ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. F. NORD

Edited by ALTON MEISTER

VOLUME 46

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CORNELL UNIVERSITY MEDICAL COLLEGE,
NEW YORK, NEW YORK

VOLUME 46

1978

AN INTERSCIENCE ® PUBLICATION

JOHN WILEY & SONS
New York • London • Sydney • Toronto

CONTRIBUTORS TO VOLUME 46

SAMUEL I. BEALE, *The Rockefeller University, New York, New York 10021*

S. GRANICK, *The Rockefeller University, New York, New York 10021*

WILLIAM B. JAKOBY, *Section on Enzymes and Cellular Biochemistry, NIAMDD, National Institutes of Health, Bethesda, Maryland 20014*

SUNG-HOU KIM, *Department of Biochemistry, Duke University Medical School, Durham, North Carolina 27710*

JUDITH P. KLINMAN, *The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111*

KLAUS MOSBACH, *Biochemical Division, Chemical Center, University of Lund, Lund, Sweden*

JACK PREISS, *Department of Biochemistry and Biophysics, University of California, Davis, California 95616*

JOHAN STENFLO, *Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden*

An Interscience ® Publication

Library of Congress Catalog Card Number: 41-9213

ISBN 0-471-02993-9

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

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VITAMIN K, PROTHROMBIN, AND γ-CARBOXYGLUTAMIC ACID

By JOHAN STENFLO, *Malmö, Sweden*

CONTENTS

I. Introduction

Prothrombin is the zymogen of the proteolytic enzyme thrombin, which by limited proteolysis converts soluble fibrinogen to insoluble fibrin, ultimately forming a blood clot. Vitamin K is required for normal biosynthesis of prothrombin (factor II)* and three other plasma proteins (factors VII, IX, and X), which are also zymogens of proteolytic enzymes involved in blood coagulation. The vitamin K-dependent modifications are required for the *in vivo* conversion of these proteins to the enzymatically active forms. The four vitamin K-dependent proteins have now been both purified and chemically characterized (1). The complete amino acid sequence of prothrombin has been determined by Magnusson et al. (2) and that of factor X by Enfield et al. (3) and Titani et al. (4). The mechanisms of *in vitro* activation of bovine prothrombin and factors IX and X have been outlined in considerable detail (5–17). Our understanding of the regulation of blood coagulation *in vivo*, however, is still incomplete.

Vitamin K activity is exhibited by 2-methyl-1,4-naphthoquinones substituted at the 3 position with a phytyl group (phylloquinone or

*The nomenclature for the coagulation factors is that recommended by an international committee [Wright, I., *J. Am. Med. Ass.*, *170*, 325–328 (1959)].

vitamin K_1) or with a multiprenyl side chain (the menaquinone series). Vitamin K was discovered by Dam (18) in the 1930s and the vitamin K-antagonist dicoumarol [3,3′-methylenebis(4-hydroxycoumarin)] was isolated and characterized by Campbell and Link in 1941 (19). Until the 1960s little progress was made in the understanding of the mechanism of action of the vitamin or the antagonist in relation to the biosynthesis of blood coagulation proteins. At this time experiments were initiated in which inhibitors of protein biosynthesis like actinomycin D and cycloheximide were used to study the regulatory effect of the vitamin on coagulant protein biosynthesis. Although early *in vivo* experiments suggested that vitamin K was involved in prothrombin biosynthesis by regulating DNA transcription, later experimental evidence favored the hypothesis that the vitamin was active in the conversion of a liver prothrombin precursor to biologically active prothrombin (20,21). This controversy was resolved when an intrahepatic precursor of prothrombin was identified in the livers of vitamin K-deficient rats (21,22) and when abnormal, biologically inactive prothrombin was isolated from blood plasma obtained from cows treated with the vitamin K-antagonistic coumarin anticoagulants (23–25). Structural comparisons of normal bovine prothrombin with abnormal, biologically inactive prothrombin led to the identification of the vitamin K-dependent structure, γ-carboxyglutamic acid in prothrombin (26). The identification of this previously unknown amino acid improved our understanding of the interactions of the vitamin K-dependent proteins with calcium ions and phospholipid. Furthermore a vitamin K-dependent carboxylase, which has now been partially characterized, was identified in rat liver (27–30). The rapid growth in our knowledge of blood coagulation biochemistry, particularly with regard to the chemistry and biochemistry of the vitamin K-dependent proteins, and our improved understanding of the involvement of vitamin K in the biosynthesis of these proteins, justify a brief review of this field.

