lower than those expected in theory, largely because some of these proteins are sacrificed in the pool used for the isolation of protein S (Figure 4.2).

The stained gels obtained from SDS-polyacrylamide gel electrophoretic examination of the final pools are shown in Figure 4.12. These gels were run in the phosphate buffer system of Shapiro *et al* (1967) using 2-mercaptoethanol as a reducing agent.

FIGURE 4.11

Chromatography of Factor X_2 and Protein Z on DEAE-Sepharose

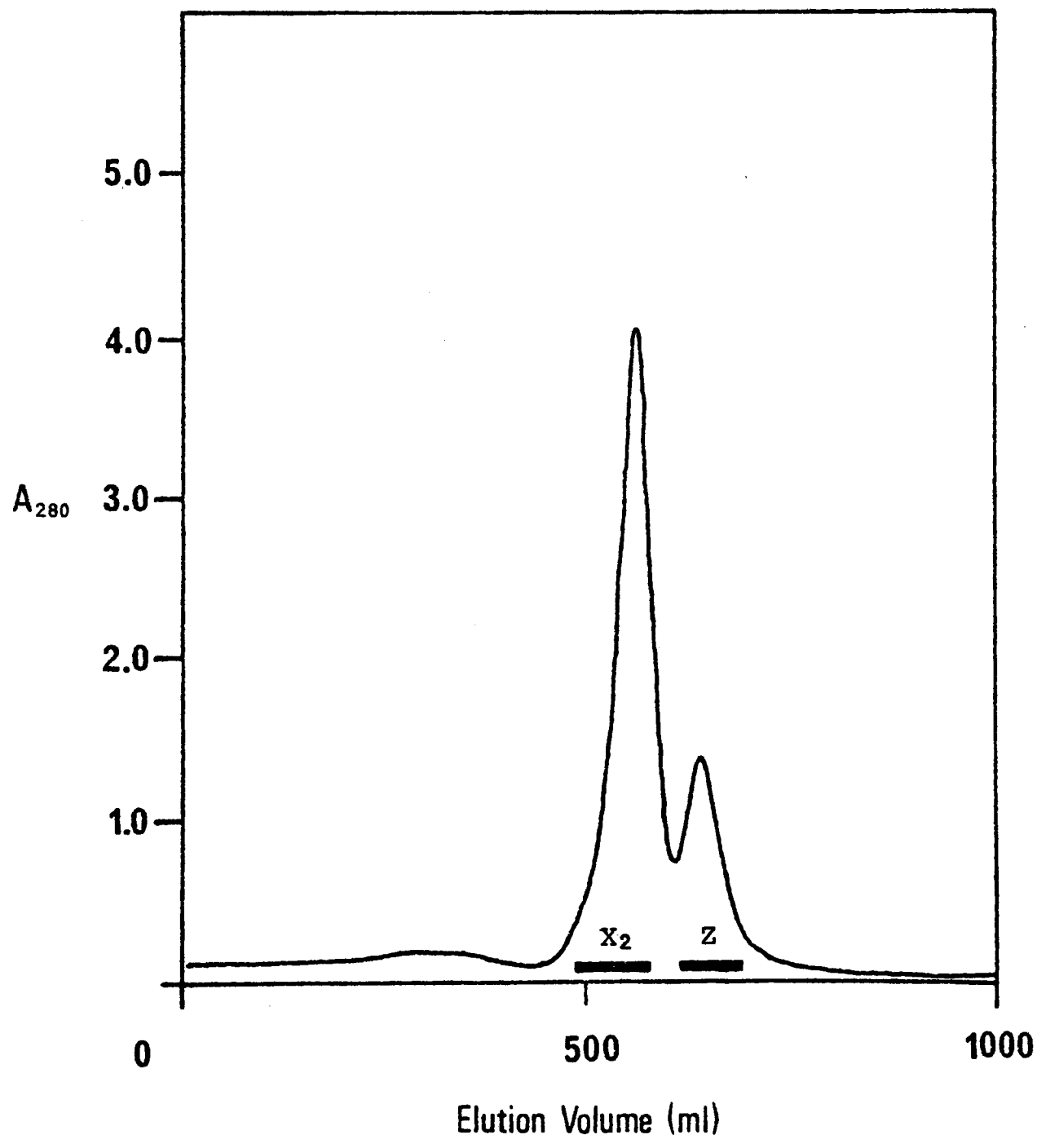


TABLE 4.1

Purification of Prothrombin

	Volume (ml)	A_{280}	Total Protein (mg)	Prothrombin (units/ml)	Prothrombin (units)	Specific Activity (units/mg)
Plasma	1×10^5	60.3	6.0×10^6	1.00	1.0×10^5	0.016
Barium Eluate	400	50.7	2.0×10^4	95	3.8×10^4	1.9
DEAE-Sephadex	425	9.8	3.0×10^3	19	8.1×10^3	2.7
Heparin-Sepharose	1000	2.5	1.8×10^3	7.1	7.1×10^3	3.9
DEAE-Sephadex	300	4.1	8.9×10^2	23	6.9×10^3	7.7

TABLE 4.2

Purification of Factor IX and Protein C

	Pool	Volume (ml)	A_{280}	Total Protein (mg)	Factor IX (units/ml)	Factor IX (units)	Specific Activity (units/mg)
Plasma		1×10^5	60.3	6.0×10^6	1.00	1.0×10^5	0.016
Barium Eluate		400	50.7	2.0×10^4	93	3.7×10^4	1.8
DEAE-Sephadex	II + IX + C	425	9.8	3.0×10^3	23	9.8×10^3	3.3
Heparin-Sepharose	IX + C	180	5.4	690	35	6.3×10^3	9.1
Heparin-Sepharose	IX	360	1.7	430	18	6.4×10^3	15.0
	C	165	0.63	100	-	-	-
QAE-Sephadex	C	160	0.29	46	-	-	-
QAE-Sephadex	C	170	0.12	20	-	-	-
DEAE-Sepharose	C	110	0.04	4	-	-	-

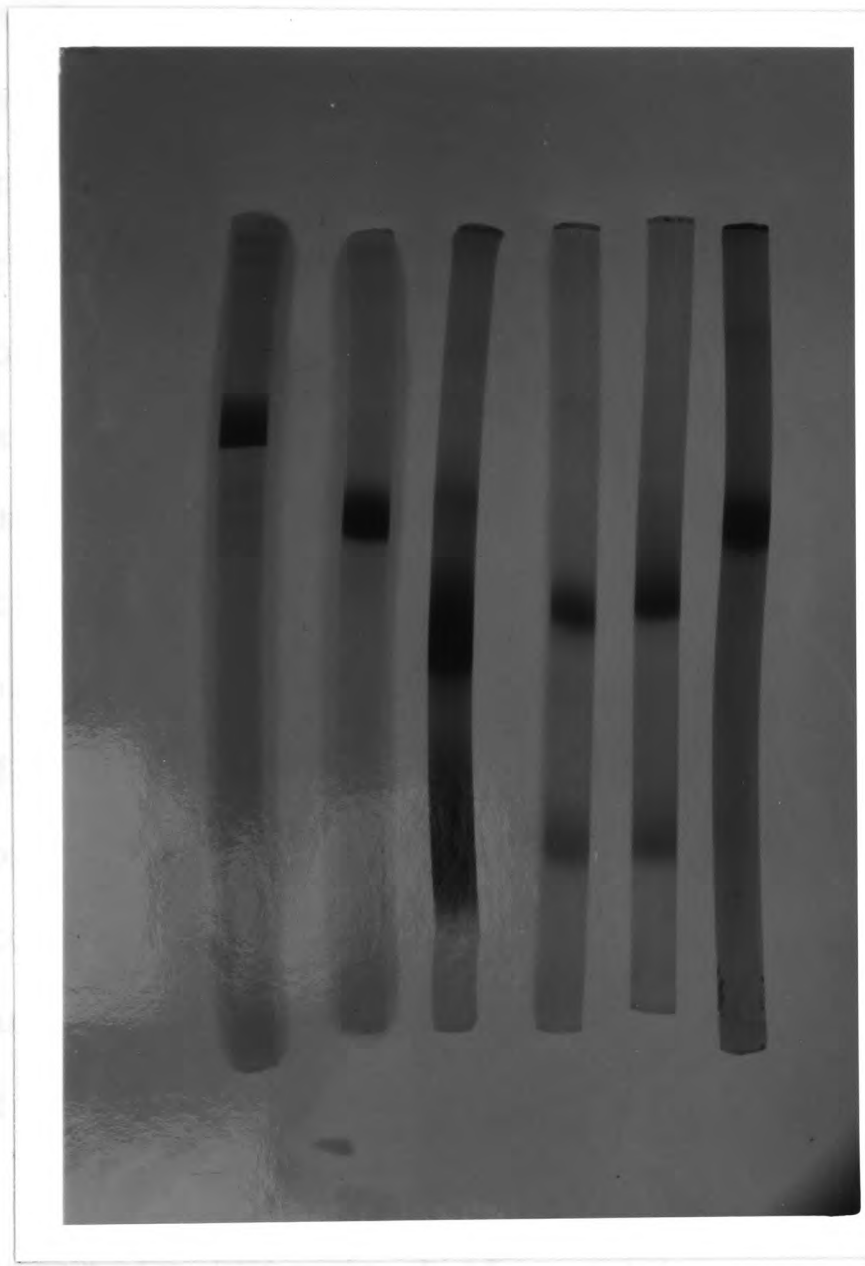
TABLE 4.3

Purification of Factor X and Protein Z

Pool	Volume (ml)	A_{280}	Total Protein (mg)	Factor X (units/ml)	Factor X (units)	Specific Activity (units/mg)
Plasma	1×10^5	60.3	6.0×10^6	1.00	1.0×10^5	0.016
Barium Eluate	400	50.7	2.0×10^4	176	7.0×10^4	3.5
DEAE-Sephadex $X_1 + X_2 + Z$	220	3.00	690	140	3.1×10^4	49
DEAE-Sephadex X_1	250	1.04	270	73	1.8×10^4	73
$X_2 + Z$	300	0.88	275	62	1.9×10^4	73
DEAE-Sephadex X_1	70	3.2	230	225	1.6×10^4	73
DEAE-Sepharose X_2	80	1.8	150	164	1.3×10^4	97
Z	100	0.66	66	-	-	-

FIGURE 4.12

SDS-PAGE of Vitamin K-dependent Proteins



1. Prothrombin (50µg)
2. Factor IX (60µg)
3. Protein C (60µg)
4. Factor X_1 (40µg)
5. Factor X_2 (40µg)
6. Protein Z (50µg)

The Preparation of Activated Factor IX

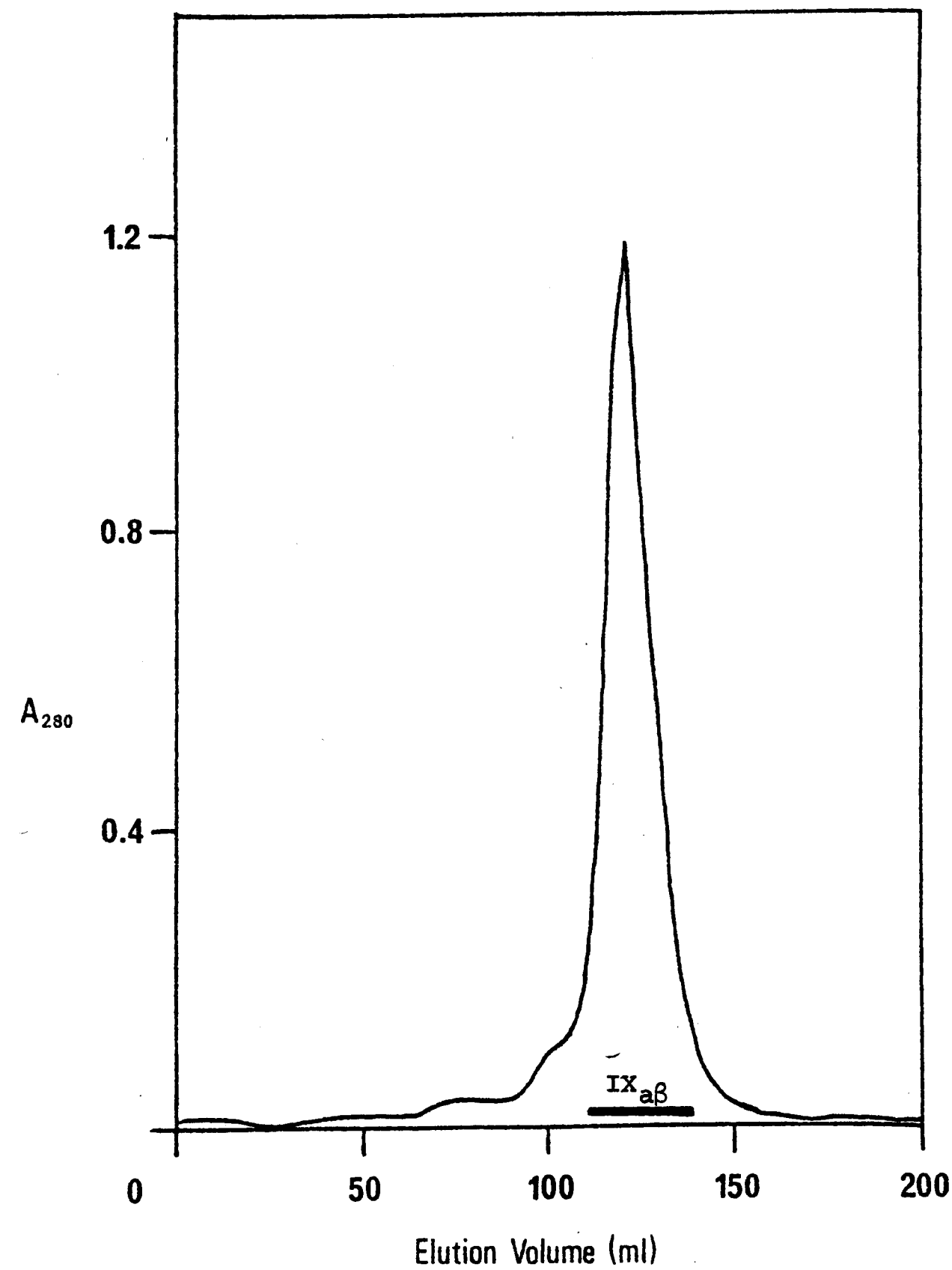
Factor IX was activated with either contact product or RVV-X.

When contact product was used as the activator, the reaction was carried out in solution. 20mg factor IX was mixed with 5mg contact product in a total volume of 50ml 0.025M tris-HCl, pH7.5, containing 0.01M calcium chloride. The mixture was incubated at 37°C for 75 minutes, and then pumped on to a column (1.5cm diameter x 20cm long) of DEAE-Sephadex A-50, equilibrated with 0.025M tris-HCl, pH7.5. The column was washed with 0.025M tris-HCl, pH7.5, 0.10M NaCl, at a flow rate of 60ml/hr, and the adsorbed proteins were then eluted with a linear gradient (2 x 100ml) from 0.20M to 0.60M sodium chloride in 0.025M tris-HCl, pH7.5. Fractions of approximately 6ml were collected; those fractions containing factor IX_a were identified by coagulation assays and were pooled as shown in Figure 4.13. The factor IX_a pool was dialysed at 4°C for 18 hours against 5000ml 0.025M tris-HCl, pH7.5. It was then examined by SDS-polyacrylamide gel electrophoresis (Figure 4.15) and by coagulation assays. Typically, this procedure yielded approximately 15mg of factor IX_a, with a specific coagulant activity of 30 to 50 units per mg.

The RVV-X employed in the activation of factor IX was insolubilized by coupling to Sepharose 4B. 6ml (packed volume) of RVV-X-Sepharose was mixed with 10mg factor IX in a total volume of 40ml 0.05M tris-HCl, pH7.5, containing 0.01M calcium chloride. The mixture was shaken gently at

FIGURE 4.13

Chromatography of Factor $IX_{a\beta}$ on DEAE-Sephadex



37°C for 30 minutes and the Sepharose was removed by filtration through a sintered glass funnel without vacuum. The filtrate was pumped on to a column (1.5cm diameter x 20cm long) of DEAE-Sephadex A-50, equilibrated with 0.025M tris-HCl, pH7.5. The column was washed with 0.025M tris-HCl, pH7.5, 0.10M NaCl, and then the adsorbed proteins were eluted with a linear gradient (2 x 125ml) from 0.10M to 0.60M sodium chloride in 0.025M tris-HCl, pH7.5 at a flow rate of 50ml/hr. Fractions of 5ml were collected during the gradient elution. Coagulation assays were used to identify the fractions containing factor IX_a which were pooled as indicated in Figure 4.14. The first peak of coagulant activity eluted from the column was due to traces of RVV-X leached from the Sepharose. The factor IX_a pool was dialysed at 4°C for 18 hours against 4000ml 0.025M tris-HCl, pH7.5, and then examined by SDS-polyacrylamide gel electrophoresis (Figure 4.15) and by coagulation assays. Typically, approximately 6mg of factor IX_a with a specific coagulant activity of about 200 units per mg was obtained by this method.

The Preparation of Activated Factor X

20mg factor X_1 was incubated at 37°C for 20 minutes with 0.10mg RVV-X in a total volume of 50ml 0.025M tris-HCl, pH7.5, containing 0.005M calcium chloride. The mixture was then dialysed at 4°C for five hours against 5000ml 0.05M tris-HCl, pH7.5, 0.10M NaCl. 5.0ml 0.10M disodium EDTA was added to the diffusate and the dialysis was continued for a further 16 hours.

FIGURE 4.14

Chromatography of Factor $IX_{a\alpha}$ on DEAE-Sephadex

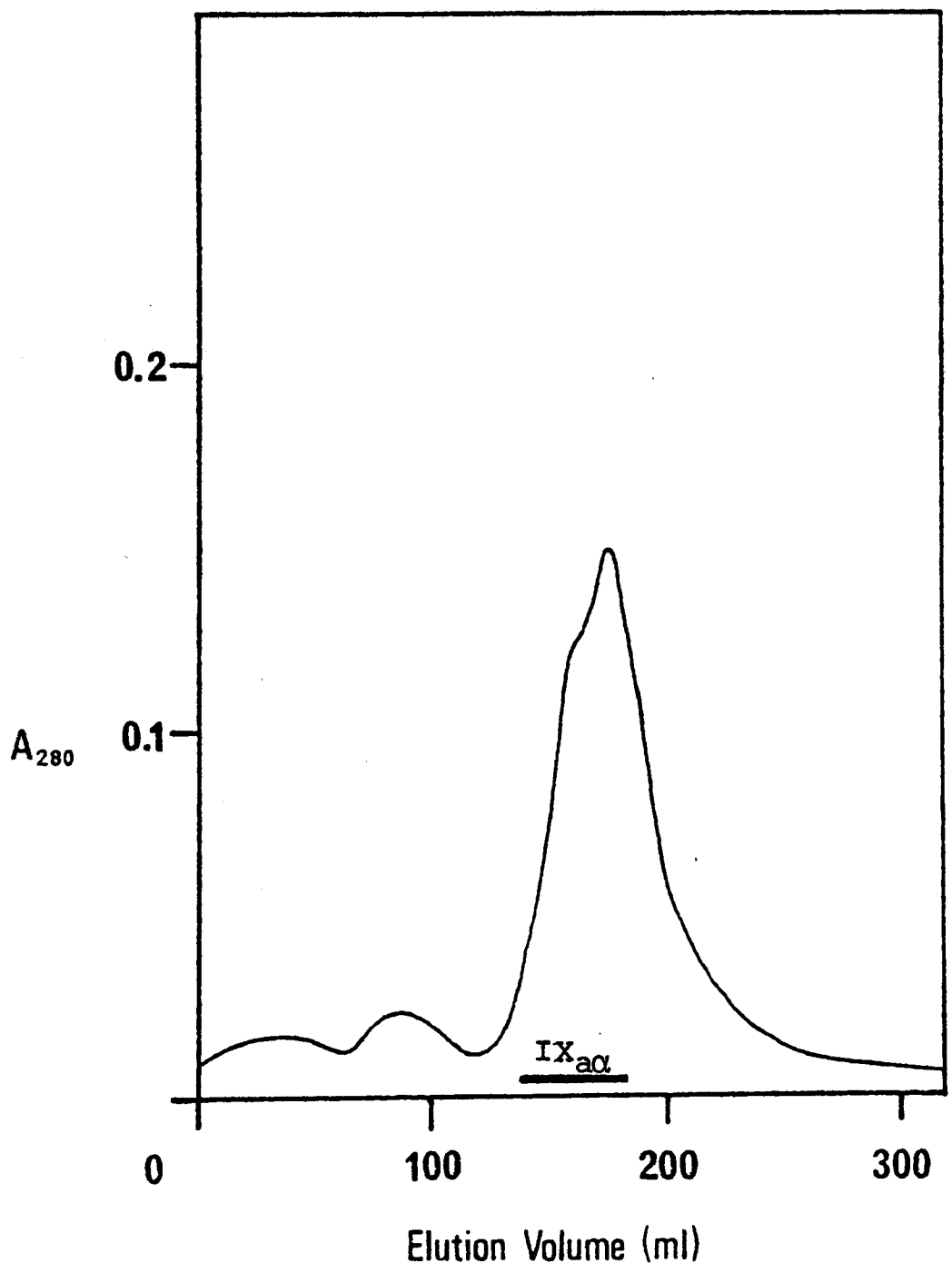
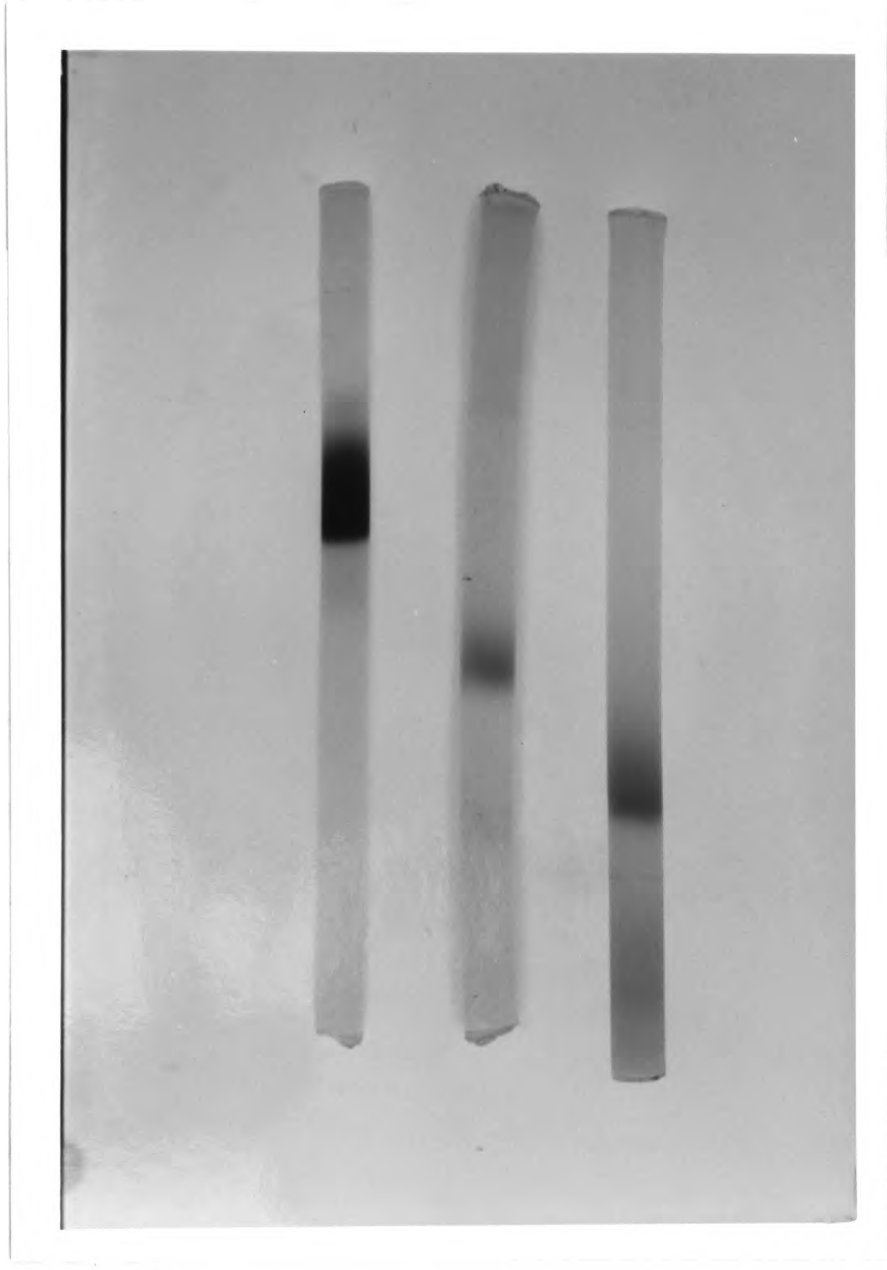


FIGURE 4.15

SDS-PAGE of Factor IX and Activated Factor IX



1. Factor IX (60µg)
2. Factor $IX_{a\alpha}$ (75µg)
3. Factor $IX_{a\beta}$ (50µg)

The dialysed pool was loaded on to a column (1.5cm diameter x 10cm long) of DEAE-Sephadex A-50, equilibrated with 0.05M tris-HCl, pH7.5, 0.10M NaCl. After the column had been washed with the same buffer, the adsorbed proteins were eluted with a linear gradient (2 x 125ml) from 0.10M to 0.75M sodium chloride in 0.05M tris-HCl, pH7.5, at a flow rate of 60ml/hr. Fractions of approximately 5ml were collected throughout and those fractions containing factor X_a were identified by their esterolytic activity using α-N-benzoyl-L-arginine ethyl ester (BAEe) as the substrate. They were pooled as shown in Figure 4.16 and dialysed at 4°C for 48 hours against 4000ml 0.025M tris-HCl, pH7.5. The dialysed pool was examined by SDS-polyacrylamide gel electrophoresis; under reducing conditions, two bands - corresponding to the light and heavy chains of the enzyme - were visible in the stained gel (Figure 4.17). This procedure resulted in the isolation of 12mg factor X_a, with a specific esterolytic activity of 4.1 millimoles BAEe hydrolysed per minute per gram of enzyme. An analysis of the amino acid composition of the factor X_{1a} indicated that the preparation contained only eight moles of proline per mole of protein. Thus, the proline-rich C-terminal peptide had been cleaved to give factor $X_{1a\beta}$ (Fujikawa *et al*, 1975).