This chapter deals mainly with the recent developments in the vitamin K and prothrombin field that led to the characterization of biologically inactive prothrombin from cows treated with the vitamin K-antagonistic coumarin anticoagulants, the identification of the intrahepatic prothrombin precursor in vitamin K-deficient rats, and the characterization of the carboxylated glutamic acid residues in prothrombin. The functional significance of these residues and the char-

acteristics of the vitamin K-dependent carboxylating enzyme system also are reviewed. Early metabolic studies on the involvement of vitamin K in the biosynthesis of blood coagulation proteins have been carefully reviewed (20,21) and are not cited here, nor do we discuss the information on the chemical properties of vitamin K and its antagonists (31) or more clinically oriented studies on vitamin K and prothrombin. For a broader perspective on blood coagulation, the reader is encouraged to consult a recent review (1).

II. Vitamin K-Dependent Proteins in Blood Coagulation

Blood coagulation is a series of consecutive conversions of zymogens of proteolytic enzymes to the corresponding active forms (1). This ultimately leads to the generation of thrombin, with subsequent conversion of soluble fibrinogen to insoluble fibrin. The product of each reaction in a sequence is the active enzyme in the following reaction. To describe such a sequence of reactions, the term "cascade" or "waterfall" reaction was introduced by Davie and Ratnoff (32) and by Macfarlane (33). In the simplified cascade scheme (intrinsic system) of Figure 1, the emphasis is on the reactions involving the vitamin K-dependent proteins. Factor X can be activated to factor Xa (the "a" is used to denote the enzymatically active forms of the coagulation proteins) either by factor IXa (1,5,34,35) in the so-called intrinsic system (Fig. 1) or in the so-called extrinsic system by factor VIIa (1,5,7–9) and a chemically uncharacterized tissue factor. The latter way of activation of factor X bypasses the earlier reactions in the intrinsic system. Both systems are triggered by cellular damage. Of the four vitamin K-dependent proteins, three (i.e., factors IX, X, and prothrombin) are active in the intrinsic system, whereas factor VII is the extrinsic factor X activator. Factors VIIa and IXa cleave the same peptide bonds in factor X (1,5,7,35).

The active forms of the vitamin K-dependent proteins are serine endopeptidases. In factors IXa, Xa, and thrombin (factor IIa) the amino acid sequences around the active sites are homologous with the corresponding sequences in trypsin and the other pancreatic serine endopeptideases (1). Trypsin cleaves almost any lysyl or arginyl bond, whereas the blood coagulation enzymes have a very restricted substrate specificity. Thus activation of prothrombin by factor Xa involves the cleavage of only two peptide bonds in prothrombin (1,9–17).

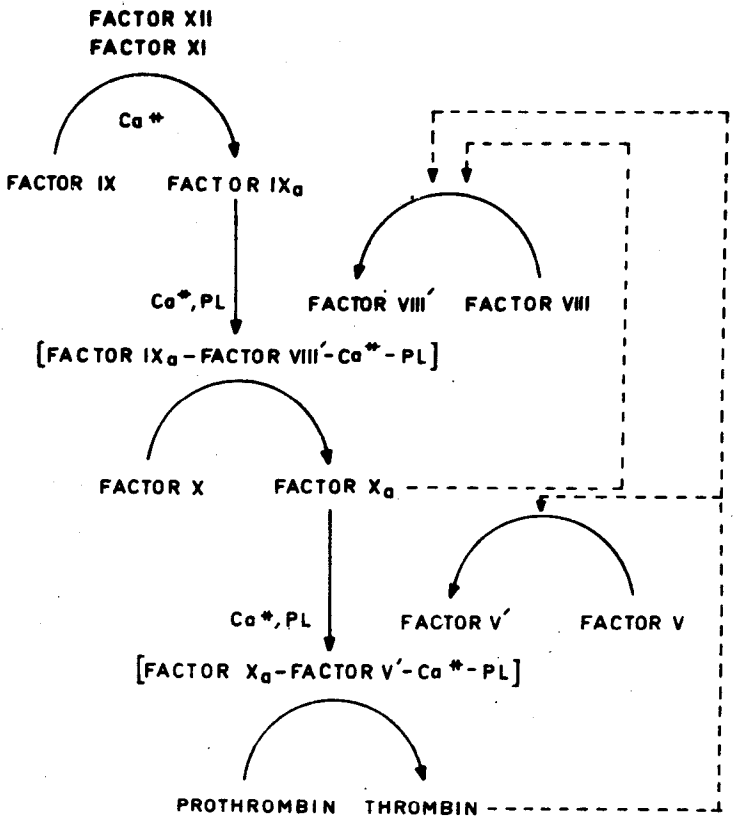

Fig. 1. Initiation of blood coagulation in the intrinsic system according to the "cascade" scheme. Emphasis is on the reactions involving vitamin K-dependent proteins; PL denotes phospholipid. Modified from Davie and Fujikawa (1).

Each zymogen activation in the blood coagulation cascade is the result of such limited proteolysis. When the system is triggered by tissue damage, the sequential arrangement of the individual blood coagulation reactions gives rise to consecutive limited proteolytic events, thereby producing an enormous potential for enzymatic amplification. The reaction rate in the cascade is influenced by accessory factors, such as phospholipid and the two high molecular weight proteins, factors VIII and V, which have no known enzymatic activities (1).

Factors V and VIII accelerate the rates of activation of prothrombin and factor X respectively (1).

The accessory factors that interact directly with the vitamin K-dependent parts of the coagulation proteins are calcium ions and phospholipid. Prothrombin and factors IX and X have been shown to bind Ca^{2+} ions in solution (17,36–40), whereas the biologically inactive forms of these proteins, which are synthesized after administration of the vitamin K-antagonistic coumarin anticoagulants do not (see below). It is clear from Figure 1 that the reactions in the cascade where the vitamin K-dependent proteins participate require calcium ions, and the activation of factor X and prothrombin, also require phospholipid. In the presence of calcium ions, prothrombin binds to phospholipid, whereas in the absence of calcium ions no binding is demonstrable (13,41–49). The part of prothrombin engaged in the calcium and lipid binding is the NH_2-terminal, fragment 1 part (43, Fig. 2). By comparison, the carboxyterminal part of the prothrombin molecule (prethrombin 1), from which thrombin is derived, does not bind to phospholipid, nor does enzymatically active thrombin (43), whose substrate is circulating as well as platelet-bound fibrinogen (1). An important difference between thrombin and factor Xa is that pro-

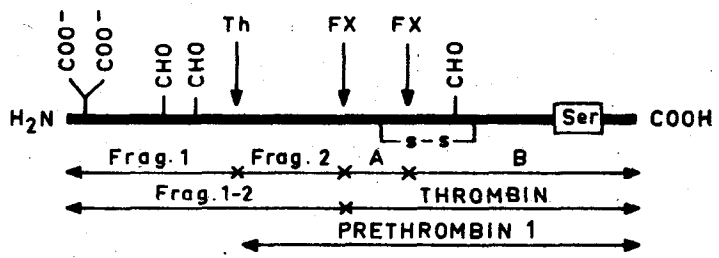

Fig. 2. Schematic diagram of the prothrombin molecule: COO^-, γ-carboxyglutamic acid residues; CHO, a carbohydrate side chain; Ser, the active site serine residue; Th, the single peptide bond in prothrombin cleaved by thrombin; FX, the two peptide bonds cleaved by factor X; A, the A or light chain of thrombin; B, the B or heavy chain. The nomenclature used to denote the peptide fragments generated from prothrombin by thrombin and factor X is that proposed by a task force of the International Committee on Thrombosis and Hemostasis. References for a comparison of this nomenclature to those used previously can be found in Myrmel et al., *Biochemistry, 15,* 1767–1773 (1976).