FIGURE 4.16

Chromatography of Factor X_a on DEAE-Sephadex

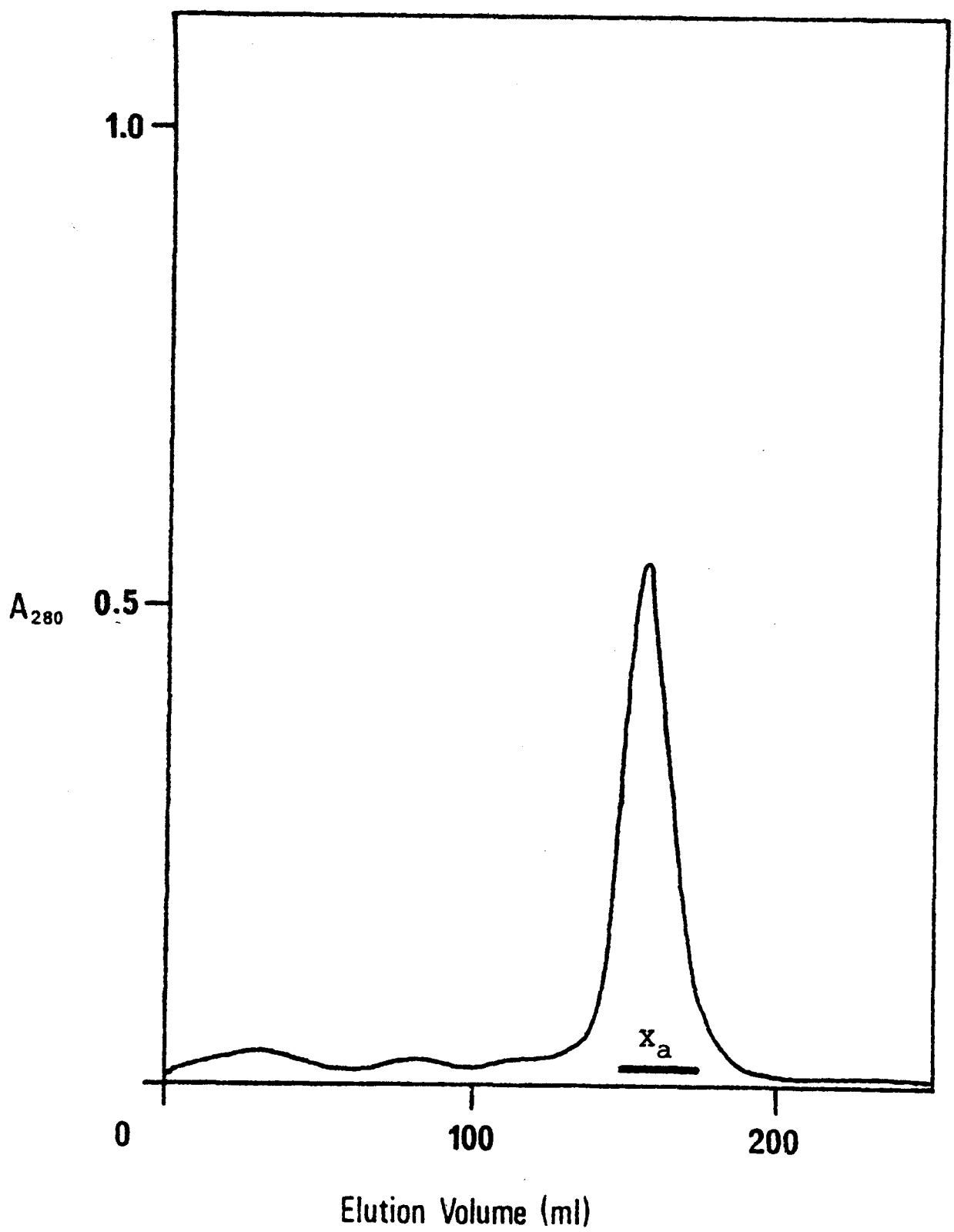
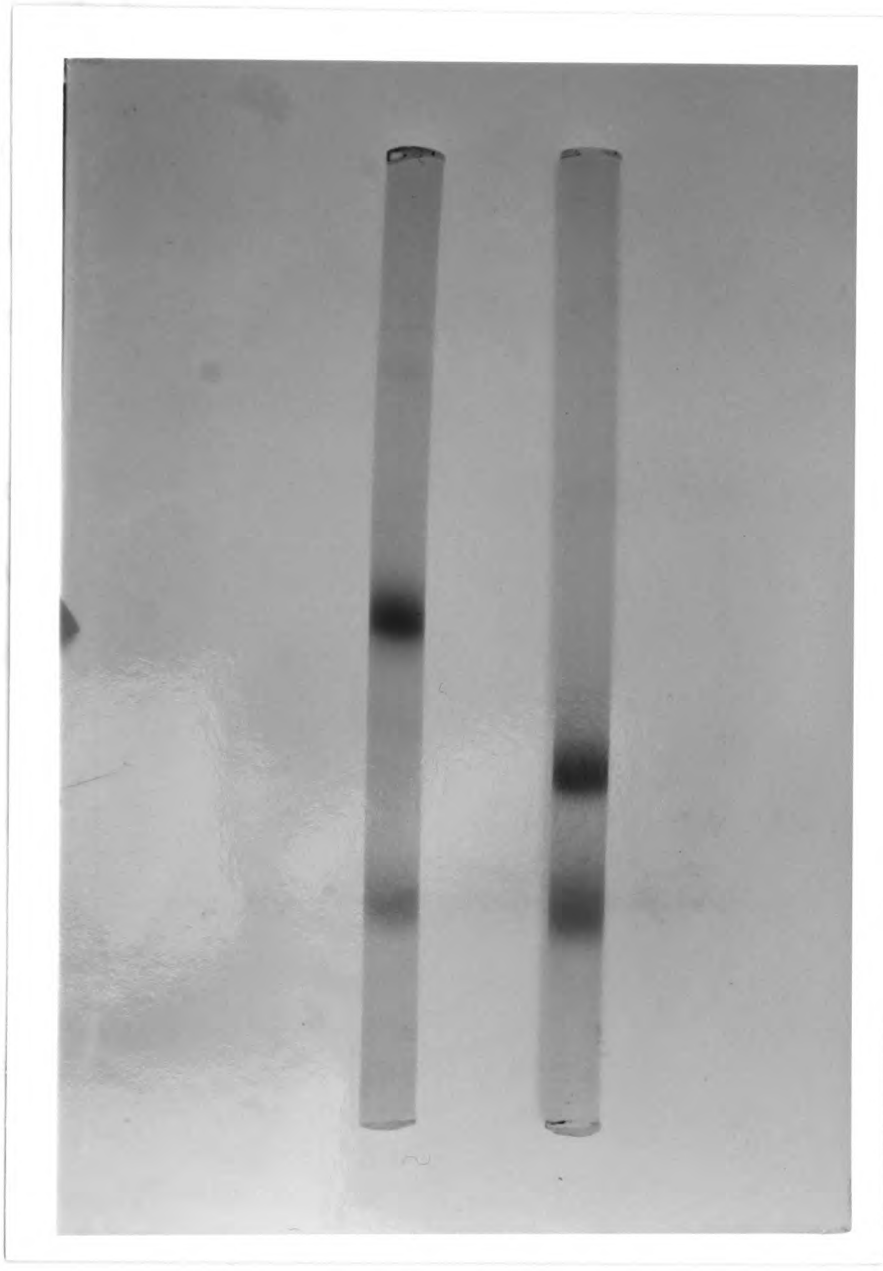


FIGURE 4.17

SDS-PAGE of Factor X and Activated Factor X



1. Factor X_1 (40µg)
2. Factor $X_{1a\beta}$ (40µg)

DISCUSSION

The use of the methods described in this Chapter permits the simultaneous isolation of prothrombin, factors IX, X_1, and X_2, and proteins C and Z from bovine plasma. In addition, protein S can be recovered from the pool corresponding to the leading side of the peak of prothrombin eluted from DEAE-Sephadex A-50 during the chromatography of the barium citrate-adsorbable material from plasma (Figure 4.2). No attempt was made during the present work to recover factor VII. Theoretically, this can be achieved by passing the pool containing prothrombin, factor IX and protein C through a column of benzamidine-Sepharose, to which factor VII is adsorbed more tightly than the other components of the pool. Thus, it should be possible to purify all of the vitamin K-dependent proteins from bovine plasma simultaneously.

If no attempt is made to recover any of the other proteins, it is possible to isolate approximately 12g of prothrombin from 200 litres of blood. It is, however, necessary to sacrifice some of this yield of prothrombin in order to obtain factor IX and proteins C and S. Approximately 3g of prothrombin can be obtained from the factor IX-protein C pool, while a further 6g of prothrombin is recoverable from the protein S pool. The prothrombin separated from factor IX and protein C migrates as a single homogenous band on SDS-polyacrylamide gel electrophoresis under both non-reducing and reducing conditions. Provided that the prothrombin pool from the first heparin-Sepharose chromatography is left in imidazole buffer for as short as time as possible the auto-

activation of the protein can be kept to an undetectable level. This can be achieved most simply by dialysing the protein against citrate buffer immediately it is eluted from the heparin-Sepharose. If the prothrombin remains in the imidazole buffer, which may contain traces of free calcium, it is converted into prethrombin 1 and fragment 1. Similarly, any prothrombin in the factor IX pool - which is subsequently rechromatographed on heparin-Sepharose in the presence of 2.5mM calcium chloride - is completely degraded and hence lost.

In the presence of calcium ions, factor IX is adsorbed by heparin-Sepharose very much more strongly than any of the other vitamin K-dependent proteins. It is only eluted from this resin at sodium chloride concentrations of 0.30M or greater and is, therefore, obtained as a homogenous peak which is apparently free from contamination by other vitamin K-dependent proteins.

Two enzymes will activate factor IX: factor XI_a (Fujikawa *et al*, 1974b) and RVV-X (Lindquist *et al*, 1978). The former reaction leads to the formation of a two-chain molecule, referred to as factor $IX_{a\beta}$, in which an activation peptide containing residues 147 to 181 of factor IX has been cleaved from the heavy chain. During the activation of factor IX by RVV-X, however, only the arginyl-valine bond between residues 181 and 182 is cleaved resulting in the formation of factor $IX_{a\alpha}$ in which the activation peptide remains attached to the light chain. The two forms of factor IX_a function in qualitatively the same manner in the

activation of factor X (Hultin and Nemerson, 1978), but it has been reported (Lindquist *et al*, 1978) that factor $IX_{a\alpha}$ has only 50% of the specific activity of factor $IX_{a\beta}$ in a coagulant or esterase assay. It is, therefore, a cause for concern that the factor $IX_{a\alpha}$ prepared during the present work usually had a higher specific activity than the factor $IX_{a\beta}$. Thus, it is possible that minute traces of RVV-X may have been leached from the RVV-X-Sepharose and have failed to separate from the factor IX_a during chromatography on DEAE-Sephadex. In order to avoid any difficulties which might arise as a result of this possible contamination of the factor $IX_{a\alpha}$, it was used only in preliminary studies of the activation of factor X. Subsequently, more detailed work was carried out exclusively with factor $IX_{a\beta}$.

The possible presence of RVV-X in solutions of factor $IX_{a\alpha}$ suggest that this reagent should be employed with great caution especially in studies on the activation of factor X where the RVV-X can itself catalyse the reaction being examined. It might be possible to overcome the problems associated with leaching of the enzyme from RVV-X-Sepharose by using 1,1'-carbonyldiimidazole (Bethell *et al*, 1979) rather than cyanogen bromide as the coupling agent: the former reagent is claimed to give a less labile linkage between the protein and the gel. Alternatively, it might be possible to add antivenom to the factor $IX_{a\alpha}$ and thus inhibit the RVV-X.

It was found to be very difficult to prepare protein C in a completely pure form. The use of a citrate buffer for the first chromatography of the eluate from barium citrate on

DEAE-Sephadex results in a less efficient separation of protein C from prothrombin than that reported by Stenflo (1976) who employed a phosphate buffer at this stage. Chromatography on heparin-Sepharose in the presence of calcium ions resolves factor IX and protein C, but this procedure is accompanied by the activation of any contaminant prothrombin in the pool. Some of the fragments of prothrombin which this releases have a tendency to co-chromatograph with protein C at subsequent stages of the purification. A secondary effect of the activation of the prothrombin is the activation of the protein C (Kisiel *et al*, 1977) by the thrombin which is generated. Hence, the protein C which is obtained by this procedure is in an activated form.

During the first chromatography of the vitamin K-dependent proteins on DEAE-Sephadex (Figure 4.2) the factor X and protein Z were eluted as a single peak when the column was washed with 0.60M citric acid-NaOH, pH6.9. It was found that this technique produced considerably better yields of these two proteins than were obtained by continuing with the gradient elution which frequently resulted in marked 'trailing' on the descending side of the factor X_2-protein Z peak. As originally reported by Jackson and Hanahan (1968), factors X_1 and X_2 were separated from one another by rechromatography on DEAE-Sephadex (Figure 4.9), but the protein Z was only poorly resolved, forming a slight shoulder on the descending side of the factor X_2 peak. The factor X_2 and protein Z were, however, well separated by the rechromatography on DEAE-Sepharose (Figure 4.11).

The activation of factor X by RVV-X depends on the cleavage of the same arginyl-isoleucine bond between residues 51 and 52 of the heavy chain as that which is cleaved during the activation of factor X by the components of the intrinsic and extrinsic pathways (Fujikawa *et al*, 1974a; Radcliffe and Barton, 1973). This single cleavage is sufficient to activate factor X completely: the molecule which is formed is referred to as factor $X_{a\alpha}$. Factor X_a will, however, also cleave the peptide bond between arginine residue 290 and glycine residue 291 in the heavy chain of factor X or factor X_a. The removal of the C-terminal region by this cleavage produces factor X_β or factor $X_{a\beta}$ (Fujikawa *et al*, 1975). As factor $X_{a\alpha}$ and factor $X_{a\beta}$ have identical coagulant activities (Fujikawa *et al*, 1975), no attempt was made in the present work to distinguish between the two forms of activated factor X or to separate them. The migration of the factor X_a on reduced SDS-polyacrylamide gel electrophoresis, as shown in Figure 4.17, would suggest that the predominant form of the molecule is factor $X_{a\beta}$; as would the results of the amino acid analyses.

Chapter 5

THE INTERACTION OF FACTORS VIII, IX AND X

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THE INTERACTION OF FACTORS VIII, IX AND X

INTRODUCTION

In the original 'cascade' (Macfarlane, 1964) or 'waterfall' (Davie and Ratnoff, 1964) hypothesis of blood coagulation, it was proposed that the initiation of the intrinsic pathway eventually resulted in the conversion by factor IX_a of factor VIII into an active enzyme, factor $VIII_a$, which, in turn, activated factor X. Currently, however, it is thought that the activation of factor X requires the concerted action of factor IX_a and factor VIII (Jackson and Nemerson, 1980; Nemerson and Furie, 1980). Much of the early evidence for the interaction of factors VIII and IX_a in this manner was based on changes in the elution of the activities from gel-filtration columns (Hougie *et al*, 1967; Chuang *et al*, 1972) or on changes in clotting assay times (Biggs *et al*, 1965; Irwin *et al*, 1975). These techniques do not, however, permit an unambiguous distinction to be made between a model involving the consecutive actions of factor IX and factor VIII, and one requiring the formation of a complex between factors IX_a and VIII in the presence of calcium ions and phospholipid. Clotting assays in particular cannot be made completely specific since they are susceptible to changes in the reactivity of coagulation factors other than that which the assay is designed to measure, to the effects of inhibitors such as antithrombin III, and to positive and negative feedback reactions occuring at various stages in the clotting process.

Factor X_a is a serine proteinase in which the amino acid sequence of the active site is homologous with that of trypsin, chymotrypsin and elastase (Titani *et al*, 1972); it is inhibited by diisopropyl fluorophosphate (Leveson and Esnouf, 1969) or by phenylmethane sulphonyl fluoride (Fujikawa *et al*, 1972b). Activated factor X also has esterase activity, hydrolysing small molecules such as tosyl-L-arginine methyl ester (Jackson and Hanahan, 1968) and α-N-benzoyl-L-arginine ethyl ester (Esnouf *et al*, 1973). The esterase activity of factor X_a can be utilised to follow the activation of the zymogen. This type of assay is convenient because it permits a direct assessment of the factor X_a activity rather than the indirect measurement obtained in clotting assays where factor X_a must activate prothrombin before the thrombin formed is measured via its action on fibrinogen. The introduction of the synthetic chromogenic substrate, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide (substrate S-2222, Kabi Diagnostica), by Aurell *et al* (1975) provided a specific assay which differentiates factor X_a from thrombin and other activated clotting factors. The use of esterase assays thus distinguishes between the requirements of the reaction in which factor X is activated and those of the subsequent reactions in the coagulation process.

One disadvantage of using esterase assays to measure the concentration of an activated clotting factor is that there is not always a direct correlation of the esterase activity with the proteinase, and hence coagulant, activity.

An example of this is provided by the two forms of factor X which can be isolated from bovine plasma (Jackson and Hanahan, 1968). Although the two forms have different specific clotting activities (Esnouf *et al*, 1973), this is apparently the result of a difference in the rates at which the zymogens are activated, since both Jackson and Hanahan (1968) and Esnouf *et al* (1973) found that the same esterase activity was generated when either factor X_1 or factor X_2 was completely activated by Russell's viper venom.

Despite the possible disadvantages and drawbacks associated with the use of measurements of the esterase activity of factor X_a, several groups have recently employed this technique in investigations of the activation of factor X. Kosow *et al* (1974) used N-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester as a substrate for the factor X_a, while Suomela *et al* (1977), Brown *et al* (1978) and Ofosu *et al* (1981) all used substrate S-2222.

An alternative technique for the examination of the activation of factor X by factor IX_a and VIII was utilised by Hultin and Nemerson (1978). This method, which was originally used to study the extrinsic activation of factor X (Silverberg *et al*, 1977), requires the labelling of the sialic acid residues of the zymogen with tritium. 80% of the label is incorporated into the activation peptide region of factor X, and the formation of factor X_a can therefore be followed by measuring the release of trichloroacetic acid-soluble radioactivity.

The various studies of the activation of factor X by

the components of the intrinsic pathway have failed to provide a definitive model for the reaction. There is general agreement that factor IX_a is the enzyme responsible for the cleavage of factor X, but until recently (Suomela *et al*, 1977; van Dieijen *et al*, 1981) there has been little experimental evidence for a direct activation of factor X by factor IX_a in the absence of the other elements of the system. Similarly, the other components of the reaction - factor VIII:C, metal ions and phospholipids - tend to be described as 'cofactors' of factor IX_a, but their exact functions remain ill-defined.

The role of factor VIII:C in the activation of factor X is a particular source of controversy. Factor VIII:C may act as a cofactor, with a function analogous to that of factor V in the activation of prothrombin by factor X_a (Esnouf, 1977). It has, however, been reported by Vehar and Davie (1977, 1980) that thrombin-activated factor VIII:C is inhibited by diisopropyl fluorophosphate, implying that the active form of the molecule is a serine proteinase. The requirement for factor VIII:C to be 'activated' by thrombin before it participates in the activation of factor X is not clearly established. Some workers (Hultin and Nemerson, 1978; van Dieijen *et al*, 1981) claimed that it was essential for the factor VIII:C to be pre-treated with thrombin while others (Varadi and Hemker, 1976; Suomela *et al*, 1977) were able to use unactivated factor VIII:C.

A study of the activation of factor X was therefore undertaken, using assays of the esterase activity of factor

X_a to follow the reaction. This investigation of the conditions required for the reaction was designed to clarify the roles of the various components, and particularly that of the factor VIII:C, in the activation of factor X.

MATERIALS AND METHODS

Preparation of Clotting Factors

Factors IX and X were prepared as described in Chapter 4. Factor X_1 was used exclusively in the experiments as it could be separated more completely than factor X_2 from any traces of factor X_a generated during the purification procedure. A typical preparation of factor X_1 had a specific clotting activity of 70 units/mg while factor IX preparations had specific activities of approximately 15 units/mg.

Factor IX was activated with either contact product or Russell's viper venom, and the factor IX_a was purified by chromatography on DEAE-Sephadex, as described in Chapter 4. The former procedure gave factor $IX_{a\beta}$ with a specific coagulant activity of between 30 and 50 units/mg whereas the latter procedure gave a factor $IX_{a\alpha}$ preparation with a specific activity of 200 units/mg. This very high specific activity of the factor $IX_{a\alpha}$ could indicate that the preparation contained traces of contaminating RVV-X.

'Partially purified factor VIII:C' was prepared as described in Chapter 2. Some preliminary experiments were carried out with less pure factor VIII:C which had not been subjected to gel-filtration or chromatography on sulphate-Sepharose. This material had a specific activity of 2 units/mg, and the results obtained with it were subsequently confirmed by repeating selected experiments in which this

factor VIII:C preparation was replaced with 'partially purified factor VIII:C' having a specific activity of 100 units/mg.

A standard preparation of human thrombin (M.R.C. Reagent 66/305) was obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, U.K. It was dissolved in distilled water to a final concentration of 40iu/ml, and stored in small aliquots at -20°C.

Bovine thrombin was prepared in this laboratory by activating purified prothrombin with bovine factor X_a and isolating the thrombin on SP-Sephadex. This thrombin had a specific activity of approximately 1700iu/mg; it was dissolved in 0.05M tris-HCl, pH7.5 and stored in small aliquots at -20°C.

The specific thrombin inhibitor, hirudin, was prepared by the method of Markwardt (1970), as described in Appendix 10.

Phospholipids

A chloroform extract of acetone-dried brain (Bell and Alton, 1954) was obtained from Diagnostic Reagents Ltd., Thame, Oxon, U.K., and used routinely as a source of phospholipid. A stock suspension of this 'cephalin' was prepared by reconstituting the contents of one vial with 5.0ml of 0.02M tris-HCl, pH7.5; the concentration of phospholipid

in the stock suspension was 200μM.

Further studies of the phospholipid requirement of the activation of factor X were carried out with phosphatidyl serine (Sigma London Chemical Co Ltd., Poole, Dorset, U.K.), supplied as a 10mg/ml solution in 95% chloroform - 5% methanol. Aliquots of this solution were mixed with 0.02M tris-HCl, pH7.5, and the organic solvents were removed by rotary evaporation; the resultant aqueous suspension was then adjusted to the desired volume with buffer.

Assay Substrates

α-N-Benzoyl-L-arginine ethyl ester (BAEe) was purchased from Sigma London Chemical Co Ltd., Poole, Dorset, U.K. It was dissolved in 0.05M tris-HCl, pH8.0, to a concentration of 0.003M and stored at room temperature for periods of no longer than six hours.

N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide hydrochloride (S-2222) was obtained from Kabivitrum Ltd., London, U.K. It was dissolved in distilled water at a final concentration of 0.004M, and stored at 4°C. It was found to be inadvisable to store stock solutions of S-2222 for periods longer than a few days.

Factor X_a Assays

Factor X_1 was activated with RVV-X and purified as described in Chapter 4. The esterase activity of the factor X was measured with both BAEe and S-2222 as the substrate.

Mixtures containing BAEe were made up as follows in

10mm path-length cuvettes in a Gilford Model 250 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio, U.S.A.), with the cuvette chamber maintained at 37°C:

1.00ml 0.003M BAEe

1.90ml 0.05M tris-HCl, pH8.0

0.10ml factor X_{1a} in 0.025M tris-HCl, pH7.5.

Individual mixtures were prepared with from 0 to 400pmoles factor X_a in the cuvettes and the change in A_{253} was recorded in each case.

Mixtures containing S-2222 were also made up in 10mm path-length cuvettes in the Gilford Model 250 Spectrophotometer:

0.10ml 0.004M S-2222

1.87ml 0.05M tris-HCl, pH8.0

0.01ml 0.01M $CaCl_2$

0.01ml 0.02M EDTA

0.01ml factor X_{1a} in 0.025M tris-HCl, pH7.5

In this case, mixtures were prepared with between 0 and 10 pmoles factor X_a in the cuvettes. The change in A_{405} in the mixtures at 37°C was recorded.

Assays of Factor X Activation

The activation of factor X was carried out in polyethylene tubes in a 37°C water bath. The reactants were added to the tube in the following order: 0.02M tris-HCl, pH7.5, calcium chloride, phospholipid, factor X_1, 'partially purified factor VIII:C' and factor IX_a. The addition of the factor IX_a was taken as the zero time point: partial activa-

tion mixtures were not preincubated at 37°C before initiating the reaction. If it was necessary to dilute a stock protein solution before adding it to the activation mixture, this dilution into 0.02M tris-HCl, pH7.0, was made immediately before the activation mixture was set up as the proteins were not stable when stored in dilute solutions for long periods of time.

In those cases where BAEe was used as the substrate for the factor X_a assays, the reaction in the activation mixtures was stopped by diluting 150µl aliquots of the mixtures into cuvettes containing 1.00ml stock 0.003M BAEe solution and 1.85ml 0.05M tris-HCl, pH8.0, at 37°C. The hydrolysis of BAEe by factor X_a was followed by recording the change in the A_{253} using the Gilford Model 250 spectrophotometer.

The reaction in activation mixtures for which S-2222 was used as a substrate in the factor X_a assays was stopped by adding an aliquot of the mixture to an equal volume of ice-cold 0.02M disodium EDTA. Aliquots of the EDTA-containing mixture were then transferred to a cuvette at 37°C containing 0.10ml 0.004M S-2222, and sufficient 0.05M tris-HCl, pH8.0, to give a total volume of 2.00ml. The increase in the A_{405} was again recorded using the Gilford Model 250 spectrophotometer.

With both substrates, the background level of ester hydrolysis by the components of the various activation mixtures was determined by an assay on an aliquot taken from the mixture immediately after the addition of factor IX_a. The

very low background rate of absorbance change, which was due to the esterase activity of factor IX_a, to traces of factor X_a in the factor X_1 preparation, and to spontaneous hydrolysis of the substrate, was then subtracted from the rate of change in the absorbance observed with other samples from the activation mixture.

Electrophoretic Analysis of Factor X Activation

Aliquots taken from factor X activation mixtures at different stages during the incubation at 37°C were mixed with an equal volume of 0.02M EDTA in an ice-bath and stored at 0°C until all of the samples were available. The mixtures were then boiled with 'sample buffer' containing SDS and 2-mercaptoethanol and analysed by SDS-polyacrylamide slab gel electrophoresis using the discontinuous buffer system of Laemmli and Favre (1973), as described in Appendix 8.

RESULTS

Assays of Factor X_a

The absorbance changes due to the hydrolysis of BAEe and S-2222 by factor X_a are listed in Tables 5.1 and 5.2 respectively. The data were plotted in the calibration graphs (Figures 5.1 and 5.2) which were used to relate the rates of substrate hydrolysis caused by aliquots from the activation mixtures to the concentrations of factor X_a in these samples.

Under the conditions used for the relevant substrate, the mean change in A_{253} with BAEe was 67.5 absorbance units per minute per micromole factor X_a and the mean change in A_{405} with S-2222 was 5.2 absorbance units per minute per nanomole factor X_a. That is, the specific chromogenic substrate was approximately one hundred times more sensitive than BAEe for the assay of factor X_a.

Activation of Factor X by Factors IX_a and VIII:C

Figure 5.3 shows the activation of factor X by factor $IX_{a\alpha}$ in the presence and absence of factor VIII:C. The activation mixtures contained 2μM factor X_1, 0.2μM factor $IX_{a\alpha}$, 50μM 'cephalin', 0.010M calcium chloride, and - where appropriate - 5 units/ml 'partially purified factor VIII:C'. Activation mixtures containing all of the reagents at the concentrations listed above were used throughout as 'standard' mixtures where the factor X_a being generated was to be assayed with BAEe. The concentrations of the various compon-

TABLE 5.1

Hydrolysis of BAEe by Factor X_a

Factor X_a in Sample (picomoles)	ΔA_{253} (units per minute) x 10^3
0	0.00
20	0.75
40	3.00
60	5.00
80	6.50
100	7.75
120	8.25
140	9.75
160	11.00
200	13.00
240	15.75
280	19.00
320	21.50
360	23.00
400	25.50

Factor X_a was mixed with 0.001M BAEe in a total volume of 3.00 ml 0.05M tris-HCl, pH8.0, and the change in A_{253} (ΔA_{253}) was recorded using a Gilford Model 250 spectrophotometer.