thrombin activation to thrombin involves cleavage at two peptide bonds with subsequent removal of the vitamin K-dependent part of the molecule from the enzymatically active part (1,2,11–17), whereas in factor Xa the vitamin K-dependent part is linked to the enzymatically active part by disulfide bridges (1,2,5–10). Thus unlike thrombin, factor Xa binds calcium ions and phospholipid. The increase in the rate of activation of prothrombin caused by the accessory factors has been illustrated *in vitro* in experiments that demonstrated that prothrombin activation in the complete system (factor Xa, Ca^{2+}, factor V, and phospholipid) was some 20,000-fold faster than the rate of activation in an identical mixture from which factor V and phospholipid were omitted (14,17).

The importance of the lipid binding *in vivo* has been proved in experiments (50,51) where factor Xa was infused into an experimental animal, either with or without calcium ions and phospholipid. In these experiments thrombus formation required that factor Xa be infused together with phospholipid (thromboplastin). The plasma concentration of the vitamin K-dependent proteins is low (i.e., prothrombin, 100–200 mg/liter; factors IX and X, 5–10 mg/liter; factor VII < 1 mg/liter) (1,8,52–53). It is thus apparent that the membrane affinity of these proteins is of vital importance for the blood coagulation cascade, which otherwise would be stifled because of dilution of the reactants and the inhibitory effect of circulating protease inhibitors, notably antithrombin III. This has been demonstrated experimentally with the abnormal prothrombin induced by coumarin anticoagulants (see below).

The close phylogenetic and functional relationship of the vitamin K-dependent proteins is reflected in their similar behaviors during purification. Several purification procedures are available for the vitamin K-dependent proteins, and most of them depend on the initial adsorption to and elution from barium citrate or some similar salt of divalent cations (54). This very effective purification step is usually followed by ammonium sulfate fractionation and chromatography on DEAE cellulose or DEAE Sephadex (Fig. 3), after which various forms of affinity chromatography are used to obtain the pure proteins (1,8,53). The proteins are assayed during purification either by coagulation bioassays (54,55) or with the aid of monospecific antisera (24,56). Because of the low plasma concentration of factor VII, purification of this protein is far more demanding than purification of the