FIGURE 5.1

Hydrolysis of BAEe by Factor X_a

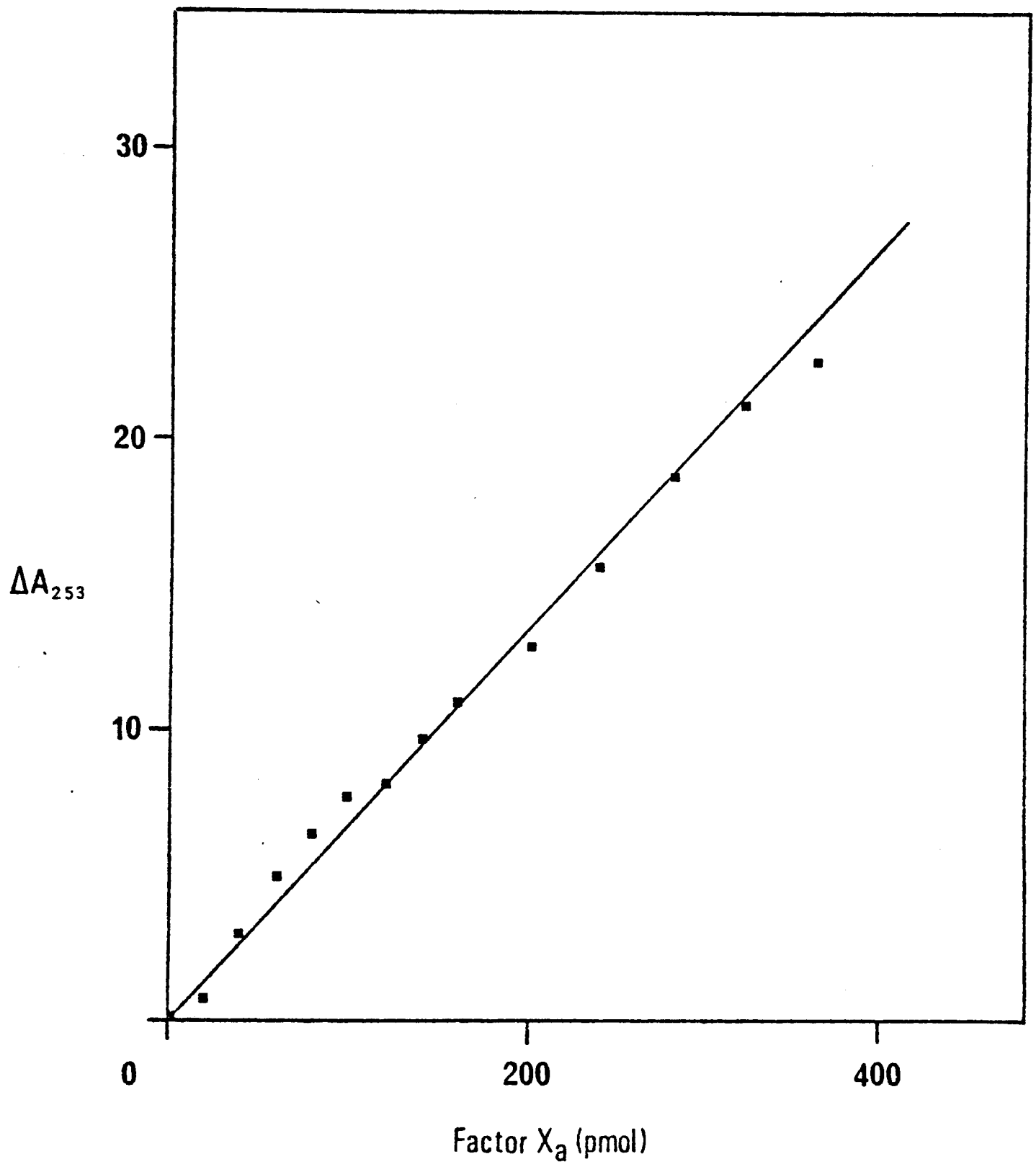


TABLE 5.2

Hydrolysis of S-2222 by Factor X_a

Factor X_a in Sample (picomoles)	ΔA_{405} (units per minute) x 10^3
0	0.00
1	5.66
2	8.66
3	15.00
4	19.33
5	24.00
6	31.66
7	39.00
8	43.00
9	47.00
10	54.66

Factor X_a was mixed with 0.2mM S-2222 in a total volume of 2.00 ml 0.05M tris-HCl, pH8.0, and the change in A_{405} (ΔA_{405}) was recorded using a Gilford Model 250 spectrophotometer.

FIGURE 5.2

Hydrolysis of S-2222 by Factor X_a

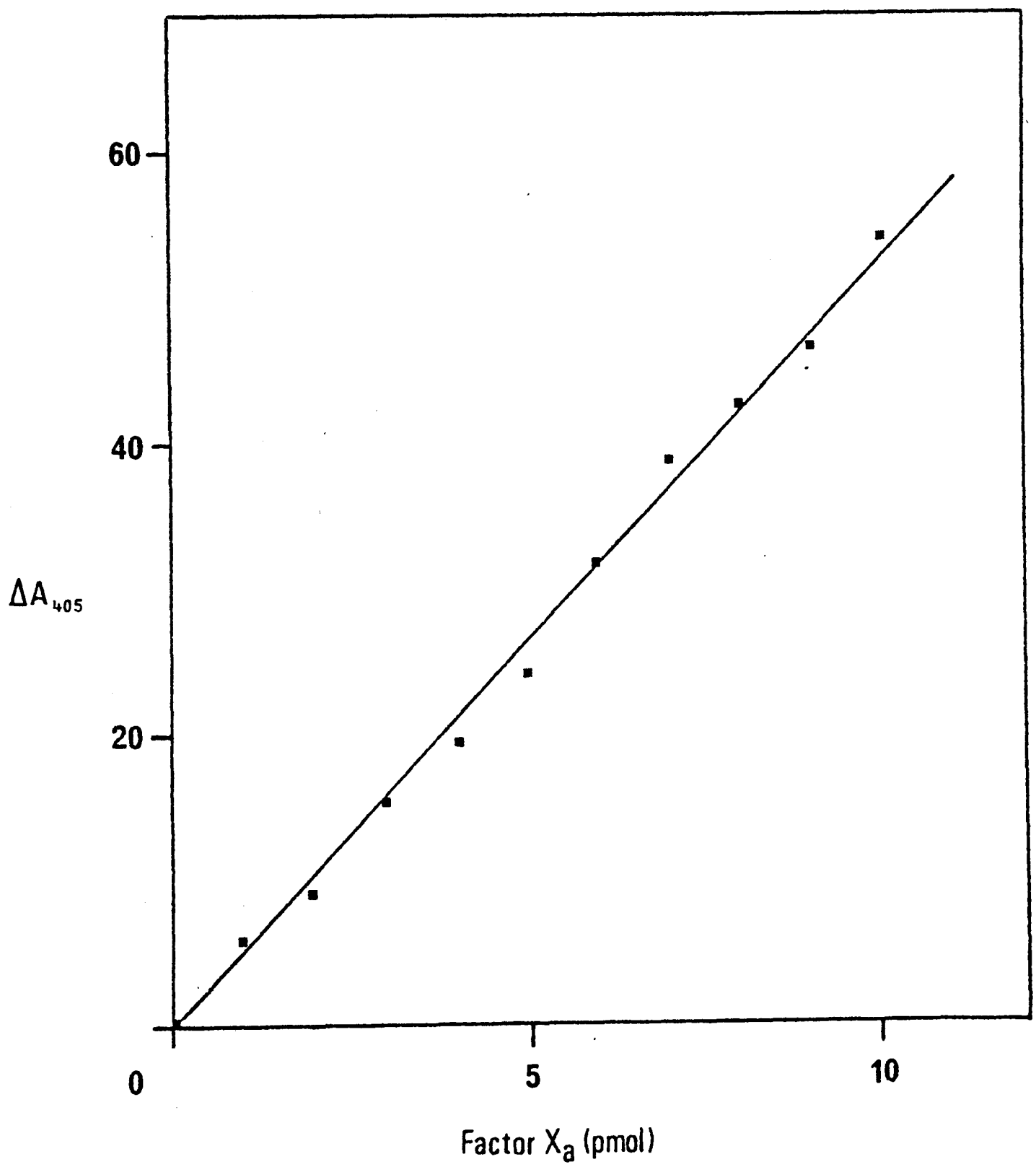
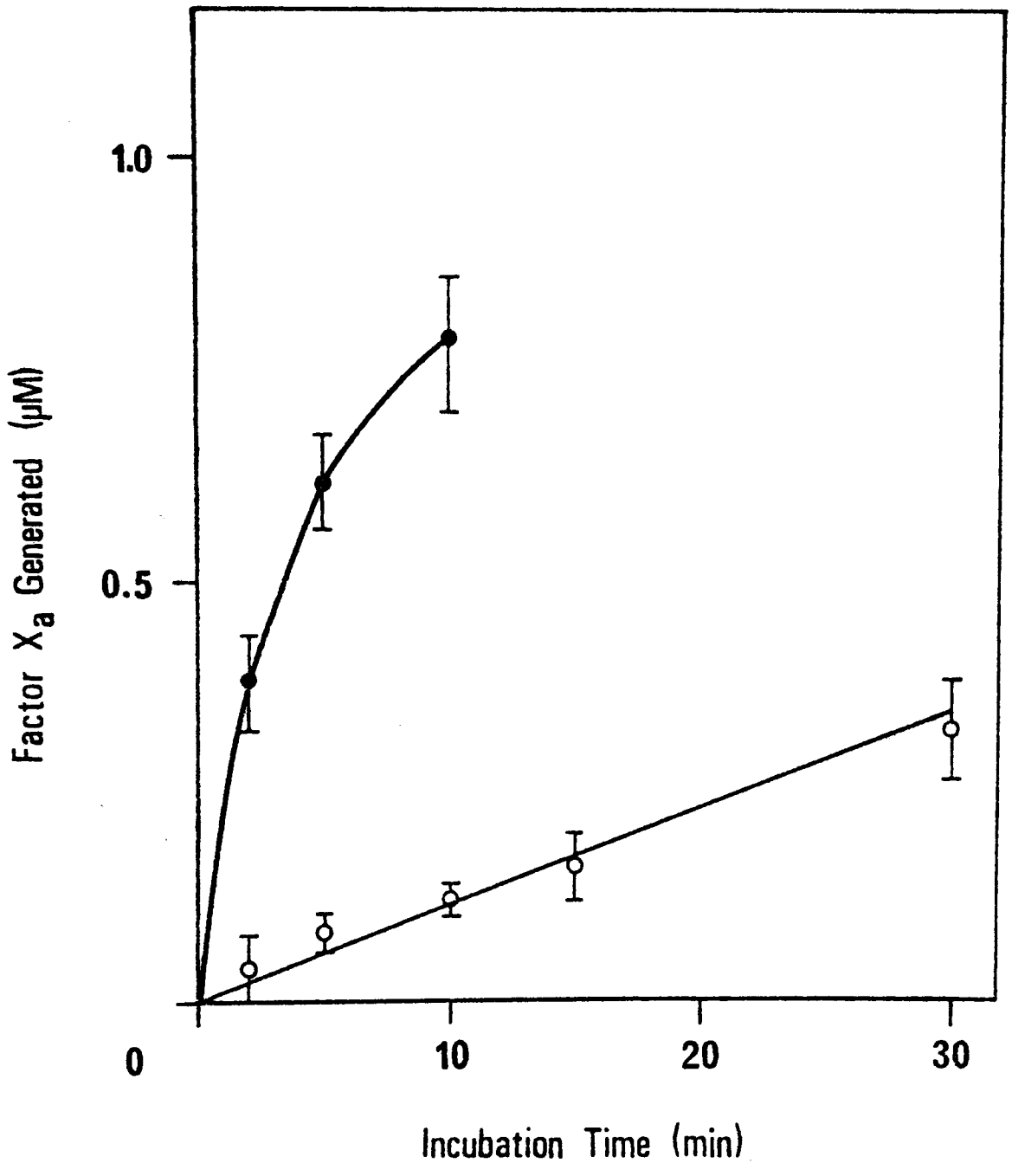


FIGURE 5.3

Activation of Factor X by Factors $IX_{a\alpha}$ and VIII:C



2μM factor X_1 was incubated at 37°C with 0.2μM factor $IX_{a\alpha}$, 50μM 'cephalin' and 10mM $CaCl_2$ in the absence (○) or presence (●) of 5 units/ml 'partially purified factor VIII:C'. The factor X_a generated was assayed with BAEe; results are shown as Mean ± SD for ten determinations at each incubation time.

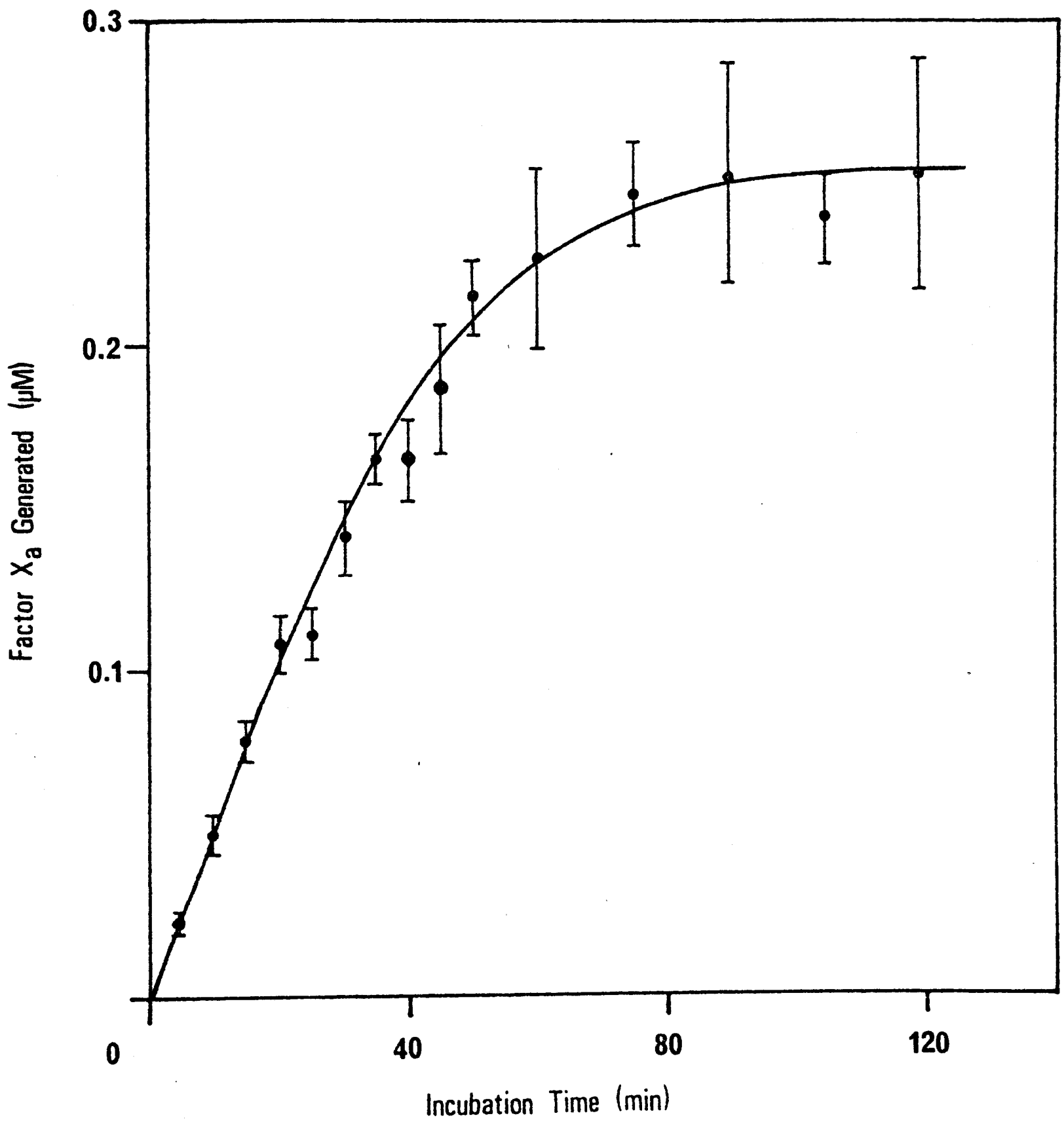
ents of these standard mixtures were chosen to provide a system in which the activation of factor X could be followed both in the presence and absence of factor VIII:C. An increase in the initial rate of the reaction from 0.05 to 0.70 moles factor X_a formed per minute per mole factor $IX_{a\alpha}$ resulted from the presence of 'partially purified factor VIII:C' in the mixture. No initial lag period was seen in either case, but the fast initial rate seen in the presence of factor VIII:C was sustained only until approximately 30% of the factor X had been activated, and then it declined.

In contrast, no factor X_a was generated in mixtures containing 2μM factor X_1, 50μM 'cephalin', 5 units/ml 'partially purified factor VIII:C' and 0.010M calcium chloride, but no factor IX_a. Factor VIII:C preparations which had been 'activated' by a prior incubation with bovine thrombin also failed to activate factor X in the absence of factor IX_a.

The activation of factor X by factor $IX_{a\beta}$ without factor VIII:C is shown in Figure 5.4. The activation mixture, which was subsequently used as the standard for assays with S-2222, contained 1μM factor X_1, 0.5μM factor $IX_{a\beta}$, 65μM 'cephalin' and 0.010M calcium chloride. When S-2222 was used as the substrate for the assay of factor X_a, standard mixtures for the activation of factor X by factors $IX_{a\beta}$ and VIII:C were made up with 1μM factor X_1, 2nM factor $IX_{a\beta}$, 3 units/ml 'partially purified factor VIII:C', 65μM 'cephalin' and 0.010M calcium chloride. Figure 5.5 shows the generation of factor X_a in the standard mixture. The initial rate of formation of factor X_a in the standard mixtures was 0.01 and

FIGURE 5.4

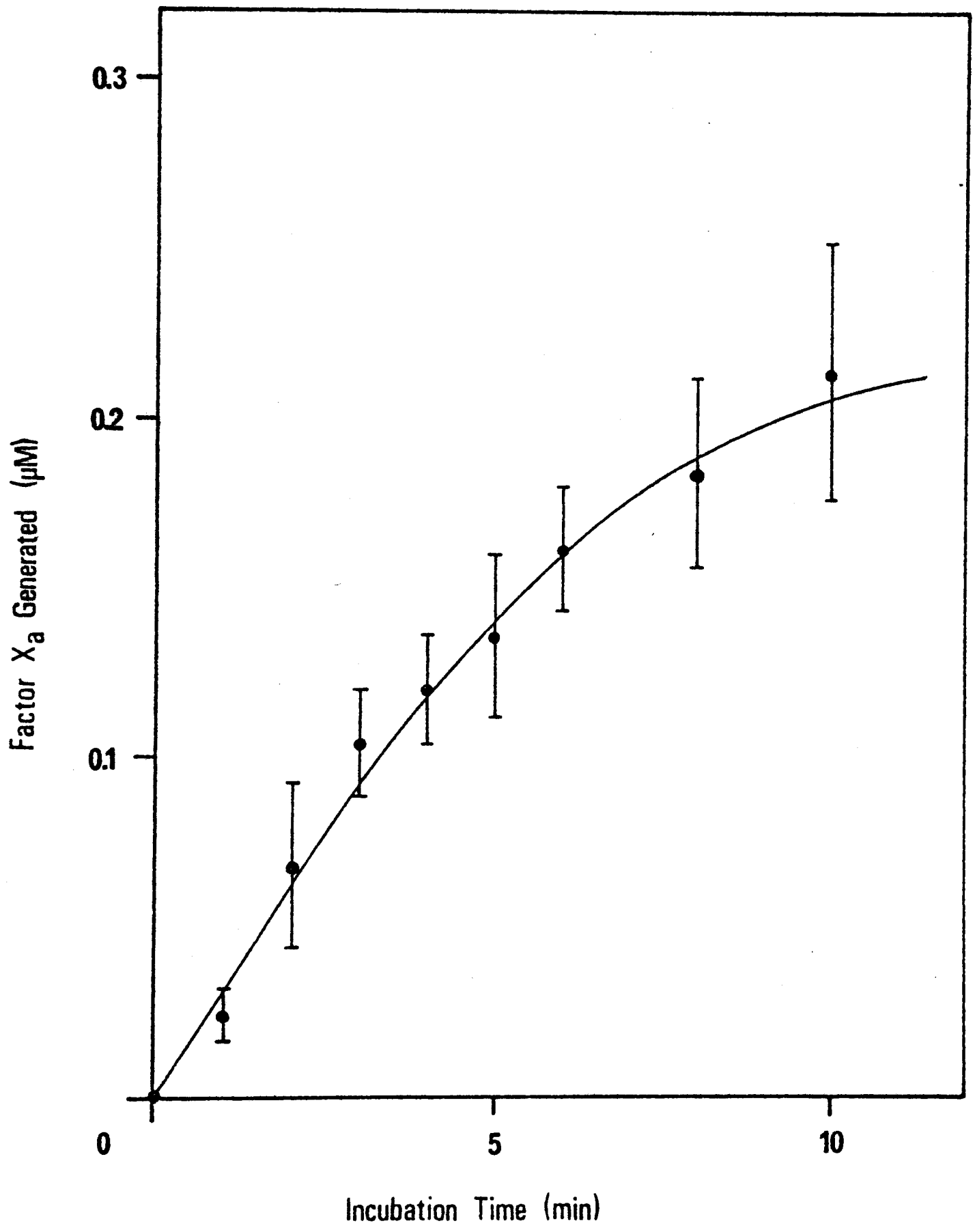
Activation of Factor X by Factor $IX_{a\beta}$



1μM factor X_1 was incubated at 37°C with 0.5μM factor $IX_{a\beta}$, 65μM 'cephalin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222; results are shown as Mean ± SD for five determinations at each incubation time.

FIGURE 5.5

Activation of Factor X by Factors $IX_{a\beta}$ and VIII:C



1μM factor X_1 was incubated at 37°C with 2nM factor $IX_{a\beta}$, 3 units/ml 'partially purified factor VIII:C', 65μM 'cephalin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222; results are shown as Mean ± SD for five determinations at each incubation time.

15.6 moles factor X_a formed per minute per mole factor $IX_{a\beta}$ in the absence and presence of factor VIII:C respectively.

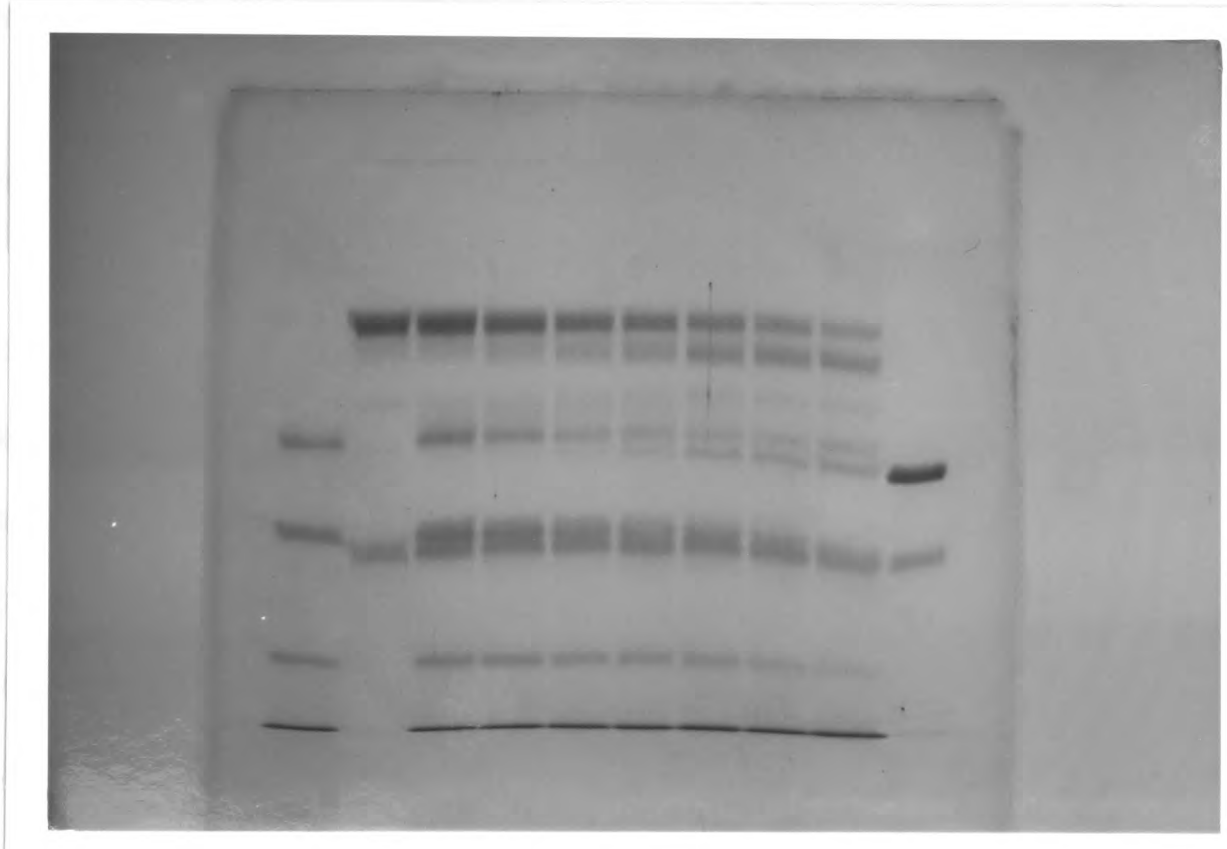
Having defined 'standard' mixtures in which the activation of factor X could be followed conveniently by esterase assays of factor X_a with either BAEe or S-2222, the effect of each individual component on the reaction was investigated by changing the concentration of that particular reactant in the activation mixture while keeping the concentrations of the other components constant.

In parallel with the experiments in which the activation of factor X was followed by means of factor X_a assays, samples from the activation mixtures were examined by SDS-polyacrylamide slab gel electrophoresis.

The gel shown in Figure 5.6 indicates that a series of changes occur in the polypeptide chain composition of an incubation mixture in which factor X_α is activated by factor $IX_{a\beta}$ in the presence of phospholipid and calcium ions. During the reaction, the band corresponding to the heavy chain of factor X_α gradually disappears and is replaced by three new bands, which correspond to the heavy chains of factor X_β, factor $X_{a\alpha}$ and factor $X_{a\beta}$. The light chain of factor X does not change during the incubation. The changes in the pattern of the bands on the gel reflect the action of both factor IX_a and the newly formed factor X_a in the mixture. The former enzyme converts factor X_α to factor $X_{a\alpha}$ or factor X_β to factor $X_{a\beta}$, whereas the latter converts factor X_α to factor X_β and factor $X_{a\alpha}$ to factor $X_{a\beta}$.

FIGURE 5.6

Activation of Factor X by Factor $IX_{a\beta}$



1. Factor $IX_{a\beta}$ (10µg)
2. Factor X_{α} (8µg)
3-9. Samples taken from incubation mixture containing 1.0µM factor $X_{1\alpha}$, 0.5µM factor $IX_{a\beta}$, 50µM 'cephalin' and 10mM $CaCl_2$ after 0, 10, 20, 30, 45, 60 and 90 minutes at 37°C
10. Factor $X_{a\beta}$ (8µg)

This gel also shows that factor IX_a is destroyed in the incubation mixture. Factor X_a will degrade factor IX_a in a mixture containing the two proteins, calcium ions and phospholipid (Figure 5.7) and it is, therefore, probable that the disappearance of the factor IX_a from the activation mixture is the result of the action of factor X_a.

When 'partially purified factor VIII:C' was present in the activation mixture, a similar series of changes occured in the polypeptide chains. The conversion of factor X_α into factor $X_{a\alpha}$ and factor $X_{a\beta}$ can be seen in the gel shown in Figure 5.8; little factor X_β is visible in this case. The concentration of factor IX_a in these mixtures was much lower than in mixtures containing no factor VIII:C and this component cannot be seen on the gel. No changes are visible in the bands arising from the 'partially purified factor VIII:C'.

Figures 5.6 and 5.8 show that relatively little uncleaved factor X_α heavy chain remained in the activation mixtures at the completion of the incubation, especially when factor VIII:C was present. The measurements of the esterase activity of the factor X_a, however, indicated that only 30% of the original factor X had been activated in the mixtures, whether or not factor VIII:C was present.

Effects of Factor IX_a on Factor X Activation

In the absence of factor VIII:C, varying the concentration of factor $IX_{a\alpha}$ without making any other alteration to the composition of the activation mixture had a pronounced effect on the course of the reaction. As is shown in Figure

FIGURE 5.7

Degradation of Factor $IX_{a\beta}$ by Factor X_a



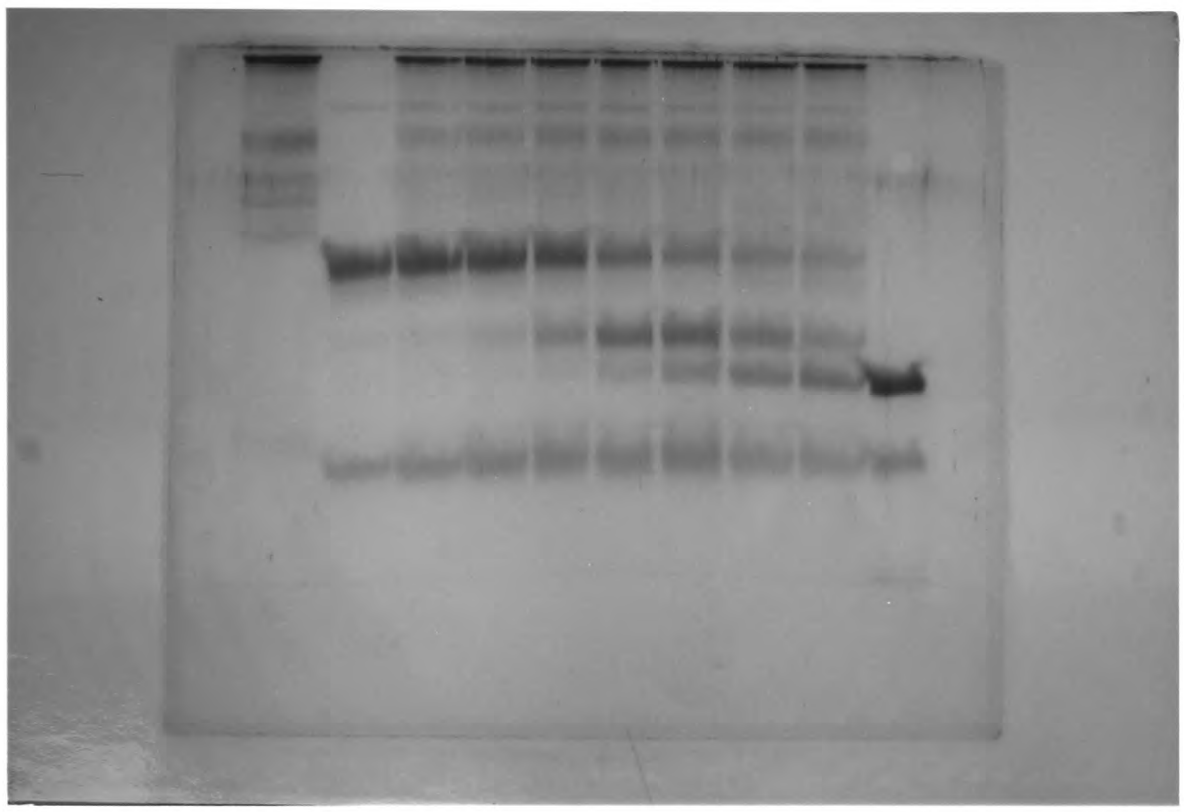
1. Factor $IX_{a\beta}$ (10µg)

2-9. Samples taken from incubation mixture containing 2.0µM factor $IX_{a\beta}$, 0.5µM factor $X_{a\beta}$, 50µM 'cephalin' and 10mM $CaCl_2$ after 0, 10, 20, 30, 45, 60, 90 and 120 minutes at 37°C

10. Factor $X_{a\beta}$ (8µg)

FIGURE 5.8

Activation of Factor X by Factors $IX_{a\beta}$ and VIII:C



1. 'Partially purified factor VIII:C' (6µg)
2. Factor $X_{1\alpha}$ (8µg)
3-9. Samples taken from incubation mixture containing 1.0µM factor $X_{1\alpha}$, 3.0nM factor $IX_{a\beta}$, 2 units/ml 'partially purified factor VIII:C', 50µM 'cephalin' and 10mM $CaCl_2$ after 0, 1, 2, 5, 10, 20 and 30 minutes at 37°C
10. Factor $X_{a\beta}$ (8µg)

5.9, no generation of factor X_a could be detected during the first twenty minutes incubation of mixtures containing 0.04μM factor $IX_{a\alpha}$ and no factor VIII:C, although continued incubation at 37°C did result in the appearance of measurable levels of factor X_a. There was a similar, but shorter, lag period in activation mixtures containing 0.1μM factor IX_a. The 'reaction rate' quoted for these mixtures in Table 5.3 is that measured during the linear portion of the time course following the initial lag period. Table 5.3 also includes the values obtained for the initial rate of the reaction when factor $IX_{a\beta}$ was used to activate factor X; in these mixtures, the initial rate of the reaction was linear, with no lag phase.

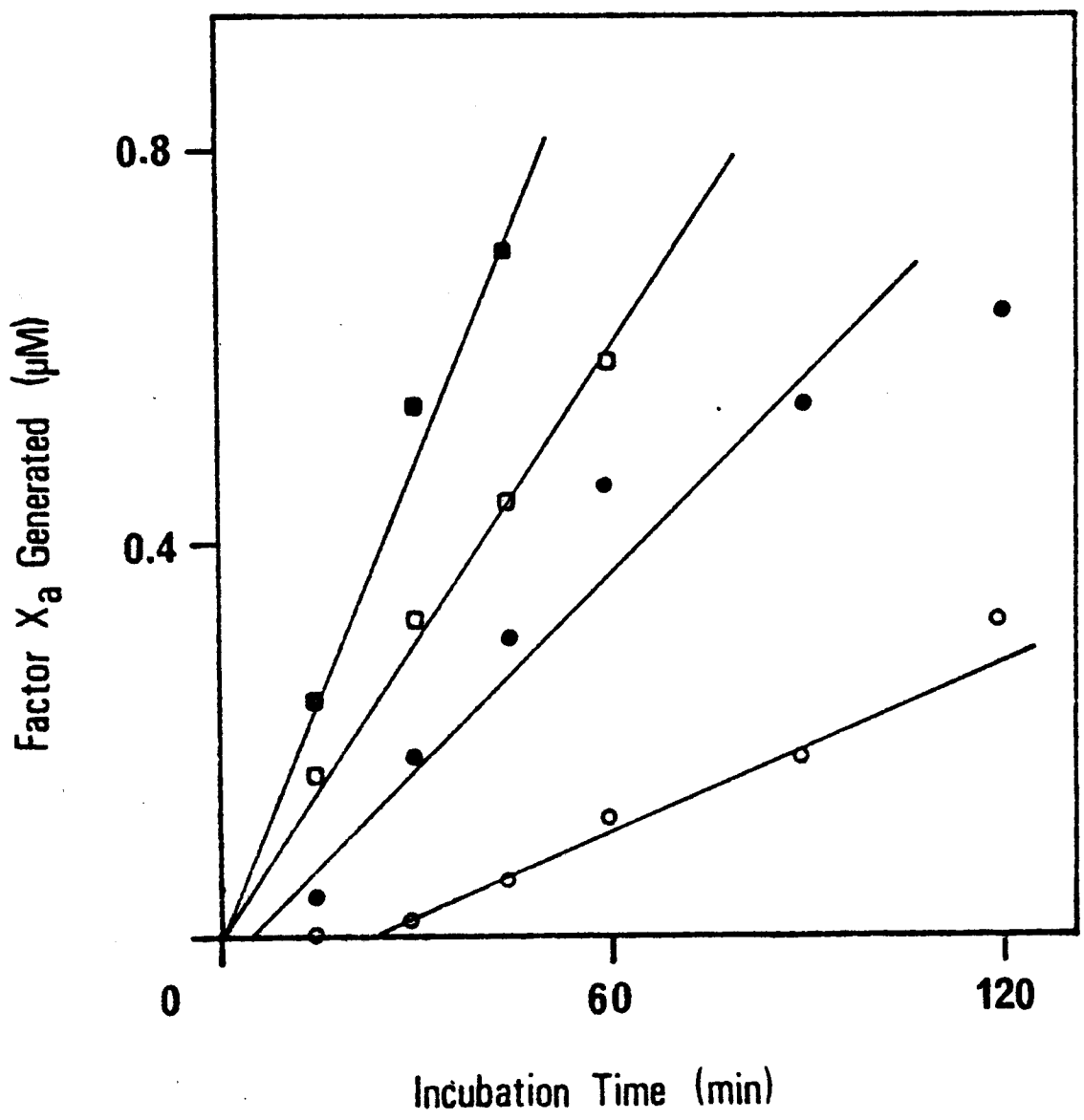
The rate at which factor X was activated by mixtures containing both factor IX_a and 'partially purified factor VIII:C' was dependent on the concentration of factor IX_a (Table 5.4) when the 'partially purified factor VIII:C' concentration was kept constant. No initial lag period was seen during the activation of factor X in any of these mixtures.

Effects of Factor VIII:C on Factor X Activation

The stimulatory effect of 'partially purified factor VIII:C' is clearly apparent from the rate of activation of factor X in the 'standard' incubation mixtures used with BAEe or with S-2222, and also from a comparison of the rates in Tables 5.3 and 5.4.

FIGURE 5.9

Activation of Factor X by Factor $IX_{a\alpha}$



2μM factor X_1 was incubated at 37°C with 50μM 'cephalin', 10mM $CaCl_2$ and 0.04 (○), 0.1 (●), 0.2 (□) or 0.4 (■) μM factor $IX_{a\alpha}$. The factor X_a generated was assayed with BAEe.

TABLE 5.3

Activation of Factor X by Factor IX_a

a) Factor $IX_{a\alpha}$

Factor IX_a (μM)	Rate of Reaction (pmoles X_a formed/ml /minute)	Rate of Reaction (moles X_a formed/minute /mole IX_a)
0.0	0.0	0.00
0.04	3.0	0.08
0.1	6.6	0.07
0.2	10	0.05
0.4	16	0.04

b) Factor $IX_{a\beta}$

Factor IX_a (μM)	Rate of Reaction (pmoles X_a formed/ml /minute)	Rate of Reaction (moles X_a formed/minute /mole IX_a)
0.0	0.0	0.000
0.2	5.0	0.025
0.7	10.0	0.014

2μM factor X_1 was incubated at 37°C with 50μM 'cephalin', 10mM $CaCl_2$ and varying concentrations of factor IX_a. The factor X_a generated was assayed with BAEe.

TABLE 5.4

Activation of Factor X by Factors IX_a and VIII:C

Factor $IX_{a\alpha}$ (μM)	Rate of Reaction (pmoles X_a formed/ml /minute)	Rate of Reaction (moles X_a/min/mole IX_a)
0.00	0	0.00
0.04	68	1.70
0.10	86	0.86
0.20	136	0.68
0.40	174	0.44

2μM factor X_1 was incubated at 37°C with 5units/ml 'partially purified factor VIII:C', 50μM 'cephalin', 10mM $CaCl_2$ and varying concentrations of factor $IX_{a\alpha}$. The factor X_a generated was assayed with BAEe.

Table 5.5 illustrates the effect of varying the concentration of factor VIII:C in activation mixtures in which the other components remained constant. There is a strong indication from these results that a 'saturation' effect can be obtained under the conditions of the experiment: increasing the concentration of 'partially purified factor VIII:C' from 1 to 5 units/ml resulted in less than a two-fold increase in the rate of the reaction. This is shown in Figure 5.10 in which the activation of factor X by factor $IX_{a\beta}$ at factor VIII:C concentrations of 0, 1 and 5 units/ml is plotted.

Effects of Calcium on Factor X Activation

No activation of factor X was detectable when 1μM factor X_1, 1μM factor $IX_{a\beta}$ and 40μM 'cephalin' were incubated together at 37°C in the absence of calcium ions. In similar mixtures containing 0.01M calcium chloride, the initial rate of formation of factor X_a was approximately 8 picomoles/ml/minute.

The activation of factor X by factors IX_a and VIII:C resembled the reaction in the absence of factor VIII:C in exhibiting a requirement for calcium ions. The effect of calcium ion concentration on the initial rate of factor X activation is shown in Table 5.6. There was an increase in the rate of the reaction with increasing calcium ion concentration throughout the range tested; no evidence of saturation or inhibition of the reaction at high levels of calcium was observed.

TABLE 5.5

Activation of Factor X by Factors IX_a and VIII:C

a) Factor $IX_{a\alpha}$ and Factor VIII:C

Factor VIII:C (units/ml)	Rate of Reaction (moles X_a formed/min/mole IX_a)
0.0	0.05
0.8	0.49
2.5	0.55
5.0	0.70

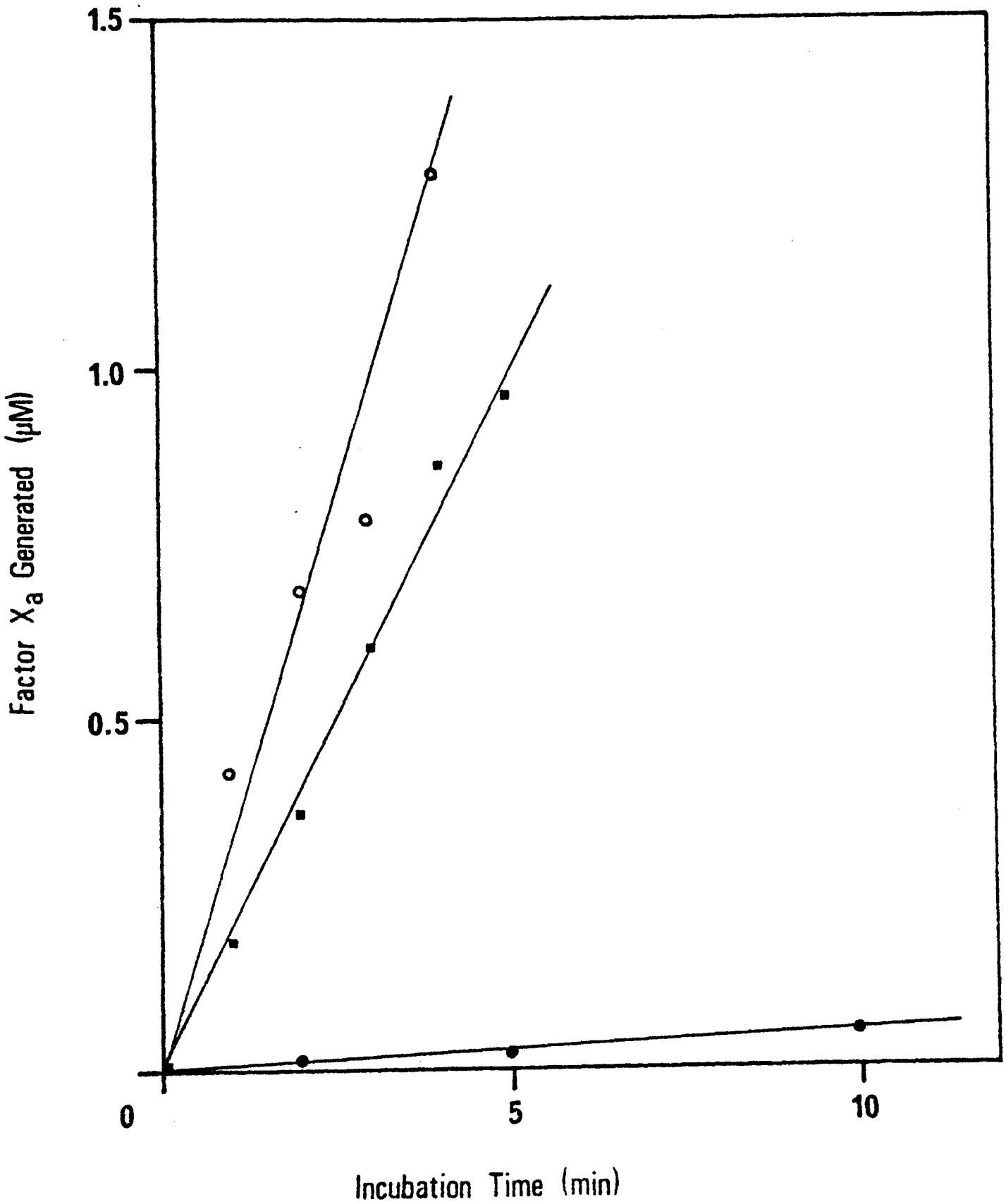
b) Factor $IX_{a\beta}$ and Factor VIII:C

Factor VIII:C (units/ml)	Rate of Reaction (moles X_a formed/min/mole IX_a)
0.0	0.025
1.0	0.95
5.0	1.56

2μM factor X_1 was incubated at 37°C with 0.2μM factor IX_a, 65μM 'cephalin', 10mM $CaCl_2$ and varying concentrations of 'partially purified factor VIII:C'. The factor X_a generated was assayed with BAEe.

FIGURE 5.10

Activation of Factor X by Factors $IX_{a\beta}$ and VIII:C



2μM factor X_1 was incubated at 37°C with 0.2μM factor $IX_{a\beta}$, 65μM 'cephalin', 10mM $CaCl_2$ and 0(●), 1(■) or 5(○) units/ml factor VIII:C. The factor X_a generated was assayed with BAEe.

TABLE 5.6

Effects of Calcium on Factor X Activation

$CaCl_2$ Concentration (mM)	Initial Rate of Reaction (pmoles X_a formed/ml/min)
0.0	0
0.5	32
1.0	48
2.0	78
5.0	110
10.0	136
20.0	220

2μM factor X_1 was incubated at 37°C with 0.2μM factor $IX_{a\alpha}$, 50μM 'cephalin', and 5 units/ml 'partially purified factor VIII:C', at different concentrations of calcium chloride. The factor X_a generated was assayed with BAEe.

Effects of Phospholipid on Factor X Activation

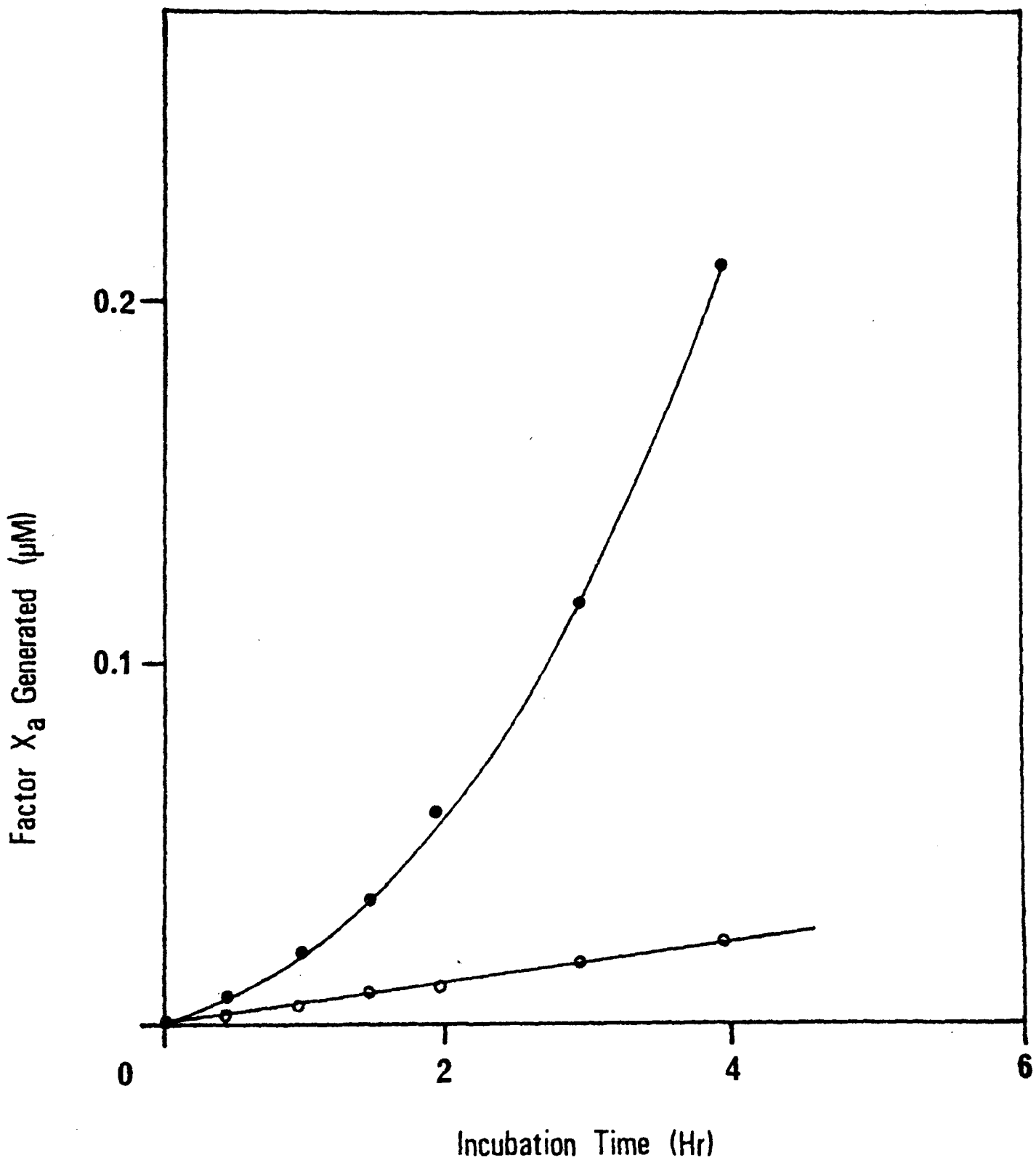
Analyses of the concentration of phospholipid in the proteins used in these experiments indicated that the factor IX_a and factor X were free from organic-extractable phosphorus; the 'partially purified factor VIII:C' preparations contained approximately 0.1nmole phospholipid per unit of factor VIII coagulant activity, and the less pure factor VIII:C preparations contained relatively higher levels of phospholipid. Thus, the addition of a factor VIII:C preparation to the activation mixtures also resulted in the addition of some phospholipid.

In the absence of factor VIII:C, the activation of factor X did not have an absolute requirement for phospholipid, but was very slow in mixtures containing no lipid. Only 0.2pmoles factor X_a/ml/minute was formed when 1μM factor X_1, 1μM factor $IX_{a\beta}$ and 0.01M calcium chloride were incubated at 37°C. This rate was approximately forty times slower than that seen in similar mixtures containing 40μM 'cephalin'.

The effects of widely varying concentrations of phospholipid - in this case 'cephalin' - on the activation of factor X by factor IX_a are shown in Figure 5.11. At 'cephalin' concentrations at or below the apparent optimum level, the rate at which factor X_a was formed did not change during the incubation, even when this was continued for up to four hours. When 'cephalin' concentrations above the optimum were present in the activation mixture, however, factor X was activated relatively slowly in the early stages of the reaction, and then the rate gradually increased with increasing time.

FIGURE 5.11

Effects of 'Cephalin' on the Activation of Factor X



2μM factor X_1 was incubated at 37°C with 0.25μM factor $IX_{a\beta}$, 10mM CaCl and 7 (○), or 170 (●) μM 'cephalin'. The factor X_a generated was assayed with S-2222.

Hence, the initial rates of activation of factor X quoted in Table 5.7 refer only to the rate of the reaction during the early period of the incubation and do not take into account any subsequent increase in the rate at which factor X was activated in the presence of high concentrations of phospholipid.

The effect of phospholipid on the activation of factor X by factor IX_a and VIII:C was dependent upon its composition as well as its concentration. The rate at which factor X_a was generated in the presence of different concentrations of crude 'cephalin' is shown in Table 5.8, while the effect of pure phosphatidyl serine is indicated in Table 5.9. In the former case, there was clearly an optimum concentration of 'cephalin', above which the initial rate of the reaction was inhibited, but this did not occur with phosphatidyl serine, even when it was present at levels five-fold higher than the optimum 'cephalin' concentration.

Effects of Thrombin and Hirudin on Factor X Activation

Neither thrombin nor its specific inhibitor, hirudin, affected the rate of activation of factor X by factor $IX_{a\alpha}$ or $IX_{a\beta}$ in the absence of factor VIII:C. Thrombin was added to the activation mixtures to a final concentration of 0.1iu/ml, which was sufficiently low for its esterase activity not to interfere with the factor X assays, even when BAEe was employed as the substrate. The final concentration of hirudin in the activation mixtures was 70 antithrombin units

TABLE 5.7

Effects of Phospholipid on Factor X Activation

'Cephalin' (μM)	Initial Rate of Reaction (pmoles factor X_a formed/ml/min)
20	2.6
40	4.0
65	4.6
130	3.8
200	2.4

1μM factor X_1 was incubated at 37°C with 0.5μM factor $IX_{a\beta}$ and 0.01M $CaCl_2$, in the presence of different concentrations of 'cephalin'. The factor X_a generated was assayed with S-2222.

TABLE 5.8

Effects of 'Cephalin' on the Activation of Factor X by Factors IX_a and VIII:C

'Cephalin' Concentration (μM)	Initial Rate of Reaction (pmoles X_a formed/ml/min)
0	34
30	112
65	198
130	208
200	118
330	52

2μM factor X_1 was incubated at 37°C with 0.2μM factor $IX_{a\beta}$, 1 unit/ml 'partially purified factor VIII:C' and 0.01M $CaCl_2$, in the presence of various concentrations of 'cephalin'. The factor X_a generated was assayed with BAEe.

TABLE 5.9

Effects of Phosphatidyl Serine on the Activation of Factor X by Factors IX_a and VIII:C

Phosphatidyl Serine Concentration (μM)	Total Phospholipid* (μM)	Initial Rate of Reaction (pmoles X_a formed/ml/min)
0	5	120
45	50	140
130	135	160
265	270	200
530	535	230
800	805	300

*Including endogenous phospholipid in Factor VIII preparation

1μM factor X_1 was incubated at 37°C with 0.1μM factor $IX_{a\alpha}$, 5 units/ml factor VIII:C (of low specific activity containing 1nmole phospholipid per unit factor VIII:C) and 0.01M $CaCl_2$ in the presence of various concentrations of phosphatidyl serine. The factor X_a generated was assayed with BAEe.

(atu) per ml, representing a large excess over the levels of the coagulation factors employed in these experiments; this concentration of hirudin had no effect on the esterase activity of factor X_a.

The effect of thrombin on the activation of factor X by factors IX_a and VIII:C was dependent upon the concentration of the other proteins. In the case of mixtures containing 5 units/ml 'partially purified factor VIII:C', together with 2μM factor X_1 and 0.2μM factor $IX_{a\alpha}$, the addition of thrombin at a final concentration of 0.1iu/ml had no effect on the rate at which factor X_a was generated. The addition of 'partially purified factor VIII:C', which had been pretreated with bovine thrombin, to mixtures containing 2μM factor X_1 and 0.2μM factor $IX_{a\beta}$ resulted in a slower rate of activation of factor X than that observed with unactivated factor VIII:C (Table 5.10). In contrast, adding lower concentrations of factor VIII:C, pretreated with thrombin, to mixtures containing 80nM factor X_1 and 1nM factor $IX_{a\beta}$ produced an enhanced rate of activation of factor X in comparison with that seen with unactivated factor VIII:C (Table 5.11). Prolonged incubation of the 'partially purified factor VIII:C' with thrombin again led to a fall in the rate at which factor X_a was formed in the activation mixture.

The principal effect of hirudin on the activation of factor X by factors IX_a and VIII:C was to reduce the rate of formation of factor X_a in the early stages of the reaction, giving rise to an initial 'lag' period, as shown in Figure 5.12. In the presence of hirudin, the initial rate of form-

TABLE 5.10

Effects of Thrombin on the Activation of Factor X by Factors IX_a and VIII:C, I.