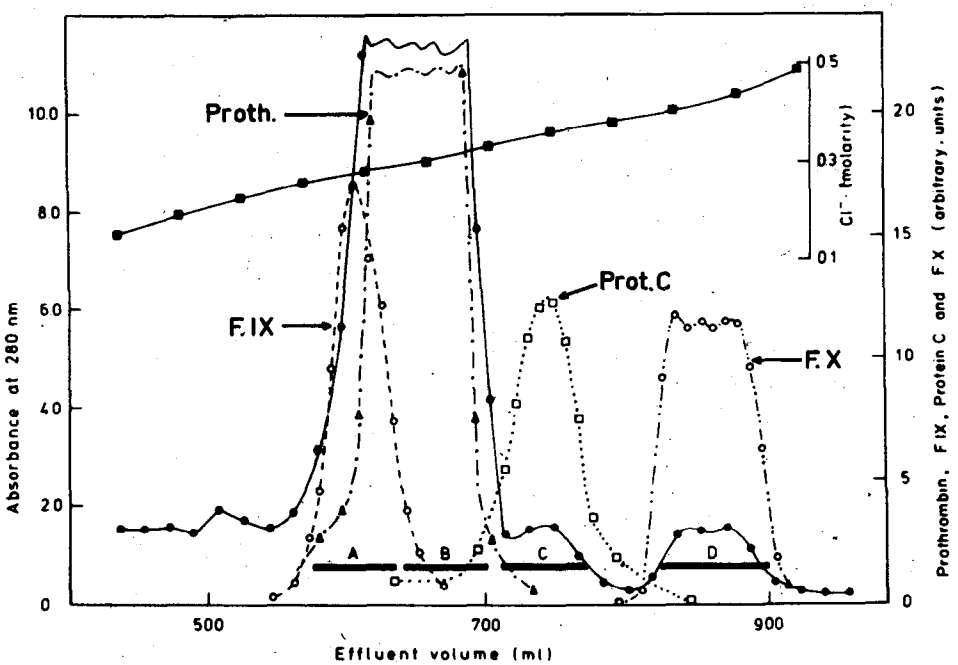

Fig. 3. Chromatogram of vitamin K-dependent proteins on a column of DEAE-Sephadex A-50 (2.5 × 40 cm). The column was equilibrated with 0.1 *M* phosphate buffer, 1 m*M* benzamidine, pH 6.0. Elution was accomplished with a linear gradient of NaCl (0.15–0.55 *M*). Curves: ●—●, absorbance at 280 nm; ■—■, Cl^- concentration. Factor VII was eluted with the peak between prothrombin and protein C. The concentrations of the vitamin K-dependent proteins were determined immunochemically. Modified from Stenflo (56).

other vitamin K-dependent proteins. To obtain approximately 2 mg of pure factor VII, 50 liters of plasma must be processed (8,53).

III. Abnormal Plasma Prothrombin Induced by the Vitamin K-Antagonistic Coumarin Anticoagulants

The first observation that dicoumarol treatment leads to the biosynthesis of abnormal prothrombin was made by Hemker et al. (57), who indirectly demonstrated the occurrence of an inhibitor of prothrombin activation in plasma from patients treated with the anti-

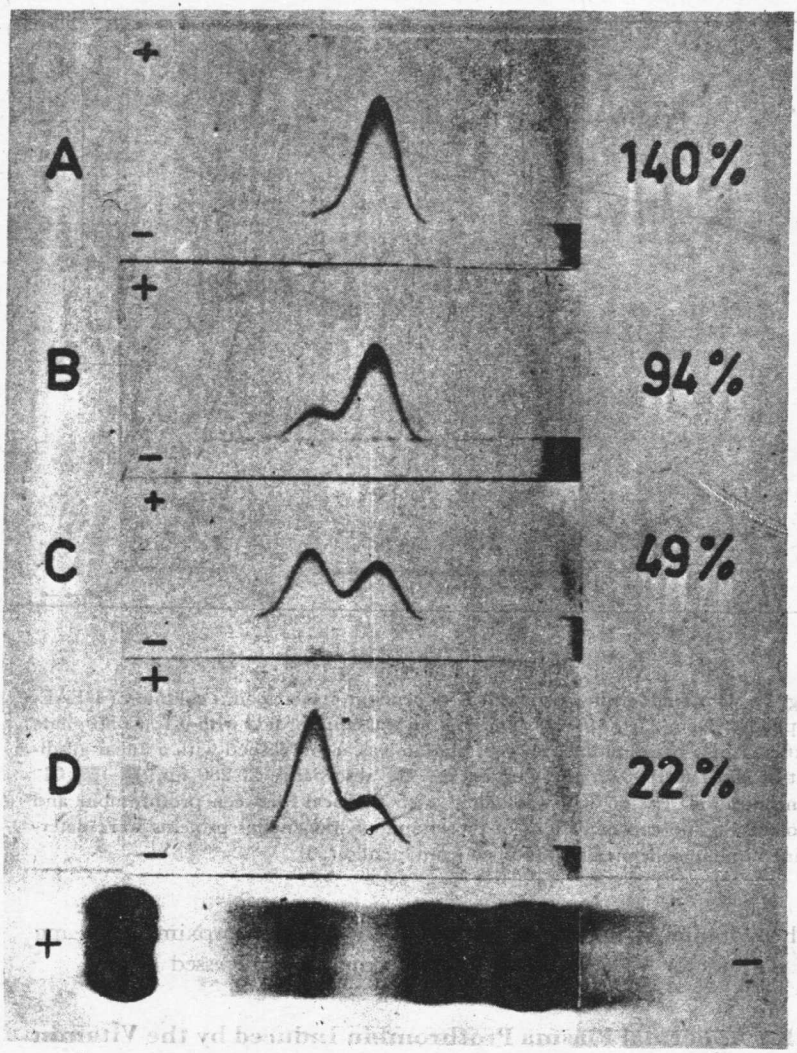

Fig. 4. Crossed immunoelectrophoresis (61) of bovine plasmas containing abnormal prothrombin induced by administration of coumarin anticoagulants. The electrophoresis was performed at pH 8.6 in 0.075 *M* barbital buffer containing 2 m*M* calcium lactate. -A monospecific antiserum against bovine prothrombin was used. (*a*) Immunoprecipitate obtained before administration of dicoumarol to the cow. (*b*)–(*d*) Precipitates given by samples obtained 2, 4, and 7 days after the beginning of dicoumarol administration. The prothrombin activity in the