Period of Preincubation (Minutes)	Initial Rate of Reaction (pmoles X_a formed/ml/minute)
Untreated factor VIII:C	192
2	124
5	108
10	52
90	14

12 u/ml 'partially purified factor VIII:C' was incubated at 37°C with 0.2 iu/ml thrombin for the required time. Aliquots were then diluted tenfold into mixtures containing factor X_1 (final concentration 2μM), factor $IX_{a\beta}$ (0.2μM), 'cephalin' (65μM) and 0.01M $CaCl_2$. The factor X_a formed was assayed with BAEe.

TABLE 5.11

Effects of Thrombin on the Activation of Factor X by Factors IX_a and VIII:C, II.

Period of Preincubation (minutes)	Initial Rate of Reaction (pmoles X_a formed/ml/minute)
0	4.0
1	7.0
2	4.4
4	3.2
6	3.8
8	1.2
10	1.6
15	1.0

5 u/ml 'partially purified factor VIII:C' was incubated at 37°C with 0.5 iu/ml bovine thrombin for the required time. Aliquots were then diluted twenty-five-fold into mixtures containing factor X_1 (final concentration 80nM), factor $IX_{a\beta}$ (1nM), 'cephalin' (10μM) and 0.01M $CaCl_2$. The factor X_a generated was assayed with S-2222.

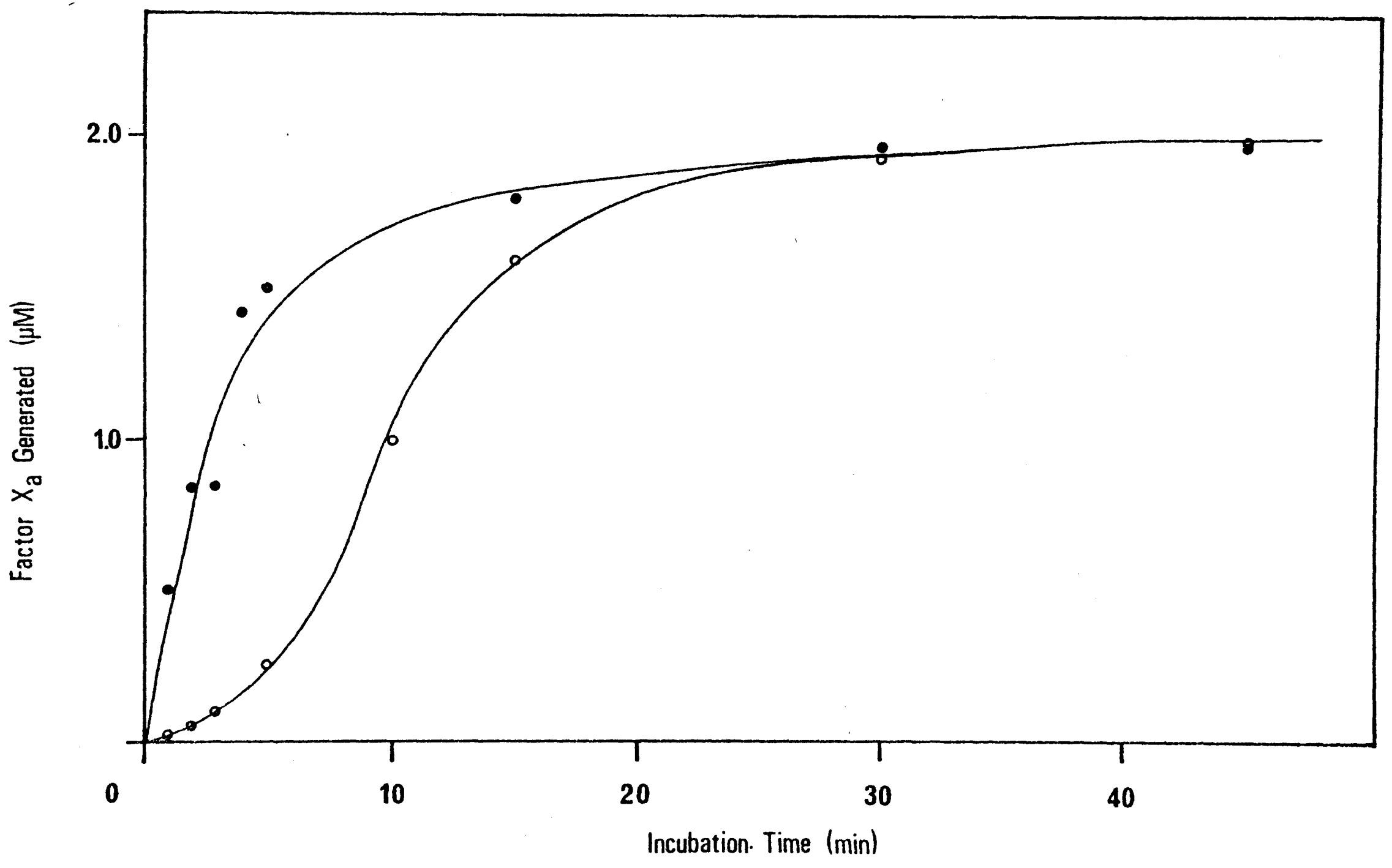
FIGURE 5.12

Effects of Hirudin on Factor X Activation

2μM factor X_1 was incubated at 37°C with 0.2μM factor $IX_{a\beta}$, 8 units/ml 'partially purified factor VIII:C', 65μM 'cephalin' and 10mM $CaCl_2$ in the presence (○) or absence (●) of 70 atu/ml hirudin. The factor X_a generated was assayed with BAEe.

FIGURE 5.12

Effects of Hirudin on Factor X Activation



ation of factor X_a was 30pmoles/ml/minute compared with the 500pmoles factor X_a generated per ml per minute in identical mixtures containing no hirudin. The presence of hirudin did not, however, affect the final concentration of factor X_a which was generated in the activation mixtures.

DISCUSSION

It is now widely believed that factor IX_a is the enzyme responsible for the activation of factor X and that factor VIII:C is a high molecular weight cofactor in the reaction. There has, however, been little unequivocal evidence that factor IX_a can activate factor X in the absence of factor VIII:C. Studies using coagulation assays (Davie *et al*, 1975; Irwin *et al*, 1975) suggested that this reaction can occur, but the low specificity of such assays make it desirable that the conclusions drawn should be confirmed by an alternative procedure. Further evidence that factor IX_a is capable of a direct action on factor X has recently been provided by Suomela *et al* (1977) and van Dieijen *et al* (1981), who used a chromogenic peptide substrate to assay factor X_a, and by Hultin and Nemerson (1978), who utilised an assay based on the release of a tritium-labelled activation peptide from factor X.

The results described in this Chapter confirm that either factor $IX_{a\alpha}$ or factor $IX_{a\beta}$ can activate factor X, provided that a sufficiently high concentration of the enzyme is used. The use of low levels of factor IX_a to activate factor X frequently resulted in there being an initial lag period in the reaction before a linear rate became established. There was a strong indication that the occurence of such initial lag periods was dependent not only upon the factor IX_a concentration itself but also on the ratio of the concentrations of protein and phospholipid. An initial

lag period in the activation has also been described by Suomela *et al* (1977) and by Hultin and Nemerson (1978). At high concentrations of factor IX_a, the rate of factor X_a formation was linear with no indication of an initial lag period.

Different authors have reported a variable effect of factor VIII:C on the activation of factor X. A variety of techniques have been employed for the purification of factor VIII:C, however, and the resulting preparations have had different specific activities and characteristics, which might account for some of the inconsistencies in the published results. Davie *et al* (1975), for example, reported that the activation of factor X could be stimulated several thousandfold, whereas Suomela *et al* (1977) observed only a fivefold increase in the initial rate of the reaction.

The results of the present work show that a 'partially purified factor VIII:C' preparation stimulates the rate of activation of factor X by either form of factor IX_a. The degree of stimulation observed is profoundly influenced by the concentration of factor IX_a in the activation mixture: the higher the factor IX_a concentration, the more rapidly the reaction proceeds in the absence of factor VIII:C and the less the rate is enhanced by factor VIII:C. Suomela *et al* (1977) have claimed that the major effect of factor VIII:C is to reduce the initial lag period during the activation of factor X. The present results confirm that the initial lag seen during the activation of factor X by low concentrations of factor IX_a is abolished by the presence of 'partially purified

factor VIII:C' in the activation mixtures. In general, however, the principal effect of factor VIII:C appears to be to stimulate the rate at which factor X is activated.

In general, no more than 30% of the factor X in the activation mixtures was converted into factor X_a, as measured by the esterase assays, in the absence or presence of 'partially purified factor VIII:C'. The SDS-polyacrylamide gels, in contrast, indicated that very little uncleaved factor X_α remained in the mixtures. No bands corresponding to possible products other than factor X_β and factor X_a appeared on the gels, suggesting that any such products were probably of low molecular weight.

The SDS-polyacrylamide gels also indicate that factor IX_a can be degraded by factor X_a. Clearly, this reaction could be a major cause of the declining rate of formation of factor X_a which was seen when the activation mixtures were incubated at 37°C for prolonged periods. The change in rate may also reflect the apparent conversion of factor X into low molecular weight fragments.

Preliminary studies in this laboratory (S J Walter, personal communication) using the release of a tritiated activation peptide to follow the conversion of factor X to X_a (Silverberg *et al*, 1977; Hultin and Nemerson, 1978) confirm that factor IX_a will activate factor X in the presence of calcium ions and phospholipid, and that the rate of the reaction is greatly stimulated by the presence of 'partially purified factor VIII:C'.

The interpretation of the role of factor VIII:C in the

activation of factor X is further complicated by the phenomenon of 'activation' of factor VIII:C by thrombin (Rapaport *et al*, 1963) or factor X_a (Davie *et al*, 1975). The physiological significance of the transient enhancement of the factor VIII coagulant activity by thrombin is unknown, although Hultin and Nemerson (1978) suggested that unactivated factor VIII:C had only a slight effect on the activation of factor X by factor IX_a while thrombin-treated factor VIII:C enhanced the rate of the reaction five hundredfold.

The addition of thrombin to activation mixtures containing 5 units/ml 'partially purified factor VIII:C' did not affect the rate at which factor X_a was formed. That is, the factor VIII:C preparation used in the experiments did not require treatment with thrombin in order for it to enhance the activation of factor X. The pretreatment of the 'partially purified factor VIII:C' with thrombin before it was added to the activation mixtures at a concentration equivalent to 1.2 units/ml for the unactivated material resulted only in a decline in the rate of activation of factor X as the period of exposure of the factor VIII:C preparation to thrombin was prolonged. The use of 'partially purified factor VIII:C' at concentrations equivalent to 0.2 units/ml for the unactivated material, in activation mixtures containing lower concentrations of factors IX_a and X, did, however, result in an increased rate of activation of the factor X when the factor VIII:C preparation was exposed to thrombin for a brief period prior to its addition to the other components of the mixture. Neither Varadi and Hemker (1976) nor Suomela *et al* (1977)

indicated that it was necessary to 'activate' their factor VIII preparations before using the material in studies on the activation of factor X, whereas both Hultin and Nemerson (1978) and van Dieijen *et al* (1981) found it necessary to pretreat factor VIII with thrombin. In the light of the present work it is conceivable that the factor VIII:C concentration may play an important part in determining whether it is necessary for a thrombin 'activation' of the coagulant activity to precede its participation in the production of factor X_a. High concentrations of factor VIII:C appear to 'saturate' the system, and under such conditions neither an increase in the actual concentration of factor VIII:C protein nor an increase in activity caused by 'activation' with thrombin has a significant effect on the overall rate of the reaction. Where lower, possibly rate-limiting, concentrations of factor VIII:C are present, thrombin 'activation' can produce an enhancement of the rate of activation of factor X.

The addition of hirudin reduced the rate at which factor X_a was generated in activation mixtures containing both factor IX_a and 'partially purified factor VIII:C', but had no effect in the absence of the latter. An inhibition by hirudin of the rate at which factors IX_a and VIII:C activate factor X has been reported previously (Pitlick *et al*, 1969; Brown *et al*, 1980) but the effect was attributed to a direct action of the inhibitor on factor IX_a. This seems unlikely to be the case in view of the lack of inhibition by hirudin of the activation of factor X by factor IX_a in the absence of factor VIII:C, which would appear to rule out any direct effect of the inhibi-

tor on factor IX_a or factor X. The cause of the inhibition by hirudin of the rate at which factors IX_a and VIII:C activate factor X is not clear. It is possible that this effect is a reflection of the inhibition of endogenous contaminant thrombin in the activation mixture, but this is difficult to reconcile with the absence of any effect observed when thrombin was added to mixtures containing similar concentrations of the proteins. The possibility also exists that hirudin might have a direct effect on the 'partially purified factor VIII:C' itself.

Reactions involving the vitamin K-dependent coagulation factors invariably require calcium ions, which are bound by the γ-carboxyglutamic acid residues (Esnouf, 1977), and the activation of factor X conforms to this pattern. The results of the experiments described in this Chapter confirmed that the activation of factor X requires calcium ions and that the cation concentration influences the rate of the reaction. The dependence on calcium ions was seen in the absence and presence of 'partially purified factor VIII:C' and it was therefore impossible to determine whether the interaction of factor VIII:C with the other coagulation factors itself requires calcium ions, or whether the calcium dependence of the reaction in the presence of 'partially purified factor VIII:C' merely reflects the requirements of the reaction in its absence.

Although it is generally believed that exogenous phospholipid is required for the activation of factor X, the experimental evidence for this is not well documented. Studies based

on coagulation assays (Davie *et al*, 1975; Irwin *et al*, 1975; Varadi and Hemker, 1976) do not provide a clear differentiation between the requirements of the reaction in which factor X is activated and the subsequent reaction in which factor X_a activates prothrombin. Studies using esterase assays to follow the activation of factor X (Kosow *et al*, 1974; Suomela *et al*, 1977; Brown *et al*, 1978; van Dieijen *et al*, 1981) have yielded contradictory results with regard to the effects of phospholipid on the reaction. This might be due to the presence of different quantities of lipid in the proteins utilised by the various groups, none of whom reported any details of the presence or absence of phospholipids in their proteins, although factor VIII preparations especially tend to contain some phospholipid. The discrepancies in the published results may also be due, in part, to the use by the different groups of a variety of types of phospholipid.

The results reported in this Chapter do not permit any firm conclusions about the requirements for phospholipid in the activation of factor X, principally because the 'partially purified factor VIII:C' contained some phospholipid. There was no requirement for phospholipid to be added to the reaction mixtures, even in the absence of factor VIII:C, but under these conditions the rate at which factor X was activated was increased markedly by the addition of crude 'cephalin'. Van Dieijen *et al* (1981) also found that factor IX_a would activate factor X only very slowly in solution. The addition of 5 units/ml 'partially purified factor VIII:C' to the activation mixtures resulted in less than 5μM endogenous phospholipid

being present. This level of phospholipid is considerably lower than the concentrations of exogenous phosphatidyl serine which led to an enhanced rate of activation of factor X both in the present study and those of Suomela *et al* (1977) and Brown *et al* (1978). It is, however, considerably higher than the concentrations of inosithin which Kosow *et al* (1974) found to have a major effect on the kinetics of the reaction, and similar to the concentrations of a phosphatidyl serine-phosphatidyl choline mixture employed in their kinetic studies by van Dieijen *et al* (1981). An extrapolation from the relatively modest increases in the rate of activation of factor X seen in the present study with large changes in the concentration of phosphatidyl serine or 'cephalin' suggests that a reduction from the 5μM endogenous phospholipid in the activation mixtures to a completely lipid-free system would not totally abolish the formation of factor X_a. That is, there does not appear to be an absolute requirement for phospholipid in the activation of factor X by factors IX_a and VIII:C.

The effects of phosphatidyl serine on the activation of factor X described in this Chapter are similar to those observed by Suomela *et al* (1977) who found no absolute requirement for added phospholipid in the activation of factor X by factors IX_a and VIII:C, but also found that inosithin stimulated the rate of the reaction. In contrast, Brown *et al* (1978) found that virtually no activation of factor X occurred in the absence of phospholipid, and that there was an optimum level of between 40 and 70μM phosphatidyl serine, above which the

rate of formation of factor X_a was inhibited. Similarly, van Dieijen *et al* (1981) found that high concentrations of their phosphatidyl serine-phosphatidyl choline mixture inhibited the activation of factor X as a result of an increase in the value of K_m for factor X in the system. These latter observations are more akin to the results obtained in the present study with 'cephalin', which had a markedly inhibitory effect at high concentrations both in the absence and presence of 'partially purified factor VIII:C'. These results, however, all await confirmation by a technique other than the measurement of the factor X_a esterase activity, since Hultin and Nemerson (1978) did not examine the effect of omitting phospholipid from their system using tritium-labelled factor X, and the studies using coagulation assays (Irwin *et al*, 1975; Davie *et al*, 1975) included phospholipid in the activation mixtures, largely because it is required in the later stages of the coagulation system.

The results presented in this Chapter are consistent with the following model for the activation of factor X. Factor IX_a will convert factor X to factor X_a at a very slow rate in solution, in a reaction with a requirement for calcium ions. Relatively high concentrations of the enzyme are required in order for the reaction to proceed at a detectable rate. The rate of the reaction is considerably enhanced by suitable phospholipids and by factor VIII:C. *In vivo*, therefore, where a maximum rate of activation of factor X is essential for efficient coagulation, it is necessary for the complete system, comprising factors IX_a and VIII:C, calcium

ions and phospholipid, to be present; blood coagulation is severely impaired in the absence of any component of the system which brings about the intrinsic activation of factor X.

Chapter 6

THE KINETICS OF THE ACTIVATION OF FACTOR X

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THE KINETICS OF THE ACTIVATION OF FACTOR X

INTRODUCTION

The activation of factor X by the components of the intrinsic pathway is an enzyme-catalysed process. The complexity of the system, which includes five distinct components - factors X, IX_a, and VIII:C, phospholipid and metal ions - has, however, discouraged detailed studies of the kinetics of the reaction. Varadi and Hemker (1976) used clotting assays to examine the kinetics of the formation of the 'factor X activating enzyme', that is, the complex of factors IX_a and VIII:C with calcium ions and phospholipid, which they termed 'tenase'. As these workers were using clotting assays they were not able to carry out a rigorous kinetic analysis of the actual activation reaction.

The activation of human and bovine factor X by the coagulant enzyme from *Vipera russellii* venom (RVV-X) has been examined by Kosow (1976) and Kosow *et al* (1974) respectively. A brief investigation of the kinetics of factor X activation by the intrinsic coagulation system was included in the latter report, but the effect of factor VIII:C on the reaction was not examined.

A study by Silverberg *et al* (1977) of the kinetics of the activation of factor X, using the release of a tritium-labelled activation peptide to follow the generation of factor X_a, was confined to the extrinsic coagulation system.

A preliminary examination of the kinetics of the reaction was included by Brown *et al* (1978) in their study

of the generation of factor X_a by the intrinsic coagulation system. Only a single concentration of factor IX_a and factor VIII:C was employed by these workers, whose major interest was in the possible inhibition of the activation of factor X by high concentrations of substrate.

Thus, when the present study was begun, little data had been published concerning the kinetics of the activation of factor X by any mechanism, and figures regarding the intrinsic coagulation system were especially scanty. A study of the role of phospholipid and factor VIII:C in the activation of factor X has been published very recently (van Dieijen *et al*, 1981) and the values for the kinetic constants derived by this group provide an interesting comparison with the values obtained in the present work.

MATERIALS AND METHODS

Preparation of Clotting Factors

Factors X_1 and $IX_{a\beta}$, and 'partially purified factor VIII:C' were prepared from bovine plasma as described in Chapter 5. Only factor VIII:C preparations with specific activities of approximately 100 units/mg were employed in these studies. These preparations contained phospholipid, at a concentration of 0.1 nanomoles per unit of factor VIII:C, whereas the factor X_1 and factor $IX_{a\beta}$ contained no detectable phospholipid.

Preparation of Phospholipid

A stock suspension of 'lecithin' was prepared by dissolving 100mg Centrolex P lecithin (Central Soya, Chicago, Illinois, USA) in 10ml diethyl ether and adding 10ml 0.025M tris-HCl, pH7.5. The ether was then removed by passing nitrogen through the solution at 37°C and the volume was restored to 10ml with 0.025M tris-HCl, pH7.5. The suspension was sonicated for one minute (MSE Ultrasonic Power Unit), and stored at 4°C under nitrogen.

Assays of Factor X Activation

The activation of factor X was carried out at 37°C in polyethylene tubes, following the procedure described in Chapter 5. In all cases, the factor X_a generated was assayed with S-2222.

Kinetic Analysis

Three series of experiments were carried out: in the first series, factor X_1 was activated with factor $IX_{a\beta}$, phospholipid and calcium chloride; in the second and third series, the activation was carried out in the presence of 'partially purified factor VIII:C' at concentrations of 0.4 units/ml and 2.3 units/ml respectively.

In each series, the activation was carried out at several different concentrations of factor X_1, while the concentrations of the other components of the system were the same in each mixture. Three or four independent replicate mixtures were made up for each concentration of factor X and the mean rate of formation of factor X_a was determined for use in the calculation of the kinetic parameters. In each case, the 'initial rate' of activation of factor X was determined from a plot of the factor X_a concentration against time during the initial phase of the reaction when the rate was linear. No attempt was made to extrapolate the graphs back through the origin: this would not appear to be valid in several cases where there was a strong indication of an initial 'lag period' during which the activation of factor X was very slow.

The 'initial rates' determined as described above were used to construct Lineweaver-Burk plots from which the kinetic constants K_m and V_{max} were calculated. Although it is usually stated that the Michaelis-Menten equation, and hence this method of calculating K_m and V_{max}, is valid only when the enzyme concentration is very small compared with

the original substrate concentration, Chaplin (1981) has recently shown that the relationship is also valid if the enzyme concentration is small compared with K_m, even if it is similar to or greater than the original substrate concentration. In this case, all of the data obtained in the present work can be analysed in terms of the Michaelis-Menten equation.

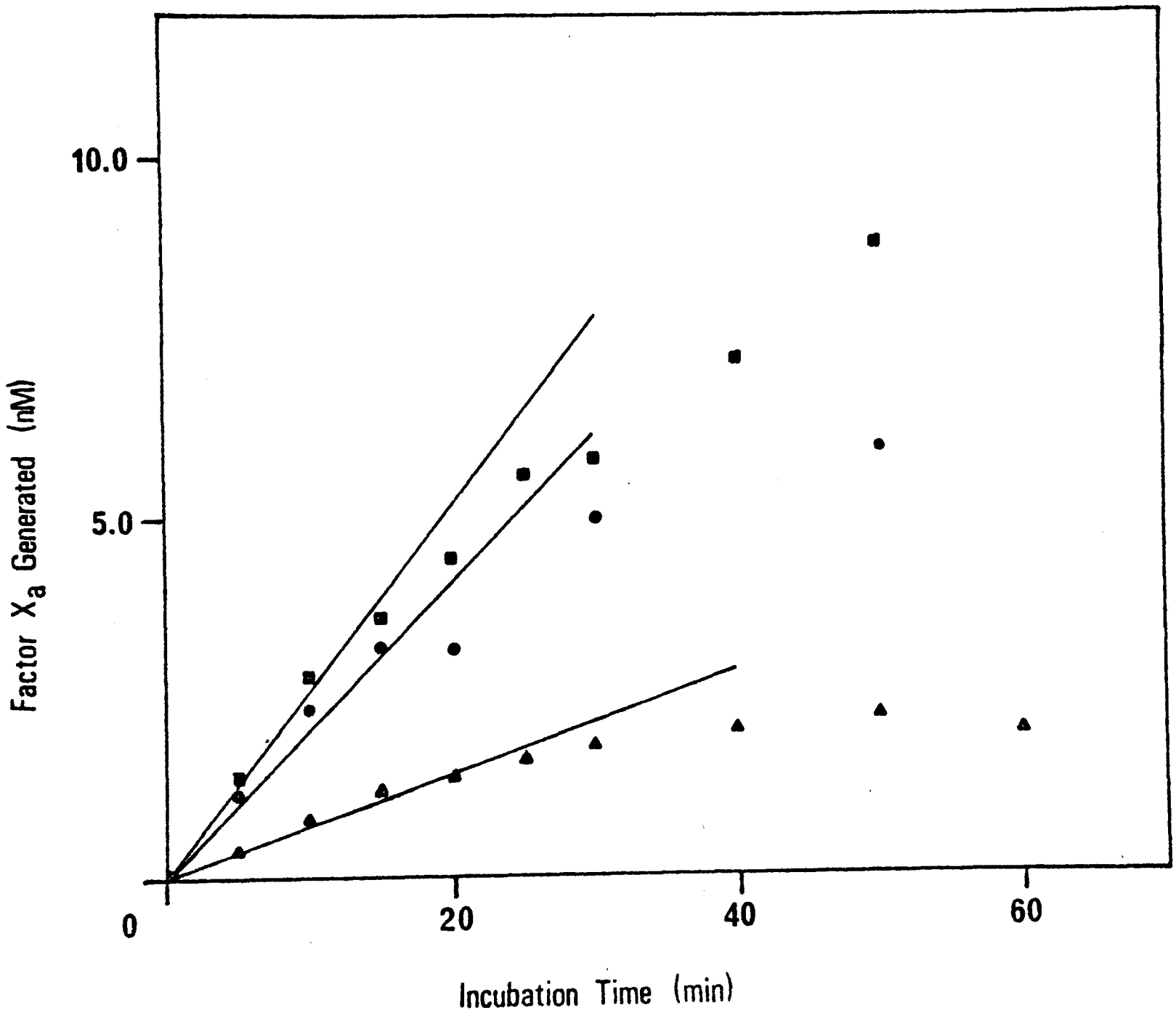
RESULTS

Activation of Factor X_1 in the Absence of Factor VIII:C

In the absence of factor VIII:C, factor X_1 was activated by incubation at 37°C with 50nM factor $IX_{a\beta}$, 13μM 'lecithin' and 10mM calcium chloride. Figure 6.1 shows the formation of factor X_a in mixtures containing several concentrations of factor X_1. The 'initial rates' of the reaction in these mixtures are listed in Table 6.1, and were used for the Lineweaver-Burk plot shown in Figure 6.2. The initial rate of the reaction appears to be slower in mixtures containing 3.0μM factor X than in mixtures where the factor X concentration is only 1.5μM. Extrapolating from the data obtained at lower concentrations of factor X, values for K_m and V_{max} in this system of 0.74μM factor X and 7.69 millimoles factor X_a formed per minute per mole of factor IX_a respectively can be calculated from the intercepts on the axes of the graph.

FIGURE 6.1a

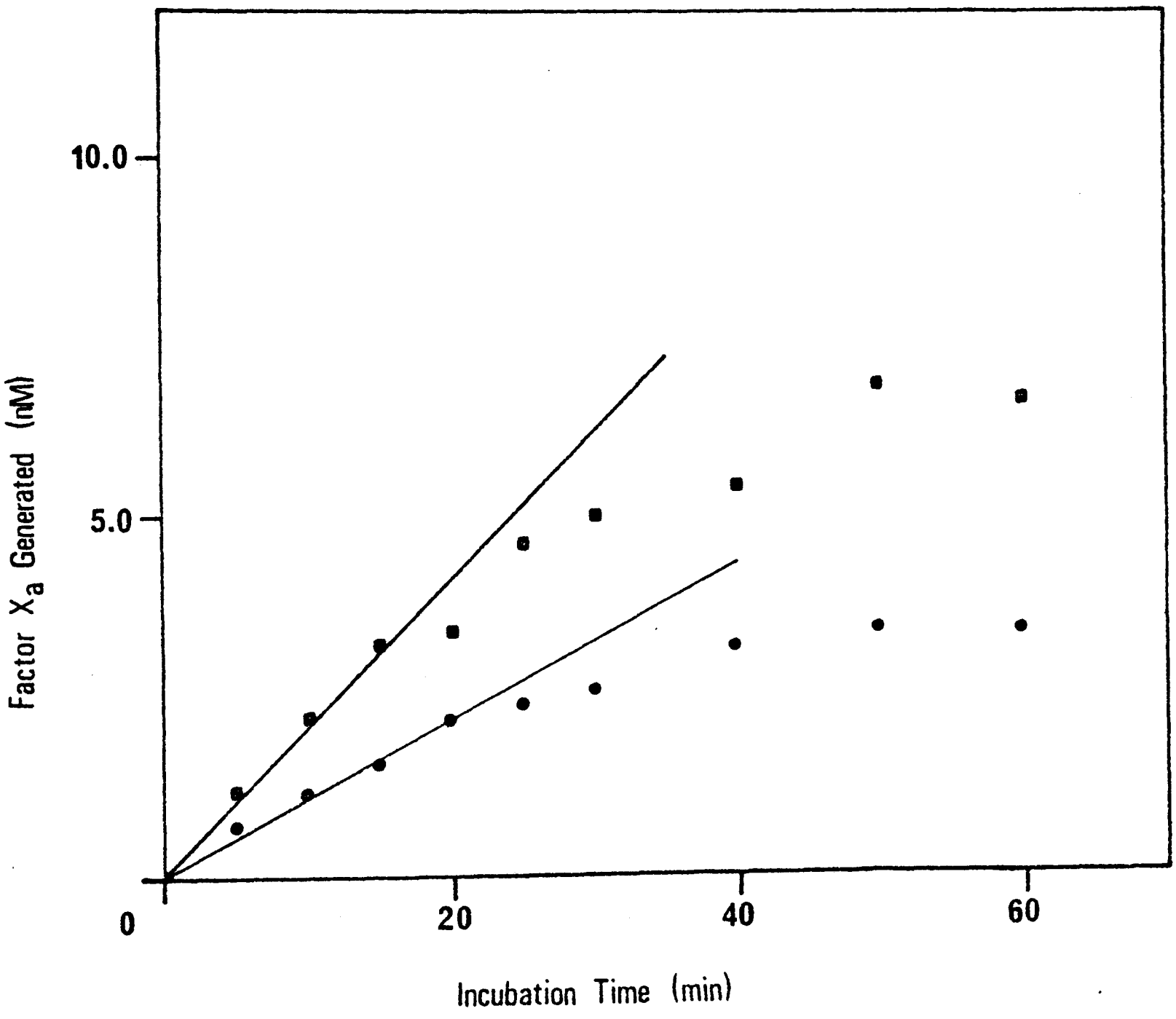
Activation of Factor X by Factor $IX_{a\beta}$



3.0 (●), 1.5 (■) or 0.15 (▲) μM factor X_1 was incubated at 37°C with 50nM factor $IX_{a\beta}$, 13μM 'lecithin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222.

FIGURE 6.1b

Activation of Factor X by Factor $IX_{a\beta}$



0.75 (■) or 0.30 (●) μM factor X_1 was incubated at 37°C with 50nM factor $IX_{a\beta}$, 13μM 'lecithin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222.

TABLE 6.1

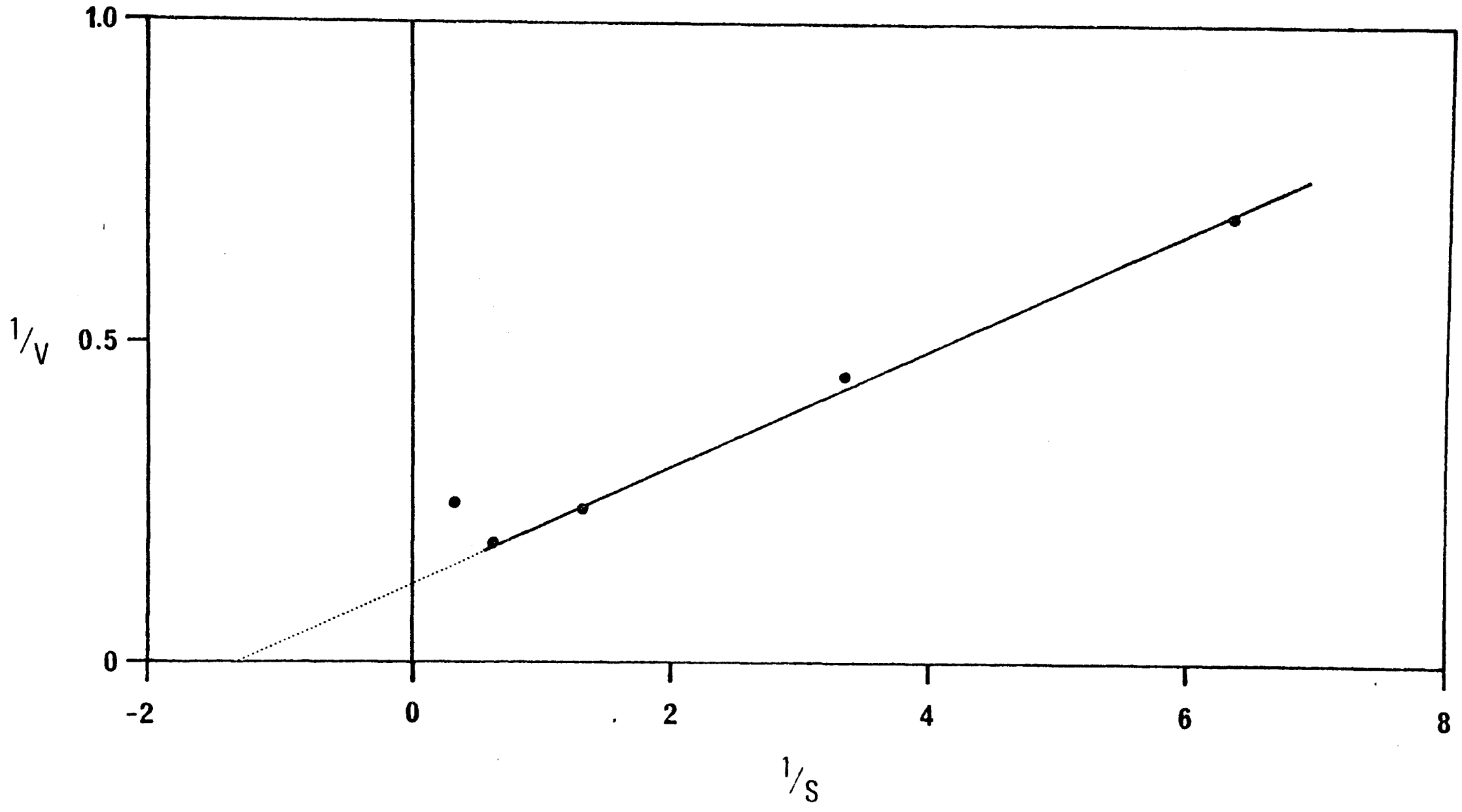
Activation of Factor X by Factor $IX_{a\beta}$

Factor X Concentration (μM)		Initial Rate of Reaction (pmoles X_a/ml/min)	(mmoles X_a/mole IX_a/min)	
's'	$^1/s$		'v'	$^1/v$
3.0	0.33	0.20	4.0	0.25
1.5	0.67	0.26	5.2	0.19
0.75	1.33	0.21	4.2	0.24
0.30	3.33	0.11	2.2	0.45
0.15	6.67	0.07	1.4	0.70

Factor X_1, at the stated concentrations, was incubated at 37°C with 0.05μM factor $IX_{a\beta}$, 13μM 'lecithin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222.

FIGURE 6.2

Lineweaver-Burk Plot of Factor X Activation by Factor $IX_{a\beta}$ in the Absence of Factor VIII:C



Activation of Factor X_1 in the Presence of 0.4 units Factor VIII:C per Millilitre

Factor X_1 was incubated at 37°C with 10nM factor $IX_{a\beta}$, 0.4 units/ml 'partially purified factor VIII:C', 1.3μM 'lecithin' and 10mM calcium chloride, and the concentration of factor X_a was measured with S-2222 as the substrate. The 'initial rates' of the reaction at various concentrations of factor X were determined from plots of factor X_a concentration against time, and are listed in Table 6.2. The data are presented in the form of a Lineweaver-Burk plot in Figure 6.3. This shows no indication of any inhibition of the reaction rate by high concentrations of substrate, but the highest factor X concentration examined in this series was only 3.4μM. The intercepts on the axes of the graph indicate values for K_m and V_{max} in this system of 0.26μM factor X and 1.2 moles factor X_a formed per minute per mole of factor IX_a respectively.

TABLE 6.2

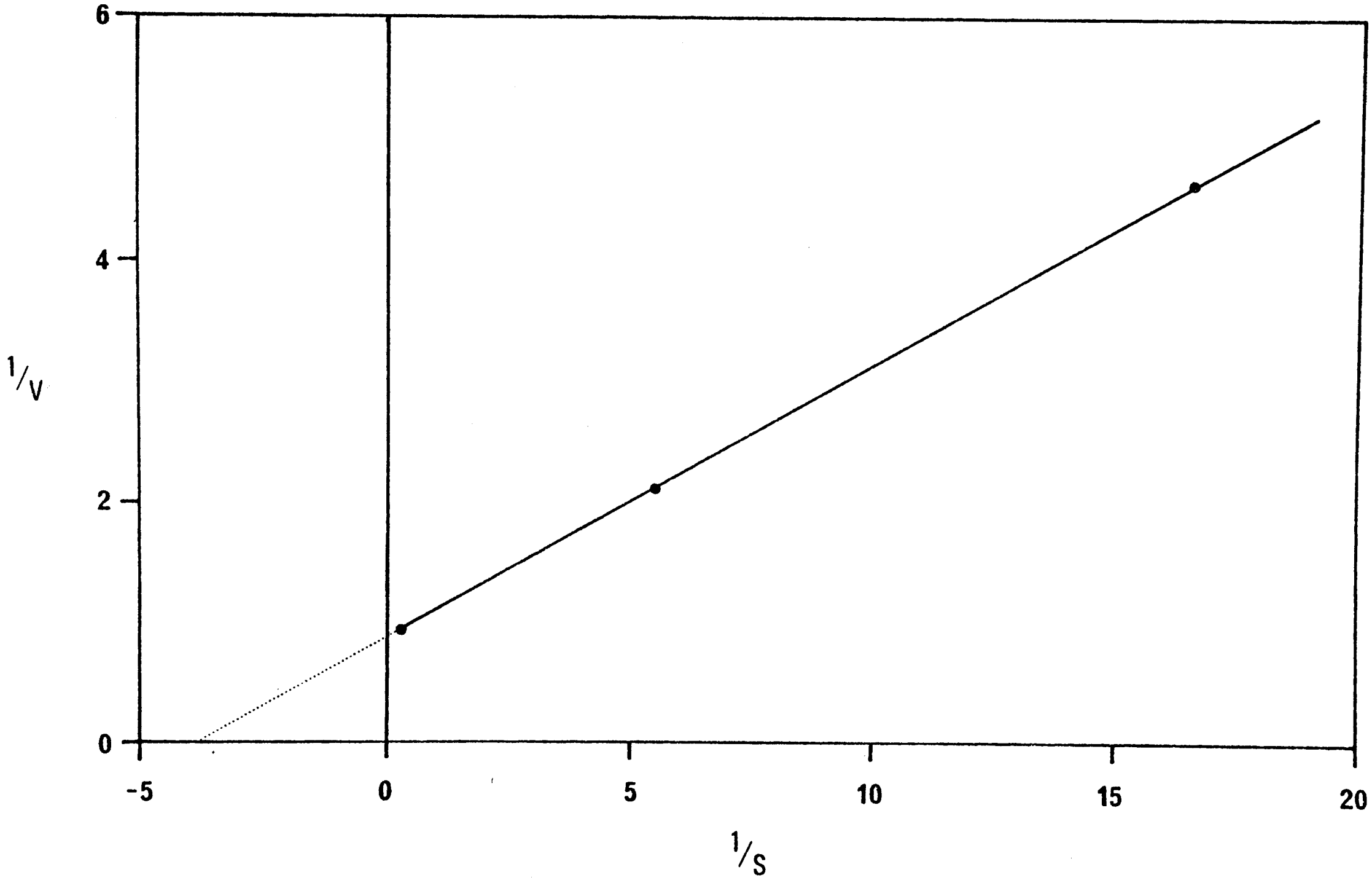
Activation of Factor X by Factors $IX_{a\beta}$ and VIII:C, I.

Factor X Concentration (μM)		Initial Rate of Reaction (pmoles X_a/ml/min) | (moles X_a/mole IX_a/min)		
's'	$^1/s$		'v'	$^1/v$
3.4	0.29	10.9	1.09	0.92
0.18	5.55	4.8	0.48	2.10
0.06	16.7	2.2	0.22	4.63

Factor X_1, at the stated concentrations, was incubated at 37°C with 0.01μM factor $IX_{a\beta}$, 0.4 units/ml 'partially purified factor VIII:C', 1.3μM 'lecithin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222.

FIGURE 6.3

Lineweaver-Burk Plot of Factor X Activation by Factor $IX_{a\beta}$ in the Presence of 0.4 units/ml Factor VIII:C



Activation of Factor X_1 in the Presence of 2.3 units Factor VIII:C per Millilitre

Factor X_1 was incubated at 37°C with 10nM factor $IX_{a\beta}$, 2.3 units/ml 'partially purified factor VIII:C', 1.3μM 'lecithin' and 10mM calcium chloride; the use of 'partially purified factor VIII:C' at this concentration resulted in the overall phospholipid concentration in the activation mixture being 1.5μM. S-2222 was used as the substrate for the assay of the factor X_a generated, and the 'initial rate' of the reaction was determined by plotting the concentration of factor X_a against time. The 'initial rates' are listed in Table 6.3 and presented as a Lineweaver-Burk plot in Figure 6.4. This graph suggests that concentrations of factor X greater than approximately 3μM may inhibit the activation. Extrapolation from the data obtained at lower concentrations of factor X indicates values for K_m and V_{max} in this system of 0.50μM factor X and 2.9 moles factor X_a formed per minute per mole of factor IX_a respectively.

The kinetic constants determined for the activation of factor X_1 by factor $IX_{a\beta}$ in the presence and absence of 'partially purified factor VIII:C' are summarised in Table 6.4.

TABLE 6.3

Activation of Factor X by Factors $IX_{a\beta}$ and VIII:C, II

Factor X Concentration (μM)		Initial Rate of Reaction (pmoles X_a/ml/min) | (moles X_a/mole IX_a/min)		
's'	$^1/s$		'v'	$^1/v$
9.09	0.11	15.5	1.55	0.65
3.45	0.29	24.3	2.43	0.41
0.91	1.10	22.5	2.25	0.44
0.18	5.55	11.8	1.18	0.85
0.09	11.0	3.6	0.36	2.75
0.06	16.7	2.95	0.30	3.38
0.02	55.5	1.02	0.10	9.78

Factor X_1, at the stated concentrations, was incubated at 37°C with 0.01μM factor $IX_{a\beta}$, 2.3 units/ml 'partially purified factor VIII:C' 1.3μM 'lecithin' and 10mM $CaCl_2$. The factor X_a generated was assayed with S-2222.

FIGURE 6.4

Lineweaver-Burk Plot of Factor X Activation by Factor $IX_{a\beta}$ in the Presence of 2.3 units/ml Factor VIII:C

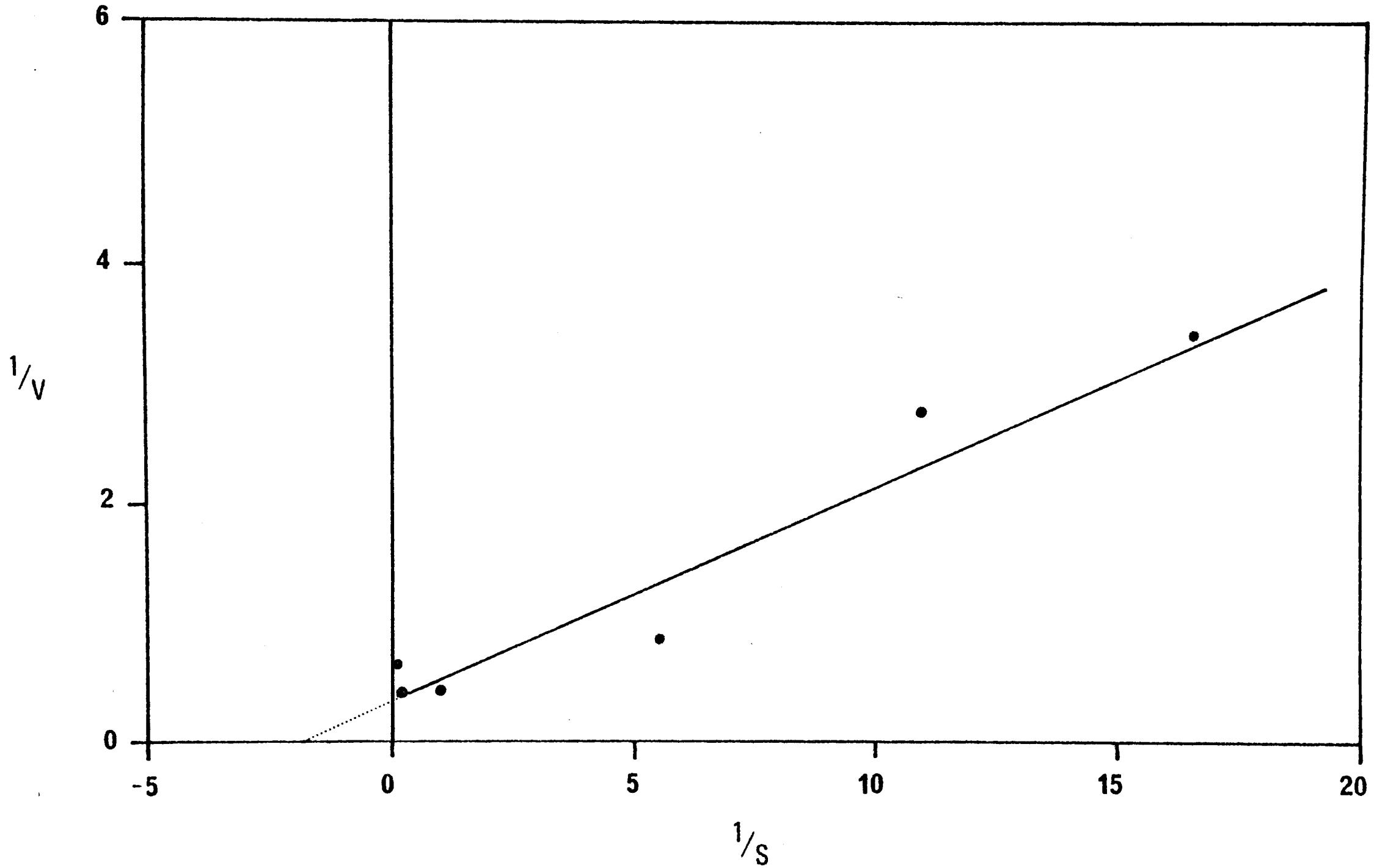


TABLE 6.4

Kinetic Constants for the Intrinsic Activation of Factor X

Activating System	K_m (μM)	V_{max} (moles X_a/min/mole IX_a)
$IX_{a\beta}$, 'lecithin' (13μM), $CaCl_2$	0.74	7.7×10^{-3}
$IX_{a\beta}$, 'lecithin' (1.3μM), $CaCl_2$, VIII:C (0.4 units/ml)	0.26	1.2
$IX_{a\beta}$, 'lecithin' (1.3μM), $CaCl_2$, VIII:C (2.3 units/ml)	0.50	2.9

DISCUSSION

The kinetics of the activation of factor X by the intrinsic or extrinsic coagulation pathways, or by RVV-X, have been investigated by Kosow *et al* (1974), Kosow (1976), Silverberg *et al* (1977), Brown *et al* (1978) and van Dieijen *et al* (1981). The data obtained by these groups are summarised in Table 6.5.

In solution, that is, in the absence of a phospholipid surface, the effect of calcium on the activation of factor X appears to depend on the enzyme which is used. Kosow (1976) found that the activation of factor X by RVV-X had an absolute requirement for calcium ions and that increasing concentrations of calcium increased the V_{max} of the reaction, exhibiting a co-operative effect, but had little effect on the K_m for factor X. In contrast, van Dieijen *et al* (1981) were able to activate factor X with factor IX_a in the absence of calcium ions, and found that the addition of 10mM calcium chloride to the activation mixture resulted in small changes in both K_m and V_{max}. Thus, it would appear that the activation of factor X in solution by factor IX_a may have a more complex mechanism than the activation by RVV-X, with calcium ions having several effects in the former case.

The values given in Table 6.5 indicate that the K_m for the activation of factor X by the complete intrinsic or extrinsic coagulation systems is in the range from 0.06μM to 0.5μM. The K_m values of 0.26μM and 0.50μM at different concentrations of 'partially purified factor VIII:C' obtained

TABLE 6.5

Published Kinetic Constants for the Activation of Factor X

Activating System	K_m (μM)	V_{max} (moles X_a/min/mole enzyme)	Reference
RVV-X (Human Factor X)	0.01	-	(Kosow, 1976)
RVV-X (Bovine Factor X)	0.25	-	(Kosow *et al*, 1974)
αVII_a, 'cephalin', $CaCl_2$	4.9	2.4×10^{-2}	(Silverberg *et al*, 1977)
αVII_a, Tissue Factor, $CaCl_2$	0.34	2.0×10^{3}	(Silverberg *et al*, 1977)
αVII_a, Tissue Factor, $CaCl_2$	0.20	-	(Brown *et al*, 1978)
IX_a	299	2.2×10^{-3}	(van Dieijen *et al*, 1981)
IX_a, $CaCl_2$	181	1.1×10^{-2}	(van Dieijen *et al*, 1981)
IX_a, Phospholipid, $CaCl_2$	0.058	2.5×10^{-3}	(van Dieijen *et al*, 1981)
IX_a, VIII:C, Phospholipid, $CaCl_2$	0.1	-	(Kosow *et al*, 1974)
IX_a, VIII:C, Phospholipid, $CaCl_2$	0.20	-	(Brown *et al*, 1978)
IX_a, VIII:C, Phospholipid, $CaCl_2$	0.063	5.0×10^{2}	(van Dieijen *et al*, 1981)

in the present study are within this range, but are slightly higher than those quoted by Kosow *et al* (1974), Brown *et al* (1978) and van Dieijen *et al* (1981) for the activation of factor X by the intrinsic coagulation system. Only van Dieijen *et al* (1981) examined the effect of different concentrations of factor VIII:C on the kinetics of the reaction: they found that increasing the concentration of factor VIII:C in the activation mixtures, while the factor IX_a and phospholipid concentrations were held constant, resulted in an increase in the K_m from 0.018μM factor X in the presence of 2 units/ml factor VIII:C to 0.063μM factor X with 11 units/ml factor VIII:C.

It was reported by both Kosow *et al* (1974) and van Dieijen *et al* (1981) that the K_m for factor X in this system is markedly influenced by the concentration of phospholipid: the former group found that an increase in the concentration of inosithin in their activation system from 1.25 to 5.0ng/ml (approximately 2 to 7nM) reduced the K_m for factor X from 0.1 to 0.04μM, whereas the latter workers found that raising the phospholipid concentration in their system from 5 to 25μM increased the K_m for factor X from 0.024 to 0.083μM. It is possible that these apparently contradictory findings are due to the use of different types of phospholipid in the two studies, with one group using a crude inosithin preparation and the other using a mixture of phosphatidyl choline and phosphatidyl serine. It is also possible that the large difference in concentration of phospholipids might lead to the different results: there might be an optimum phospholipid

concentration between 7nM and 5μM, with the K_m for factor X activation increasing at phospholipid concentrations above or below this optimum.

In the present study, the K_m for the activation of factor X was determined in the presence of two different concentrations of 'partially purified factor VIII:C'. In both cases, 1.3μM 'lecithin' was added to the activation mixtures, but the phospholipid present in the factor VIII:C preparation resulted in the total phospholipid concentration being 1.34 and 1.53μM in the mixtures containing 0.4 and 2.3 units/ml factor VIII:C respectively. Thus, the increase in the K_m from 0.26 to 0.50μM factor X as the concentration of 'partially purified factor VIII:C' was raised could be due not only to the difference in the factor VIII:C concentration but also to the increase in the phospholipid concentration. The similarity of the K_m values determined in the presence and absence of factor VIII:C, both in the present study and that if van Dieijen *et al* (1981), strongly suggests that the factor VIII:C concentration has a much lesser influence on the K_m than does the phospholipid concentration.

The values determined in the present study for the K_m for factor X being activated by factor IX_a in the presence or absence of factor VIII:C are a little higher than the published values (Kosow *et al*, 1974; Brown *et al*, 1978; van Dieijen *et al*, 1981). The latter group, however, observed a thirty-fold increase in the K_m when they increased the phospholipid concentration by a similar factor in activation mixtures containing no factor VIII:C. Thus, the small discrepancies

among the published values, and between these figures and those obtained in the present work may be explicable in terms of the concentrations and types of phospholipid employed in the different studies.

Thus, the K_m which is determined for the activation of factor X by factor IX_a in the presence of phospholipid, whether or not factor VIII:C is included in the activation mixture, is dependent upon the phospholipid concentration. A value for the K_m obtained under these conditions must, therefore, be regarded as an apparent K_m.

In the present work, the addition of low concentrations (0.4 units/ml) of 'partially purified factor VIII:C' to the activation mixtures, together with a ten-fold reduction in the phospholipid concentration, resulted in a 150-fold increase in the value of V_{max}, compared with that determined in the absence of factor VIII:C. The value of V_{max} increased only 2.5-fold when the concentration of 'partially purified factor VIII:C' was raised to 2.3 units/ml. Only one other group (van Dieijen *et al*, 1981) has examined the effect of the concentration of factor VIII:C on the V_{max} of the reaction. They found that the addition of 2 units/ml factor VIII:C to their activation mixtures increased the value of V_{max} 10 000-fold, while raising the factor VIII:C to 11 units/ml resulted in a further increase in V_{max} of only 2.5-fold. Qualitatively, therefore, the results from the two studies are in very good agreement: the V_{max} for the activation reaction increases by several orders of magnitude in the presence of factor VIII:C concentrations below 2.0 units/ml but a five-

fold increase in the factor VIII:C concentration has only a relatively small effect on the V_{max}. The magnitude of the increase in V_{max} observed by the two groups, however, differs by a factor of approximately 100. This might be a result of the use of different concentrations of phospholipid from different sources or it might reflect the different factor VIII:C preparations used. In their study, van Dieijen *et al* (1981) utilised a factor VIII/vWF preparation which they were obliged to 'activate' with thrombin in order for it to stimulate the activation of factor X. The 'partially purified factor VIII:C' used in the present study did not require pretreatment with thrombin.

Both Brown *et al* (1978) and van Dieijen *et al* (1981) observed deviations from linearity in their Lineweaver-Burk plots at high concentrations of factor X. A similar observation was made in the present work, both in the presence and absence of 'partially purified factor VIII:C'. Brown *et al* (1978) interpreted their results as showing a simple substrate inhibition of the reaction at factor X concentrations greater than 0.4μM. Van Dieijen *et al* (1981), however, found that the factor X concentration above which the initial rate of the reaction was inhibited depended on the concentration of phospholipid in the activation mixture. They, therefore, suggested that this effect was a reflection of the binding capacity of the phospholipid for factor X and that an increase in the concentration of factor X above that required to saturate the phospholipid did not affect the initial rate of the reaction.

Silverberg *et al* (1977) observed a deviation from linearity in a Lineweaver-Burk plot of the activation of factor X by the extrinsic coagulation system at substrate concentrations greater than 0.9μM. They were not able to discern whether this resulted from an inhibition of the reaction by the substrate or products, or from a proteolytic inactivation of factor VII_a by newly formed factor X_a. The latter hypothesis may also be applicable to the intrinsic coagulation system in which factor X_a appears to degrade factor IX_a proteolytically. A consideration of the kinetics of the activation of factor X by the extrinsic system is further complicated by the formation (Morrison-Silverberg and Jesty, 1981) of ternary complexes of factor VII, factor X_a and tissue factor, which will activate factor X, and inactive binary complexes of factors VII and X_a.