coagulant dicoumarol. They also suggested that the inhibitor was a precursor of prothrombin. Direct evidence of the presence of abnormal prothrombin in plasma from patients treated with the vitamin K-antagonistic coumarin anticoagulants was provided with immunochemical techniques by Ganrot and Nilehn (58,59) and by Josso et al. (60). Crossed immunoelectrophoresis (61,62) made it possible to compare the abnormal prothrombin with normal prothrombin before fractionation of plasma, since both prothrombins have the same main antigenic determinants. Three important differences were noted (59); unlike normal prothrombin, the abnormal one neither bound calcium ions nor was adsorbed to barium citrate. Furthermore, when citrate plasma from a dicoumarol-treated individual was coagulated by the addition of calcium chloride, the abnormal prothrombin was not activated to thrombin as normal prothrombin is but appeared to remain unchanged. These results have since been extended and the dicoumarol-induced human prothrombin has been partially purified (63–68). Since purified dicoumarol-induced prothrombin does not inhibit the activation of normal prothrombin (24), the relationship of the inhibitor initially observed by Hemker et al. (57) to the immunochemically demonstrated abnormal prothrombin is not clear.

Figure 4 shows the appearance of abnormal prothrombin during dicoumarol treatment of a cow. Like human dicoumarol-induced prothrombin, the bovine counterpart has the same main antigenic determinants as the normal protein (23–25). Crossed immunoelectrophoresis has also been successfully used to demonstrate abnormal, dicoumarol-induced forms of factors IX and X in the bovine species (56,69). Like dicoumarol-induced prothrombin they do not bind calcium ions. In the terminology used by Hemker and coworkers (69), the coumarin anticoagulant induced forms of the vitamin K-dependent proteins are termed PIVKA (proteins induced by vitamin K-absence; i.e., PIVKA II, VII, IX, and X).

The differences between normal and dicoumarol-induced prothrombin observed with immunochemical methods suggested that calcium binding and biological activity are related to vitamin K action. A comparison between the two prothrombins was undertaken to reveal

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samples relative to an arbitrary standard is given to the right. The electrophoretic pattern of bovine plasma in agarose gel is given below as a reference for the electrophoretic mobilities of normal (cathodal) and abnormal (anodal) prothrombin. From Stenflo, and Ganrot (24).

the chemical basis of these differences. Since biosynthesis of abnormal, biologically inactive prothrombin could be induced in cattle by administration of coumarin anticoagulants, procedures based on conventional chromatographic methods for the purification of the bovine dicoumarol-induced prothrombin were devised by Stenflo and Ganrot (23,24) and Nelsestuen and Suttie (25). These purification procedures are far more cumbersome than those used for normal prothrombin because unlike biologically active prothrombin, the dicoumarol-induced prothrombin cannot be adsorbed to barium citrate. Recently a simplified method utilizing adsorption to immobilized antibodies as an essential purification step was described by Wallin and Prydz (70). Access to plasma from coumarin-treated cattle made possible the large-scale purification process necessary to obtain enough material for structural studies on the purified proteins. Furthermore, abnormal bovine prothrombin was advantageous for investigation because it has been more carefully characterized than human prothrombin (54). Its amino acid sequence has been determined (2), and a large body of information is available on the mechanism of activation of bovine prothrombin (1,11–17).

The purified dicoumarol-induced prothrombin is not activated to thrombin under normal circumstances, nor does it inhibit the activation of normal prothrombin (24,25). Structural comparisons of the intact proteins established that the molecular weights, as judged by acrylamide gel electrophoresis in sodium dodecyl sulfate, the sedimentation coefficients, the amino acid compositions after acid hydrolysis, the carbohydrate compositions and the NH_2- and COOH-terminal amino acid residues, are identical (25,71). Equilibrium dialysis showed that the dicoumarol-induced prothrombin binds at most one calcium ion per mole (36,37), whereas normal prothrombin binds 10–12 calcium ions per mole (17,37–39) (Fig. 5). At low calcium concentrations there is a positive cooperativity in the binding of calcium ions to normal prothrombin according to all reports (17,37,39,72) but one (38).