The apparent inhibition of the activation of factor X at high substrate concentrations, which was seen in the present study and has also been reported by other workers, was observed at factor X concentrations ranging from 0.4μM (Brown *et al*, 1978) to approximately 2 to 3μM (van Dieijen *et al*, 1981) which would suggest a more complex mechanism than simple substrate inhibition. The suggestion by van Dieijen *et al* (1981) that the effect is caused by a saturation of the binding sites for factor X on the phospholipid vesicles is consistent with the initial rate of activation being virtually unchanged by increasing concentrations of factor X, as reported by this group, but cannot account for the marked fall in the rate at high substrate concentrations

observed by Brown *et al* (1978). The cause of the inhibition of the activation of factor X by high substrate concentrations therefore remains open to debate. It is probable that more than one effect is involved: once the binding sites on the phospholipid vesicles are saturated with factor X, it is unlikely that a further increase in the protein concentration would produce a significant increase in the reaction rate; the formation of high concentrations of factor X_a at a very early stage of the activation would also tend to result in the destruction of the factor IX_a, leading to a relatively low rate of reaction being measured.

The inhibition of factor X activation by substrate concentrations greater than 1μM occurs in artificial systems but is highly unlikely to be of physiological significance. The plasma concentration of factor X is approximately 0.2μM, and is therefore below the levels at which inhibition occurs in the synthetic system. In addition, it is very probable that any excess factor X_a produced *in vivo* would be neutralised by antithrombin III.

Chapter 7

GENERAL DISCUSSION

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GENERAL DISCUSSION

The Nature of Factor VIII

In many respects, factor VIII is the most enigmatic component of the blood coagulation system. Its existence - at least via its deficiency in haemophilia A - has been recognised for many years, and yet it is very much less well characterised than any other coagulation factor, even those which have been discovered relatively recently. The major problem in defining the nature of the factor VIII molecule arises from the association of the coagulant activity with the factor VIIIR:Ag and platelet aggregating factor activities. Three models have been proposed (Bloom and Peake, 1977): a single molecule responsible for all of the 'factor VIII-related' activities; two separate molecules, one possessing the coagulant activity and one responsible for both factor VIIIR:Ag and platelet aggregating factor; and a complex of factor VIII:C and factor VIIIR:Ag.

Factor VIII:C and factor VIIIR:Ag co-purify in the isolation of factor VIII/vWF (Legaz and Davie, 1976) and in the preparation of 'partially purified factor VIII:C' described in Chapter 2, suggesting that the activities are probably not carried by two separate molecules.

It is, however, also unlikely that a single polypeptide chain is responsible for all of the activities: factor VIII:C can be separated from factor VIIIR:Ag and platelet aggregating factor in both factor VIII/vWF and 'partially purified factor VIII:C' by a variety of non-proteolytic procedures described

in Chapter 3. In addition, as discussed below, it is difficult to reconcile the genetics of the hereditary deficiencies of factor VIII with a single molecule carrying all of the 'factor VIII-related' activities.

The most credible model for plasma 'factor VIII' is, therefore, one in which the factor VIII:C and factor VIIIR:Ag activities are carried by different molecules which circulate in a complex with one another. The form of this complex is itself open to debate: the 'dissociation' of the activities by buffers of high ionic strength suggests that the molecules are linked by non-covalent bonds, whereas the 'dissociation' by reducing agents implies that the linkage is via disulphide bridges. Whatever the nature of the bonds between the components, the factor VIII:C almost certainly comprises only a very small proportion of the factor VIII/vWF complex: the polypeptide of molecular weight 200 000 seen on SDS-polyacrylamide gel electrophoresis of reduced factor VIII/vWF probably corresponds to a subunit of factor VIIIR:Ag.

Genetics of Factor VIII

A deficiency of the plasma factor VIII coagulant activity occurs in both haemophilia and von Willebrand's disease (Barrow and Graham, 1974). In haemophilia, the factor VIIIR:Ag and factor VIIIR:WF activities are normal, whereas all of the 'factor VIII-related' activities are usually decreased in von Willebrand's disease, although variant forms have been described (Ekert *et al*, 1976) in which the level of one activity is reduced disproportionately to the others.

Von Willebrand's disease and haemophilia A display different modes of inheritance (Barrow and Graham, 1974). The former is an autosomal trait which is expressed in heterozygotes of either sex while the latter is a classical sex-linked recessive character. It is therefore probable that two distinct genetic loci are involved in the biosynthesis of factor VIII: one on the X chromosome, which is responsible for the production of the factor VIII:C molecule itself, and a second on an autosomal chromosome, which is responsible for the production of the factor VIIIR:Ag. In haemophilia, there is either a failure to synthesise factor VIII:C or the production of a non-functional molecule. In von Willebrand's disease, however, the defect is in the biosynthesis of the factor VIIIR:Ag molecule; in the absence of functional factor VIIIR:Ag, no factor VIII:C activity is detectable.

Factor VIII:C activity is also absent from the plasma of a small group of patients who are deficient in both factor V and factor VIII. The factor VIIIR:Ag and factor VIIIR:WF activities are, however, normal in these individuals, and the plasma contains normal levels of factor V antigen (Giddings *et al*, 1977). Bloom and Peake (1977) suggested that an autosomal gene locus might control the synthesis of a peptide required for the expression of the coagulant activity of both these factors. Marlar and Griffin (1980), however, have suggested that the combined deficiency of factors V and VIII:C might be a result of an excess of protein C activity, arising from a deficiency of a naturally-occuring plasma inhibitor of protein C. If this explanation is correct, it is not necessary

to invoke the participation of a third gene in the biosynthesis of factor VIII.

Thus, in man, the genetics of factor VIII are compatible with factor VIII:C and factor VIIIR:Ag activities being carried on different molecules with distinct gene loci coding for the respective polypeptides. The absence of factor VIII:C in von Willebrand's disease suggests an intimate association of the polypeptide chains.

Assays of Factor VIII:C

The definition of the nature of factor VIII at a molecular level would be greatly simplified by the preparation of the coagulant protein in pure form. Any assessment of the various purification procedures which have been employed is, however, hampered by the lack of a universally applicable and acceptable assay technique.

Factor VIII:C activity is usually measured by means of coagulation assays. There is normally a good agreement between the one-stage assay (Langdell *et al*, 1953) and the two-stage assay in both its original (Biggs *et al*, 1955) and modified (Denson, 1967) versions when plasma samples are compared. The agreement is, however, rather less good when concentrated samples of factor VIII:C are compared with plasma (Kirkwood and Barrowcliffe, 1978); in comparisons of this type, the two-stage assay indicates that concentrates contain approximately 20% higher levels of factor VIII:C than the one-stage assay. A second source of discrepancy between the two types of coagulation assay arises as a result of the pheno-

menon of thrombin 'activation' of factor VIII:C. An increase in the coagulant activity of factor VIII following its exposure to thrombin is observed solely in a one-stage assay; two-stage assays show only a decline in the factor VIII:C activity under these conditions.

Recently, Seghatchian (1979) has suggested that a more direct assessment of the factor VIII:C activity in a sample might be obtained by generating factor X_a in a system equivalent to the first stage of a two-stage coagulation assay, and then assaying the factor X_a with the chromogenic peptide substrate, S-2222. This method does, however, give anomalous results in some cases (Seghatchian, 1979), including a small proportion of haemophilic plasma samples which appear to contain normal levels of factor VIII:C. In addition, this technique, being based on the two-stage coagulation assay, is most unlikely to be sensitive to 'thrombin-activated' factor VIII:C.

The antibodies to factor VIII:C which arise in haemophiliacs who have received multiple blood transfusions have been used to develop immunoradiometric assays for factor VIII:C (Peake and Bloom, 1978; Lazarchick and Hoyer, 1978; Peake *et al*, 1979). These assays, however, provide a measurement of the protein carrying the factor VIII:C, which is usually termed factor VIII:CAg, rather than a measurement of the biological activity. Thus, for example, the immunoradiometric assays detect factor VIII:CAg in serum which has no factor VIII:C activity.

An assay of factor VIII:C based on the activation of

factor X in a purified system would have several advantages over coagulation assays. The direct measurement of the formation of factor X_a, using either the esterase activity of the enzyme or the release of a radioactive activation peptide from tritium-labelled factor X (Silverberg *et al*, 1977; Hultin and Nemerson, 1978), would permit the use of a more simple basic system than that required for clotting assays, and accessory components could be introduced at known, specified concentrations. Thus, for example, the role of thrombin in 'activating' factor VIII:C during coagulation could be examined in detail by assaying identical samples of factor VIII:C in the presence of various concentrations of thrombin and in its absence, with no complications arising from the generation of the enzyme during the later stages of the coagulation process.

A direct assay of this type for factor VIII:C would be invaluable for comparisons of different preparations of purified factor VIII. Assays could be carried out under conditions designed to measure the coagulant activity in both 'unactivated' and fully 'activated' forms. Thus, preparations which appeared to have a high specific activity as a result of the factor VIII:C being partially 'activated' could be distinguished from those with genuinely high specific activities resulting from the separation of the protein from contaminants, since these preparations would still retain their potential for being 'activated'.

An assay for factor VIII:C based on the activation of factor X in a purified system therefore has several advantages

over normal coagulation assays. It would also be more useful than the immunoradiometric assays for the examination of procedures for the purification of factor VIII:C as it would measure only biologically active material and would not be sensitive to inactive protein.

The Role of Factor VIII:C in Blood Coagulation

Factor VIII:C is a critically important component of the coagulation system: the deficiency of the activity in haemophilia A leads to a severe haemorrhagic disorder, in contrast to a deficiency of, for example, factor XII or factor XI. It was originally thought (Davie and Ratnoff, 1964; Macfarlane, 1964) that factor VIII:C participated only in the intrinsic coagulation system. The severity of the bleeding in haemophilia A, however, implies that, in the absence of factor VIII:C, very little factor X is activated by the components of either the intrinsic or extrinsic pathway. It is possible that the activation of factor IX by factor VII_a and tissue factor (Østerud and Rapaport, 1977, 1980) is of greater physiological importance than the activation of factor X by these components. If this is the case, the major physiological activator of factor X must be the intrinsic pathway, and specifically factors IX and VIII, which would account for the importance of these factors in normal coagulation.

Both the 'cascade' (Macfarlane, 1964) and 'waterfall' (Davie and Ratnoff, 1964) schemes of coagulation envisaged the action of factor IX_a as being the conversion of factor VIII to an enzyme which subsequently activated factor X. The

only experimental study which has purported to confirm this model appears, however, to be that of Lundblad and Davie (1964). The results of this investigation, which employed coagulation assays to show that the incubation of factor VIII with factor IX_a in the presence of phospholipid and calcium ions generated a material with the capacity to activate factor X, could, however, also be interpreted in terms of a complex of factors IX_a and VIII:C being required for the activation of factor X.

It seems most unlikely that factors IX and VIII:C act sequentially in coagulation. Factor IX_a is an enzyme which will activate factor X in the absence of any accessory components (van Dieijen *et al*, 1981). Thus, were factor VIII:C to be converted to an enzyme capable of catalysing the same reaction, a degree of redundancy would be introduced into the system, reducing the potential for amplification of the response at this stage in the cascade.

There is no unequivocal evidence that factor IX_a, or any other proteinase, can transform factor VIII:C into an active enzyme. Vehar and Davie (1977, 1980) found that the product of the reaction between thrombin and factor VIII:C rapidly lost up to 85% of its coagulant activity when it was treated with diisopropyl fluorophosphate. They interpreted this observation in terms of factor VIII:C being converted by thrombin into a serine proteinase which is susceptible to inhibition by diisopropyl fluorophosphate. Hultin and Jesty (1980), however, found that, in addition to diisopropyl fluorophosphate, other inhibitors of thrombin - including hirudin and DAPA (Nesheim *et al*, 1979a) - enhanced the rate at

which the activity of thrombin-activated factor VIII:C decayed. They suggested that all of the experimental data are consistent with the 'activation' of factor VIII:C being the result of the formation of a complex with thrombin rather than the conversion of the factor VIII:C itself into an active enzyme.

The formation of a complex between factor VIII:C and thrombin would be analogous to the formation of a complex between factor X_a and factor VII in the presence of tissue factor (Morrison-Silverberg and Jesty, 1981) during the initiation of the extrinsic coagulation system. In this case, however, a cleavage of factor VII by factor X_a results in an increase in its activity, whereas the cleavage of factor VIII:C by excess thrombin, or by activated protein C, brings about a decrease in the activity of the putative factor VIII:C-thrombin complex.

Factor V resembles factor VIII:C in that its coagulant activity is enhanced by treatment with thrombin (Rapaport *et al*, 1963). The recent preparation of pure bovine (Nesheim *et al*, 1979b; Esmon, 1979; Bartlett *et al*, 1980) and human (Dahlbäck, 1980; Kane and Majerus, 1981) factor V has permitted studies of the changes at a molecular level associated with its 'activation' by thrombin. Esmon (1979) and Nesheim and Mann (1979) have shown that thrombin cleaves the single polypeptide chain of molecular weight 330 000 into several fragments, with the factor V activity being associated with a two-chain species of molecular weight approximately 290 000. The 'activation' of factor V by thrombin clearly, therefore, involves a proteolytic action of the enzyme, but the 'activation' of factor

VIII:C may not follow a similar mechanism.

As described in Chapter 5, under appropriate conditions, treating 'partially purified factor VIII:C' with thrombin results in an enhancement of its activity in the activation of factor X by a purified system. Thus, it would appear that the discrepancy between the one- and two-stage coagulation assays referred to in Chapter 3 reflects a failing of the two-stage method in these circumstances. This might be due to the technique depending on a measurement of the total factor X_a present at the completion of the first stage incubation rather than on the initial rate of the activation. A transient increase in the factor VIII:C activity early in the incubation might not result in the activation of more factor X during the complete first stage, and if the factor X_a were produced more rapidly than usual, sufficient time might elapse for it to be neutralised by inhibitors such as antithrombin III before the second stage of the assay.

Overall, therefore, a consideration of the results obtained in the study of the activation of factor X, and in the clotting assays, together with a comparison with factor V, leads to the conclusion that the 'activation' of factor VIII:C by thrombin represents a genuine, but transient, increase in the coagulant activity.

The results described in Chapters 5 and 6, together with those reported by Suomela *et al* (1977), Brown *et al* (1978), Hultin and Nemerson (1978) and van Dieijen *et al* (1981) confirm the view that factor VIII:C participates in the activation of factor X as a cofactor for factor IX_a rather than as an enzyme

in its own right. In this respect there is a clear analogy with the role of factor V in the activation of prothrombin by factor X_a. Factor V acts as a cofactor for factor X_a (Jobin and Esnouf, 1967) and is not, itself, converted to an active enzyme. Factor X_a alone activates prothrombin in solution at a rate approximately 300 000 times slower than that which can be obtained in the presence of calcium ions, phospholipid and thrombin-activated factor V (Nesheim *et al*, 1979c). The principal stimulatory effect of factor V on the kinetics of prothrombin activation is a five hundred-fold increase in the V_{max} for the reaction. Thus, the stimulation of factor X activation by factor VIII:C, which increases the V_{max} for this reaction as indicated by the results in Chapter 6 and those reported by van Dieijen *et al* (1981), may well follow the same mechanism as the stimulation by factor V of prothrombin activation.

The activation of factor X by factors IX_a and VIII:C is a complex process. A rapid rate of reaction is dependent upon the presence of optimum concentrations of every component of the system: factors X, IX_a and VIII:C, and calcium ions and phospholipid. Increasing the concentration of factor IX_a or factor X, the enzyme and substrate respectively, produces the expected increase in the rate of the reaction. The results of the present study and those of Brown *et al* (1978), however, indicate that concentrations of factor X higher than approximately three times the K_m inhibit the activation. The rate of the reaction was not directly proportional to the factor IX_a concentration in any of the systems investigated in

Chapter 5, with the rate per unit enzyme at higher enzyme concentrations being lower than that at low concentration. This deviation from a linear relationship is most probably due to the presence of phospholipid in the activation mixtures since van Dieijen *et al* (1981) were able to show that the rate of formation of factor X_a in solution did increase linearly with the concentration of factor IX_a.

The relationship between the concentration of phospholipid in the activation mixtures and the rate of generation of factor X_a is complex. Phospholipids appear to affect the K_m for factor X in the system rather than the V_{max} of the reaction, but van Dieijen *et al* (1981) found that while low concentrations of phospholipid dramatically decreased the K_m, there was a considerable increase in the K_m for factor X at higher phospholipid concentrations.

Factor VIII:C stimulates the rate of activation of factor X by factor IX_a. The results in Chapter 5 indicate that, at a constant factor IX_a concentration, the reaction rate is dependent upon the factor VIII:C concentration. There is, however, a very much greater difference between the rates without factor VIII:C and in the presence of 1 unit/ml than there is between the rates in the presence of 1 unit/ml and higher concentrations of factor VIII:C. This observation is supported by the results of the kinetic studies described in Chapter 6 which indicate that the V_{max} for the activation of factor X is 150 times higher in the presence of low concentrations of factor VIII:C than in its absence but a five-fold increase in the factor VIII:C concentration brings about only

a three-fold increase in the value of V_{max}.

Thus, it appears that the overall rate of activation of factor X depends on the concentrations of both factor IX_a and factor VIII:C. In the case of factor VIII:C, however, there is a 'saturation' effect and very large increases in the concentration of the activity are required to raise the reaction rate significantly above that observed at relatively low levels of factor VIII:C. It is possible that this is the result of the factor IX_a becoming saturated with cofactor. The effect might also reflect a possible role for factor VIII:C in the binding of the other protein components to the phospholipid surface analogous to the role of factor V in the binding of factor X_a to phospholipids in the activation of prothrombin (Nesheim *et al*, 1979c). In that case, an increase in the factor VIII:C concentration to levels above those required for complete binding to the phospholipid surface would have little effect on the rate of activation of factor X.

Although the mechanism by which it does so is not yet conclusively established, this study of the role of factor VIII:C in blood coagulation provides clear evidence that it is responsible for the stimulation of the rate of activation of factor X by the components of the intrinsic pathway, resulting in the generation of sufficient factor X_a for normal coagulation to occur.

Appendices

MATERIALS AND METHODS

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APPENDIX 1

REAGENTS AND SUPPLIERS

Reagents for use in coagulation assays were purchased from Diagnostic Reagents Ltd., Thame, Oxon, U.K.; from DADE Division of the American Hospital Supply Corporation, Didcot, Oxon, U.K.; or from Immuno Ltd., Sevenoaks, Kent, U.K. Plasma deficient in the vitamin K-dependent proteins, which was used for the assay of prothrombin, was prepared by adsorbing normal bovine plasma containing 0.01M sodium oxalate with two 50mg/ml aliquots of barium sulphate. The addition of purified prothrombin to the barium sulphate-adsorbed plasma provided a substrate plasma for use in the assay of factor X.

Acrylamide for gel electrophoresis was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. The grade sold as 'Specially Purified for Electrophoresis' was used without further treatment. N,N'-methylene-bis-acrylamide, ammonium persulphate and 2-mercaptoethanol were also obtained from BDH Chemicals Ltd. N,N,N',N'-tetramethylethylene diamine (TEMED) and sodium dodecyl sulphate (SDS) were purchased from Sigma London Chemical Co Ltd., Poole, Dorset, U.K.

Immunological studies were carried out using agarose plates made with agarose purchased from Miles Laboratories Ltd., Slough, Bucks, U.K.

α-N-Benzoyl arginine ethyl ester (BAEe) was used as a substrate for the measurement of the esterolytic activity of enzymes such as thrombin and factor X_a. It was purchased from Sigma London Chemical Co Ltd.

The more specific synthetic chromogenic substrates developed recently for the assay of the coagulation factors were purchased from Kabivitrum Ltd., London, U.K. Factor X_a was assayed using substrate S-2222, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide; other substrates were used as appropriate for the other serine proteinases.

Other reagents were obtained from several commercial suppliers, as indicated in Table A.1. Common inorganic chemicals were purchased from BDH Chemicals Ltd. In general, the most highly purified grade available (Analar) was used. Exceptionally, however, in the case of imidazole, the BDH Laboratory grade reagent was purchased.

TABLE A.1

Reagents and Suppliers

Reagent	Abbreviation	Supplier
2-Amino-2-(hydroxymethyl)-1,3-propandiol	Tris	Sigma Chemical Co.
Bell and Alton Platelet Substitute ('Cephalin')		Diagnostic Reagents Ltd, Thame.
α-N-Benzoyl-L-arginine Ethyl Ester	BAEe	Sigma Chemical Co.
N-Benzoyl-ile-glu-gly-arg *p*-Nitroanilide	S-2222	Kabivitrum Ltd, London.
Carboxymethyl-Sephadex C-50	CM-Sephadex	Pharmacia Fine Chemicals, London.
Celite 512		Johns-Manville Co Ltd, London.
Centrolex P Lecithin		Central Soya, Chicago, Ill., USA.
Chlorosulphonic Acid		BDH Chemicals Ltd.
Coomassie Brilliant Blue G-250		Serva Feinbiochimica, Heidelberg, GFR.
Coomassie Brilliant Blue R-250		Sigma Chemical Co.
Cyanogen Bromide	CNBr	Sigma Chemical Co.
Diethylaminoethyl-Sephadex A-50	DEAE-Sephadex	Pharmacia Fine Chemicals, London.
Diethylbarbituric Acid	Barbitone	BDH Chemicals Ltd.
Dimethylformamide	DMF	BDH Chemicals Ltd.
Epichlorhydrin-triethanolamine-Cellulose	ECTEOLA-Cellulose	Whatman Ltd, Maidstone.
Ethylenediaminetetra-acetic Acid (Disodium salt)	EDTA	BDH Chemicals Ltd.
Freunds Adjuvant		Difco Laboratories, Detroit, Mich., USA.
Heparin		Sigma Chemical Co.
Kaolin (Light)		BDH Chemicals Ltd.

TABLE A.1 (CONTINUED)

Reagent	Abbreviation	Supplier
2-Mercaptoethanol		BDH Chemicals Ltd.
Naphthalene Black		George T Gurr, London.
Octan-2-ol		BDH Chemicals Ltd.
Phenylmethylsulphonyl Fluoride	PMSF	Sigma Chemical Co.
Phosphatidyl Serine	PS	Sigma Chemical Co.
Polyethylene Glycol 6000	PEG	BDH Chemicals Ltd.
Russell's Viper (*Vipera russellii russellii*) Venom	RVV	Prof P M Dalal, Haffekine Institute, Bombay, India.
Russell's Viper Venom - Cephalin	RVV-Cephalin	Diagnostic Reagents Ltd, Thame.
Sepharose CL-4B		Pharmacia Fine Chemicals, London.
Tannic Acid		BDH Chemicals Ltd.
Thrombin (Human Standard)		NIBSC, Hampstead, London.
Thrombin (Bovine)		Diagnostic Reagents Ltd, Thame.
Tiger Snake (*Notechis scutatus occidentalis*) Venom	TSV	Mr A Softly, Royal Perth Hospital, Perth, WA, Australia.
Trypsin		Sigma Chemical Co.

APPENDIX 2

BUFFER SOLUTIONS

Throughout this thesis, the same convention is used for the nomenclature of all buffer solutions. The stated molarity of the solution refers to the concentration of the first-named component. The second-named component of the buffer is used to adjust the pH to the stated value. In general, the final adjustment of the pH was carried out at the stated concentration. In the case of the citric acid-sodium hydroxide buffer, however, the pH was adjusted to 6.9 in a solution containing 1.0M citrate; this solution was then diluted to the concentration required in working buffers without further adjustment of the pH, resulting in the final 0.05M buffer having a pH of approximately 7.2.

APPENDIX 3

COAGULATION ASSAYS

Coagulation assays were used routinely to measure the concentrations of the clotting factors. In all cases, normal bovine plasma was used as a standard, and its concentration was defined as one unit per millilitre for all the coagulation factors.

Prothrombin Assay (Jobin and Esnouf, 1967)

Reagents: Barium sulphate-adsorbed bovine plasma.

TSV-phospholipid reagent, prepared by mixing one crystal of tiger snake (*Notechis scutatus occidentalis*) venom, 0.3ml of 50mg/ml Centrolex P lecithin suspension and 9.7ml 0.02M tris-HCl, pH7.5.

Method: 0.10ml of sample containing prothrombin was mixed with 0.10ml adsorbed plasma and 0.10ml TSV-phospholipid reagent. The mixture was incubated at 37^{o}C for 30 seconds before 0.10ml 0.025M calcium chloride was added, and the time taken for a clot to form was recorded.

Two-Stage Factor VIII Assay

Two-stage assays of factor VIII activity were carried out using a modified version (Denson, 1967) of the technique originally described by Biggs *et al* (1955). The reagents were purchased in kit form from Diagnostic Reagents Ltd., Thame, Oxon, U.K.

Reagents: 'Factor VIII Reagent', containing lyophilised serum, factor V and phospholipid. Each vial of this reagent was reconstituted with 10ml of a mixture of equal volumes of 0.15M sodium chloride and 0.025M calcium chloride.

'Substrate Plasma'. Each vial of lyophilised plasma was reconstituted with 5ml distilled water.

'Citrate-saline'. All samples for assay were diluted in a mixture of one volume of 0.13M tri-sodium citrate to five volumes of 0.15M sodium chloride.

Method: 0.05ml of the diluted sample containing factor VIII was mixed with 0.20ml 'factor VIII reagent' and incubated at 37^{o}C for ten minutes. 0.10ml of substrate plasma was then added, and the time taken for a clot to form was recorded.

One-Stage Factor VIII Assay (Langdell *et al*, 1953)

Reagents: 'Kaolin-Cephalin', prepared by mixing 3ml 0.15mg/ml platelet substitute (Bell and Alton, 1954) with 2ml 1% kaolin suspension.

Congenital human factor VIII-deficient plasma.

Method: 0.10ml of the factor VIII-containing sample was incubated at 37^{o}C for 120 seconds with 0.10ml 'kaolin-Cephalin' and 0.10ml factor VIII-deficient plasma. 0.10ml of 0.05M calcium chloride was then added and the clotting time was recorded.

Factor IX Assays (Elödi and Váradi, 1978)

Reagents: 'Celite-cephalin', prepared by suspending 0.10g Celite 512 in 5ml platelet substitute.
Human congenital factor IX-deficient plasma.

Method: 0.10ml of the sample containing factor IX was mixed with 0.10ml factor IX-deficient plasma and 0.20ml of 'Celite-cephalin'. The mixture was incubated at 37°C for 180 seconds and 0.10ml of 0.025M calcium chloride was added. The time required for clot formation was recorded.

A slightly modified procedure was employed for the assay of activated factor IX. In this case the Celite 512 was omitted, and the incubation at 37°C was reduced to 30 seconds.

Factor X Assays (Denson, 1961)

Reagents: 'RVV-Cephalin', containing Russell's viper (*Vipera russellii*) venom in platelet substitute.
Factor X-deficient bovine plasma.

Method: 0.10ml factor X-containing sample, 0.10ml factor X-deficient plasma and 0.10ml 'RVV-Cephalin' were incubated at 37°C for 30 seconds. 0.10ml 0.025M calcium chloride was then added and the clotting time was recorded.

In order to assay activated factor X, the RVV-Cephalin reagent was replaced with platelet substitute.

Activated Protein C Assays

A semi-quantitative assay for protein C was used, based on the activated partial thromboplastin time test.

Reagents: Kaolin suspension, prepared by mixing 1.0g kaolin with 100ml 0.15M sodium chloride solution.

'Cephalin': Platelet substitute reconstituted as directed by the manufacturer.