Although the dicoumarol-induced prothrombin has no prothrombin activity, it can be activated to thrombin by nonphysiological prothrombin activators (25,60). Thus staphylocoagulase, an exoprotein from *Staphylococcus aureus*, generates the same amount of thrombin activity from both normal and dicoumarol-induced prothrombin (60). In contrast to activation of prothrombin with its physiological activator, factor Xa (1), activation with staphylocoagulase is due not to pro-

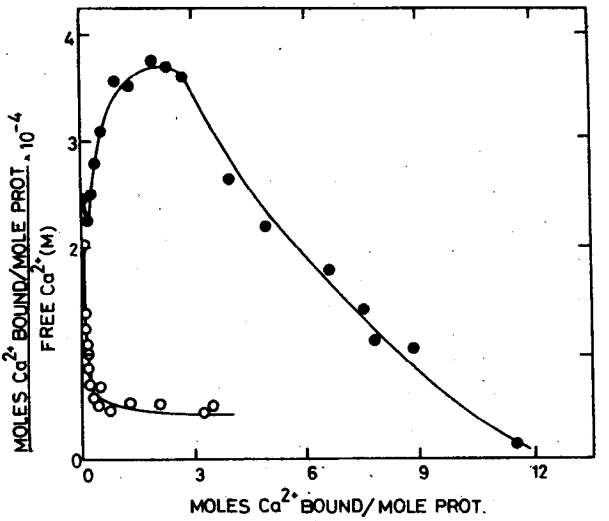

Fig. 5. Scatchard plot of Ca^{2+} binding of normal and abnormal, dicoumarol-induced prothrombin: ●, normal prothrombin; ○, abnormal prothrombin. From Stenflo, and Ganrot (37).

teolysis but to the formation of a complex of prothrombin and staphylocoagulase that has thrombin activity (73). Nelsestuen and Suttie (25) showed that both normal and dicoumarol-induced prothrombins can be activated by trypsin and by the venom from the snake *Echis carinatus* and that the same amount of thrombin activity was generated from both. These experiments indicated that the COOH-terminal (thrombin) portion (Fig. 2) of the dicoumarol-induced prothrombin molecule was intact and that the dicoumarol-induced prothrombin was inactive *in vivo* and in the standard prothrombin bioassays because of a defect in the activation mechanism. Additional evidence that the difference between the two prothrombins is in the NH_2-terminal parts of the molecules was provided when the proteins were cleaved with thrombin and the two fragments, fragment 1 and prethrombin 1, obtained from normal and dicoumarol-induced prothrombin were compared (74). Prethrombin 1 from both prothrombins had identical electrophoretic and immunochemical properties. Fragment 1 from normal prothrombin bound Ca^{2+} ions; but that from dicoumarol-induced pro-

thrombin did not. Differences were demonstrated in the peptide maps prepared from thermolysin digests of reduced and carboxymethylated fragment 1 from the two prothrombins. By digestion of normal bovine prothrombin with trypsin and subsequent adsorption of the digest with barium citrate, a calcium-binding peptide was isolated from the fragment 1 region of prothrombin (75). When dicoumarol-induced prothrombin was subjected to the same treatment, no such peptide could be isolated. It was thus firmly established that the vitamin K-dependent structures responsible for calcium binding were in the NH_2-terminal (fragment 1) part of prothrombin, and that the COOH-terminal (thrombin) part of the molecule was not modified by the action of vitamin K. Simultaneous studies on the phospholipid binding of prothrombin and fragment 1 clearly demonstrated that it was the fragment 1 portion of prothrombin that bound to phospholipid and that this binding involved Ca^{2+} ions (43). Stenflo (76) isolated and sequenced tryptic peptides obtained from digests of the NH_2-terminal cyanogen bromide fragment (residues 1–72) of the two prothrombins. The peptide containing residues 4–10 from normal prothrombin had the same amino acid sequence as a tryptic peptide isolated previously by Magnusson (77) and shown to have an anomalously high negative charge. This result was confirmed, and it was also shown that the peptide that consists of residues 4–10 in dicoumarol-induced prothrombin had lower negative charge, even though the amino acid compositions of these two peptides after acid hydrolysis were identical.