Normal bovine plasma.

Method: 0.05ml of sample containing activated protein C was added to 0.10ml 'Cephalin' and 0.10ml plasma. The mixture was incubated at 37°C for 30 seconds before adding 0.05ml kaolin suspension. The incubation at 37°C was continued for a further 120 seconds. 0.10ml 0.025M calcium chloride was then added and the clotting time was recorded.

Graphical Calculation of Coagulation Assay Results

In general, three dilutions of a standard and three dilutions of the test sample were assayed simultaneously. The clotting times were plotted against the sample dilutions on double logarithmic graph paper. The best straight line was drawn through the points obtained for the standard and a line parallel to this was then drawn through the test sample points. A convenient point on the concentration axis was chosen to be 100% and a line parallel to the axis was drawn through the point at which the test sample graph intersected with this 100% calibration. The point at which this line intersected with the standard sample graph defined the concen-

tration of the coagulation factor in the test sample. The method is illustrated for a two-stage factor VIII assay in Figure A.1.

Routinely, rather than plotting graphs by hand, a Model 9100B programmable calculator (Hewlett-Packard Co., Loveland, Colorado, U.S.A.) was used to derive the concentration of the coagulation factor in the test sample from the relationship:

$$C = Q \text{ antilog} \left[K \left(\frac{\text{Log } S_1 + \text{Log } S_2 + \text{Log } S_3 - \text{Log } T_1 - \text{Log } T_2 - \text{Log } T_3}{\text{Log } S_3 - \text{Log } S_1 + \text{Log } T_3 - \text{Log } T_1} \right) \right]$$

C is the concentration of the coagulation factor in the test sample.

Q is the concentration of the factor in the standard.

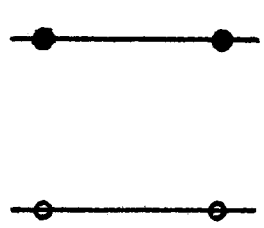
$$K = \frac{2 \times \text{Log (overall dilution range)}}{\text{number of dilutions}}$$

S_1, S_2 and S_3 are the clotting times for 3 dilutions of the standard.

T_1, T_2 and T_3 are the clotting times for 3 dilutions of the test sample.

FIGURE A.1

Calculation of Two Stage Factor VIII Clotting Assay Results



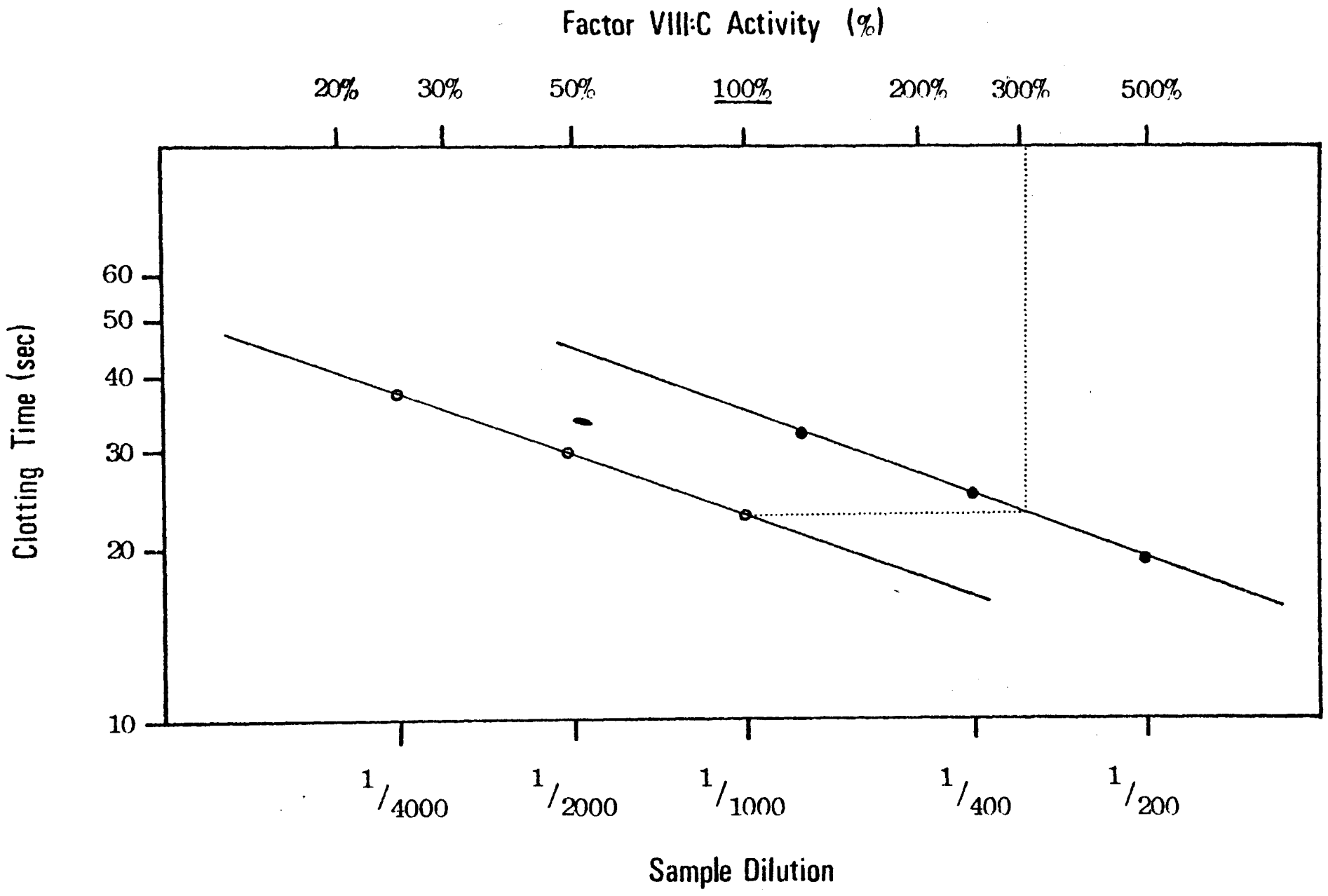
Standard plasma

Sample

The activity of the sample is 310% of the activity of the standard plasma. That is, the sample contains 3.1 units/ml factor VIII:C.

FIGURE A.1

Calculation of Two Stage Factor VIII Clotting Assay Results



APPENDIX 4

PLATELET AGGREGATING FACTOR ASSAYS

Assays of Platelet Aggregating Factor activity (Bovine factor VIIIR:WF activity) were restricted to semi-quantitative measurements only.

Reagents: Platelet rich human plasma, prepared by centrifuging freshly drawn blood containing 0.01M sodium oxalate at 250g for 120 seconds and removing the plasma layer with a pipette.

Method: 0.20ml of the sample containing platelet aggregating factor was mixed with 0.20ml of platelet rich plasma. The mixture was shaken gently for five minutes and allowed to settle. The aggregation of the platelets was assessed by eye.

APPENDIX 5

ESTIMATION OF PROTEIN CONCENTRATION

The concentration of proteins in solutions was estimated routinely by measurement of the absorbance at 280nm. In order to take into account any contribution of light scattering to this absorbance, a measurement was also made at a wavelength of 320nm. The true absorbance at 280nm was calculated from these two measurements:

$$A = A_{280} - (A_{320} \times 1.6)$$

The true absorbance at 280nm was then converted into a concentration by using the following extinction coefficients for solutions containing 1mg/ml of the particular proteins:

Prothrombin	1.38	(Esnouf *et al*, 1973)
Thrombin	1.95	(Winzor and Scheraga, 1964)
Factor IX	1.49	(Fujikawa *et al*, 1974b)
Factor $IX_{a\beta}$	1.43	(Fujikawa *et al*, 1974b)
Factor X	0.96	(Esnouf *et al*, 1973)
Factor X_a	0.94	(Jesty and Esnouf, 1973)
Protein C	1.37	(Kisiel *et al*, 1976)

A value of 1.00 was assumed for the absorbance of a 1mg/ml solution of mixtures of proteins and for those proteins, including factor VIII, for which the extinction coefficient was not known.

APPENDIX 6

PREPARATION OF ANTISERA

Antisera against the purified bovine proteins were raised in New Zealand white rabbits. 10µg of protein, dissolved in 0.50ml water, was emulsified with 0.50ml Freund's complete adjuvant and administered by intraperitoneal injection at six or eight sites. Two weeks later, a booster dose, containing 100µg of antigen in 0.50ml water emulsified with 0.50ml incomplete Freund's adjuvant, was given.

About two weeks after the second immunisation, blood was collected from the ear vein. It was allowed to clot at 37^{o}C for two hours and then left at 4^{o}C overnight. The clot was removed and the serum was stored in 1ml aliquots at -20^{o}C. No attempt was made to purify the immunoglobulin fraction from the serum.

APPENDIX 7

IMMUNOLOGICAL TECHNIQUES

Double Immunodiffusion (Ouchterlony, 1958)

A hot solution of 1% agarose in 0.025M barbitone-tris, pH8.6, was poured into a mould made by clamping a plastic former between two glass plates. This was left at room temperature for about 30 minutes until the agarose had solidified and the mould was then dismantled, leaving a slab of agarose 7cm x 7cm x 0.1cm attached to the surface of one glass plate. Circular wells 0.25cm in diameter were punched in the plate, approximately 0.5cm apart in a hexagonal pattern as shown in Figure A.2.

When several antigens were being compared, 4µl of each was added to one of the outer ring of wells, and 4µl of an appropriate antiserum was placed in the centre well. Conversely, in order to compare several antisera, these were added to the outer wells, while the antigen was placed in the centre well.

After adding the samples, the plate was left in a moist chamber at room temperature for several days. It was examined periodically and the pattern of visible precipitin lines was recorded.

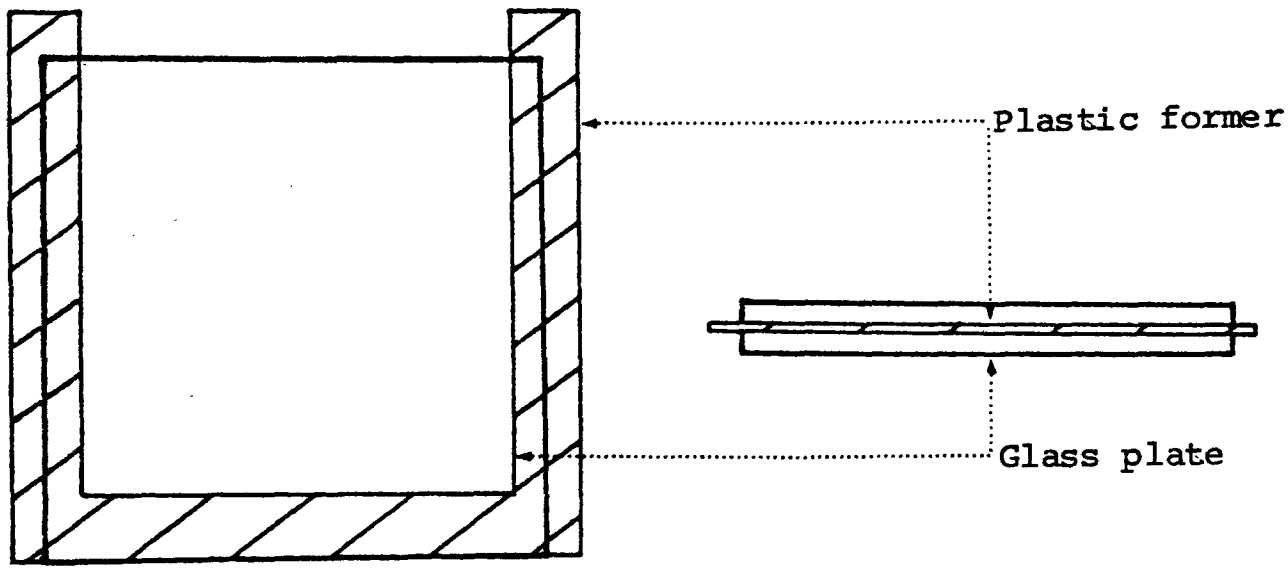
Immunoelectrophoresis

Quantitative immunoassays were carried out by the 'rocket' technique originally described by Laurell (1972). 5ml of a hot solution of 1% agarose in 0.025M barbitone-tris,

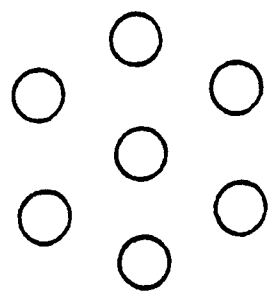
FIGURE A.2

Immunological Plates

a) Mould for Plates



b) Pattern of Wells for Immunodiffusion



pH8.6, containing 0.1mM calcium lactate, was allowed to cool to 55°C. An antiserum to the protein being assayed was added and the agarose was used to pour a plate as described above for immunodiffusion plates. In this case, a single row of wells was punched approximately 1cm from the edge of the plate.

4µl aliquots of the test samples or standard antigen solutions were added to each well, and the plate was connected to troughs containing electrophoresis buffer (0.025M barbitone-tris, pH8.6, 0.1mM calcium lactate) at either end of an electrophoresis tank (Shandon Southern Products Ltd., Runcorn, Cheshire, U.K.), by means of filter paper 'wicks'. Electrophoresis was then carried out at a constant current of 10mA per gel for five to six hours.

After staining, the heights of the peaks formed by the standard and test samples were measured. A calibration curve was constructed from the standards and the antigen concentration in the test samples was determined from this.

Staining Immunoprecipitates

The precipitin arcs in double immunodiffusion gels, and peaks in immunoelectrophoresis were frequently visible without any staining of the gel being required. Two methods were used to enhance weak bands or to stain gels in which the immunoprecipitates were not otherwise visible.

In cases where there was no desire to store the stained plate, tannic acid was used (Alpert *et al*, 1970). The completed gel was soaked in a 1% solution of tannic acid

for about 15 minutes and then rinsed in tap water. The immunoprecipitates became visible as white lines, but after about six hours, the entire plate became dark olive-green.

Coomassie brilliant blue G-250, or Naphthalene black, was used to stain those gels which were to be kept as permanent records. The plate was soaked in 0.17M sodium chloride for between 24 and 48 hours, then rinsed in distilled water for about 60 minutes. The washed gel was covered with a damp filter paper and dried in an oven at 55^{o}C. After drying, it was stained with either 2.5mg/ml Coomassie brilliant blue G-250 in water : methanol : acetic acid (9:9:2) or 0.010mg/ml Naphthalene black in methanol : acetic acid : water (5:5:1). The gel was then destained in water : methanol : acetic acid (75:4:6).

APPENDIX 8

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

The electrophoretic analysis of protein samples was carried out on both rod and slab polyacrylamide gels; the gels always contained sodium dodecyl sulphate (SDS), as did the reservoir buffers. Routinely, rod gels contained 7.5% acrylamide and electrophoresis was carried out in a phosphate buffer system as described by Weber and Osborn (1969) and Shapiro *et al* (1967). Slab gels were made up containing either 10% or 12% acrylamide in the lower (resolving) gel and 2.7% acrylamide in the upper (concentrating) gel; the discontinuous tris buffer system of Laemmli and Favre (1973) was used.

Polyacrylamide Slab Gel Electrophoresis

Reagents: Stock acrylamide; containing 222mg/ml acrylamide and 6mg/ml N,N'-methylenebisacrylamide.

Upper gel buffer; 0.5M tris-HCl, pH6.8.

Lower gel buffer; 1.5M tris-HCl, pH8.8.

Reservoir buffer; 0.05M tris-glycine, pH8.3, containing 0.1% SDS.

Sample buffer; 0.01M tris-HCl, pH6.8, containing 0.1% SDS, 0.1% 2-mercaptoethanol, 10% sucrose and 0.002% bromophenol blue.

Method: Two glass plates and perspex formers were clamped together to form a mould and the edges were sealed with 0.5% agarose. The lower gel was prepared

by mixing together (for a 10% acrylamide gel) 13.5ml stock acrylamide, 7.5ml lower gel buffer, 8.55ml water and 0.025ml tetramethylethylene diamine. This mixture was de-gassed under vacuum and 0.15ml 0.44M ammonium persulphate and 0.30ml 10% SDS were then added. The gel solution was poured into the mould and left to set at room temperature with a flat perspex rod inserted into the top of the mould. The upper gel was prepared by mixing 1.35ml stock acrylamide, 2.5ml upper gel buffer, 6.0ml water and 0.01ml tetramethylethylene diamine. After de-gassing under vacuum, 0.10ml 0.44M ammonium persulphate and 0.10ml 10% SDS were added. The top perspex rod was removed from the gel mould and the upper gel solution was poured in on top of the lower gel. A perspex 'comb' was inserted into the top of the mould to form wells in the upper gel, which was allowed to set at room temperature. The perspex formers were then removed from the top and bottom of the gel, which was inserted into the electrophoresis tank. The upper and lower reservoirs were filled with buffer and the protein samples were applied to the wells in the upper gel. The proteins were examined under reducing conditions: the samples were therefore mixed with an equal volume of sample buffer and heated in a boiling water bath for ten minutes to ensure complete

reduction. Electrophoresis was carried out at a constant P.D. of 150 volts for approximately four hours until the dye-front was 1cm from the edge of the gel. The gel was then removed from the glass plates and stained.

Polyacrylamide Rod Gel Electrophoresis

Reagents: Stock acrylamide; containing 222mg/ml acrylamide and 6mg/ml N,N'-methylenebisacrylamide.

Gel buffer; 0.2M phosphate-NaOH, pH7.1.

Reservoir buffer; 0.1M phosphate-NaOH, pH7.1, containing 0.1% SDS.

Sample buffer; 0.2M phosphate-NaOH, pH7.1, containing 1% SDS, 8M urea, and bromophenol blue.

Method: Fifteen glass tubes (0.5cm diameter x 15cm long) were inserted into a cylindrical plastic mould. A mixture of 30ml stock acrylamide, 45ml gel buffer, 10ml 10% SDS, 4.5ml 0.066M ammonium persulphate and 0.135ml tetramethylethylene diamine was poured into the mould and 0.1ml butan-1-ol was layered on to each tube. Immediately the gels had set, after about 30 minutes at room temperature, the butanol was removed as it was found that if the butanol was left in contact with the gels for longer periods, this could cause artefacts during electrophoresis. The gel tubes were inserted into the electrophoresis tank and the reservoirs were filled with buffer. The

protein samples were mixed with an equal volume of sample buffer; if the electrophoresis was to be carried out under reducing conditions, one drop of 2-mercaptoethanol was added, giving a final concentration of approximately 1%. The samples were applied to the tops of the gels and electrophoresis was carried out at 4mA/gel for about 18 hours until the dye-front reached the bottom of the gel. The gels were reamed out from the glass tubes and stained.

Staining Polyacrylamide Gels

Slab gels were stained with 2.5mg/ml Coomassie brilliant blue G-250, dissolved in water : methanol : acetic acid (9:9:2). The staining was carried out either at room temperature for 18 hours, or at 55^{o}C for 60 minutes. The gels were destained in water : methanol : acetic acid (75:4:6) at 55^{o}C.

Rod gels were stained by the same method as the slab gels, or with Coomassie brilliant blue R-250. In the latter case, the gels were fixed for 18 hours in water : propan-2-ol : acetic acid (65:25:10), stained for two hours in 1mg/ml Coomassie blue dissolved in the fixing solution, and destained in water : acetic acid : methanol (79:7:14).

APPENDIX 9

COUPLING PROTEINS TO SEPHAROSE

Proteins, and other compounds containing free amine groups, were coupled to Sepharose 4B (Pharmacia Fine Chemicals, London, U.K.) using the cyanogen bromide activation technique introduced by Cuatrecasas (1970).

The Sepharose 4B was activated by suspending 50ml of gel in 50ml water, and adjusting the pH to 10. 5g cyanogen bromide was dissolved in a minimum volume of dimethylformamide and added dropwise to the stirred gel. The pH of the solution was maintained between 9 and 11 by the addition of 6M sodium hydroxide, and the temperature was kept below 25^{o}C by the addition of crushed ice. The suspension was stirred for a further 15 minutes after the addition of the cyanogen bromide, with the pH and temperature being controlled as before.

The gel was then filtered on a sintered glass funnel and washed with 1000ml water, followed by 1000ml 0.2M citric acid-NaOH, pH6.9. The total washing time was less than 90 seconds.

0.05g of protein was dissolved in 50ml 0.2M citric acid-NaOH, pH6.9, and the activated Sepharose was resuspended in this solution. The mixture was stirred gently for 18 hours, and then filtered on a sintered glass funnel. The gel was resuspended in 200ml 0.1M glycine-NaOH, pH8.6, and stirred for a further 24 hours in order to neutralise excess imino-carbonate groups. Finally, the suspension was filtered on

a sintered glass funnel and the gel was washed with 2000ml water and 2000ml 0.05M tris-HCl, pH7.5, before being resuspended in this buffer.

APPENDIX 10

THE ISOLATION OF HIRUDIN

Ninety European leeches (*Hirudo medicinalis*), which had been starved for the preceding three months were killed by immersion in 96% ethanol for 24 hours. The anterior region of the animals was dissected from the remainder of the body, and dehydrated in 200ml fresh 96% ethanol for 24 hours. Hirudin was extracted from the dehydrated head sections as described by Markwardt (1970).

The dehydrated leech heads were chopped into small pieces with a razor blade and stirred for 30 minutes with a mixture of 40ml acetone and 60ml water. The supernatant was decanted and the residue was stirred for 30 minutes with a second 100ml volume of acetone : water (40:60). The supernatant was again decanted and pooled with the original extract. 20ml water and 80ml acetone were added to the pooled extract and the pH was adjusted to 4.4 with glacial acetic acid. The mixture was spun at 3500g in a Coolspin centrifuge (MSE Ltd., Crawley, Sussex, U.K.) for 15 minutes and the clear green supernatant was decanted. The pH of this solution was adjusted to 6.0 by the addition of 10% ammonia, and the volume was reduced to 30ml by rotary evaporation at 35^{o}C. 8ml of 30% trichloroacetic acid was added, lowering the pH to 1.8, and the protein was precipitated by the addition of 350ml acetone. The mixture was centrifuged at 3500g for ten minutes and the supernatant was discarded. The precipitate was resuspended in 50ml

acetone and again centrifuged at 3500g for ten minutes. The washings were discarded and the precipitate was stored in a vacuum dessicator for 18 hours.

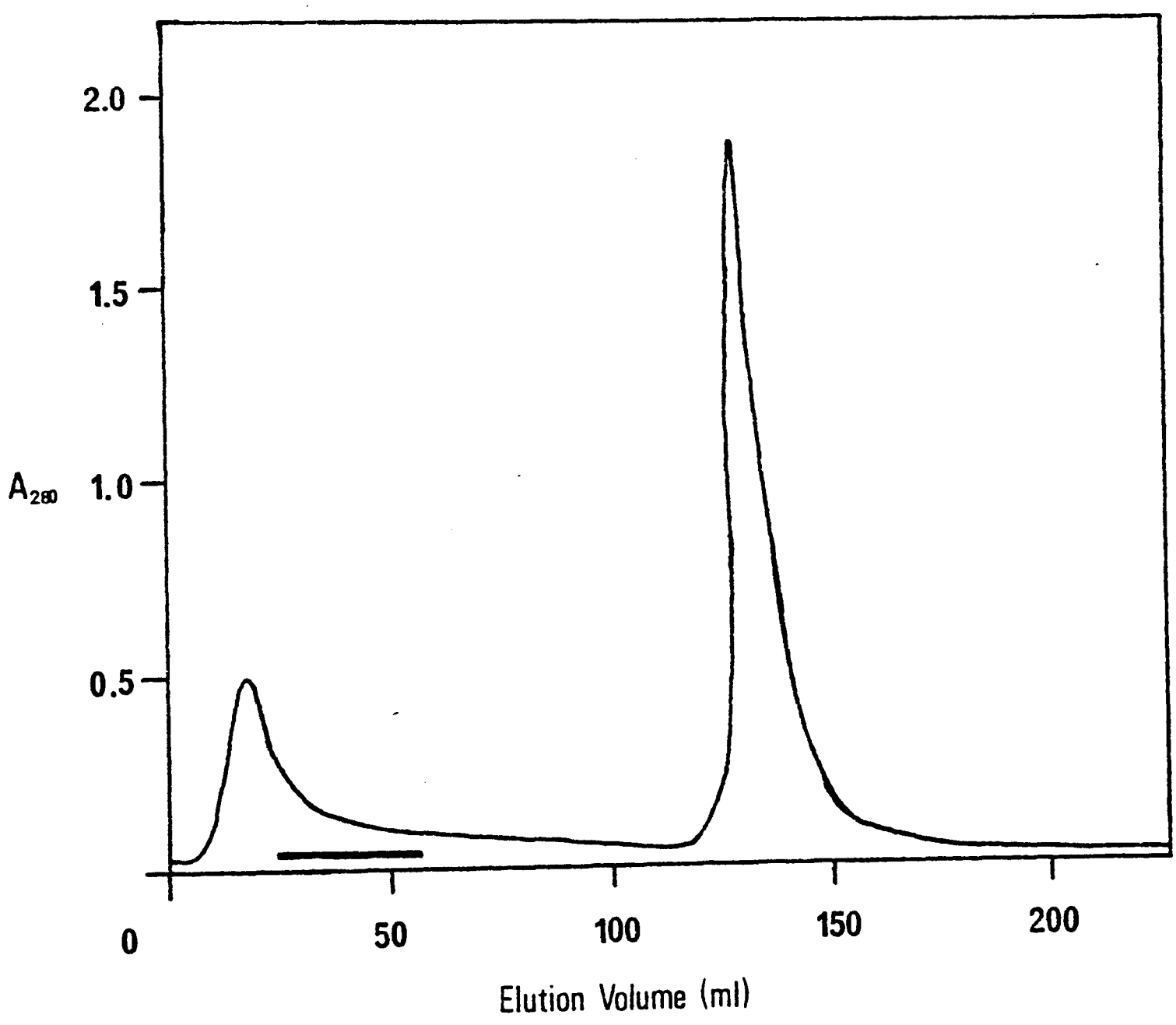
The dry brown powder thus obtained was suspended in 3.0ml water and cooled to 3°C. Six aliquots, each of 0.90ml, of ice-cold 96% ethanol were added at five minute intervals, and the mixture was left at 3°C for a further ten minutes. It was then centrifuged at 3500g for ten minutes and the supernatant was decanted. The precipitate was resuspended in 3.0ml of ice-cold water, mixed with 5.4ml cold 96% ethanol, and allowed to stand at 3°C for 30 minutes. The mixture was again centrifuged at 3500g for ten minutes, and the supernatant was decanted. The extraction of the precipitate with water and ethanol was repeated and the three supernatants were pooled. The pool was cooled to 0°C in ice and 38ml ethanol, containing 0.5% ammonium acetate, at -10°C was added. The mixture was left at 0°C for 30 minutes and then centrifuged at 3500g for ten minutes. The supernatant was discarded and the precipitate was washed with 50ml ice-cold ethanol before being dried in a vacuum dessicator for 18 hours.

The dry brown powder was dissolved in 10ml 0.01M ammonium acetate-acetic acid, pH4.6, and applied to a column (2cm diameter x 6cm long) of CM-Sephadex C-50, equilibrated with the same buffer. The column was washed with 100ml starting buffer at a flow rate of 75ml/hr, and the adsorbed proteins were then eluted from the gel with 1.0M ammonium acetate-acetic acid, pH7.0. Fractions of approximately 5ml were collected throughout, using an ISCO Model 328 Fraction

Collector and accessories (Instrument Specialities Co., Lincoln, Nebraska, U.S.A.). The fractions containing hirudin, which corresponded to the trailing side of the unadsorbed protein peak (Figure A.3), were pooled, divided into small aliquots and lyophilised.

FIGURE A.3

Chromatography of Hirudin on CM-Sephadex



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