Prothrombin activation results from interaction of the protein with phospholipid, Ca^{2+} ions, factor V, and factor Xa (Fig. 1). Since calcium is required for binding of prothrombin to phospholipid (41–49), it seemed as if the abnormal prothrombin that did not bind calcium ions would not be bound to a phospholipid surface. These assumptions have since been verified experimentally by Esmon et al. (78), who showed that the purified bovine dicoumarol-induced prothrombin did not bind to phospholipid in either the presence or absence of calcium ions (Fig. 6). Furthermore, addition of phospholipid to a mixture of factor Xa, calcium ions, and normal prothrombin resulted in an immediate drastic increase in the rate of prothrombin activation. No such increase in the rate of activation was observed in an identical experiment in which dicoumarol-induced prothrombin was used. Similar experiments in which the factor V-catalyzed increase in the rate of prothrombin activation was studied, indicated that the factor

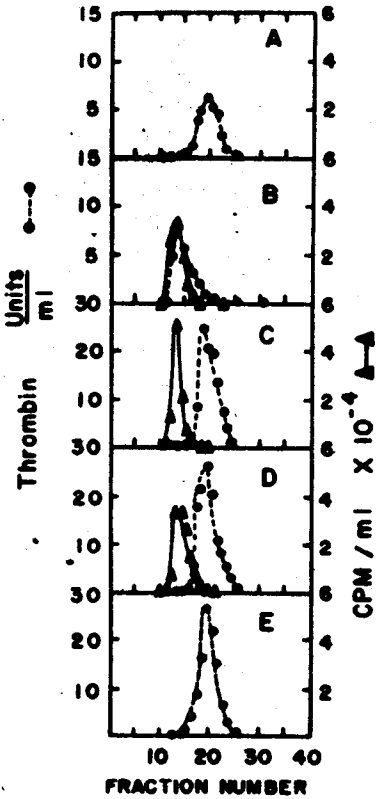

Fig. 6. Binding of normal and abnormal, dicoumarol-induced prothrombin to phospholipid (PL). Prothrombin, normal or abnormal (final concentration 0.2 mg/ml) was incubated for 30 min with 0.75 mg/ml of PL (equimolar mixture of [^{14}C] dioleoyl phosphatidylcholine and dioleoyl phosphatidyl glycerol) in 0.04 *M* Tris-HCl, 0.07 *M* NaCl, pH 7.5, in the presence or absence of 10 m*M* $CaCl_2$. After incubation, 0.4 ml samples were chromatographed on a Bio-gel A 0.5 *M* column (0.9 × 29 cm) equilibrated with buffer (see above) with or without 10 m*M* $CaCl_2$. The flow rates were 10 ml/hr and the fraction volumes were 0.55 ml. (*a*) Normal prothrombin with $CaCl_2$ but without PL. (*b*) Normal prothrombin with $CaCl_2$ and with PL. (*c*) Abnormal prothrombin with $CaCl_2$ and with PL. (*d*) Abnormal prothrombin and PL but without $CaCl_2$. (*e*) Abnormal prothrombin without PL but with $CaCl_2$. Curves: ●--●, thrombin activity; ▲—▲, PL, ^{14}C radioactivity. From Esmon et al. (78), with permission.

V-binding part (fragment 2) in dicoumarol-induced prothrombin was intact.

It has been reported that vitamin K is involved in the glycosylation of prothrombin and that there are differences in carbohydrate composition between prothrombin isolated before and during dicoumarol administration (67,79,80). This report has not been verified (81–83), and it is in disagreement with the results obtained by comparing normal and dicoumarol-induced bovine prothrombin in which no significant difference in carbohydrate composition could be observed (25,71). Furthermore, asialo- and aglycoprothrombin retain biological activity (82) and bind calcium ions (23), indicating that vitamin K is