The effect of liver disease on the vitamin K dependent factors in haemostasis.

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A thesis entitled

THE EFFECT OF LIVER DISEASE ON THE VITAMIN K DEPENDENT FACTORS IN HAEMOSTASIS

presented by
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SYNOPSIS

This thesis deals with the effect of liver disease on the vitamin K dependent clotting factors II, VII, IX, X. Chapter I is a review of the literature and gives a background to the biological importance of vitamin K and the mechanism of its action. In Chapter II the effect of liver disease on the synthesis of the vitamin K dependent clotting factors is described, in particular the effect on factor VII. It was concluded that there is a relationship between the synthetic ability of the liver and circulating levels of the vitamin K dependent clotting factors. This synthetic ability is disturbed in liver disease.

The study of serial changes in the vitamin K dependent clotting factors following the infusion of vitamin K is described in Chapter III. The study was concerned with assessing the effect of treatment on clotting factor levels as well as the pathways involved in the adsorption and utilisation of the vitamin K. In addition the study indicated that during the initial treatment with vitamin K the four vitamin K dependent clotting factors were synthesized at the same rate. The rate of synthesis during the 24 hour period of analysis appeared to be biphasic, indicating possible feed back mechanism on the synthesis pathway. Principal component analysis of the data derived during the 24 hour response indicated that an analysis at 16 - 18 hours may provide adequate information on the ultimate response to vitamin K.

Proteins induced by vitamin K absence (PIVKA) in liver disease were investigated in Chapter IV, using the modified thrombotest as well as immunological techniques. The presence of PIVKA and PIVKA II was demonstrated in some forms of liver disease. Following

the administration of vitamin K normal factor II appeared within one hour and a $T_{\frac{1}{2}}$ for PIVKA II of 17 hours was established.

Furthermore, the appearance and disappearance of normal factor II and PIVKA II are identical. It was concluded that such a finding was related to a conformational change in the intracellular PIVKA II and this finding is discussed in the context of its effect on the haemostatic mechanisms.

CONTENTS

CHAPTER I

BIOLOGICAL IMPORTANCE OF VITAMIN K AND THE MECHANISM OF ITS ACTION

1.1 INTRODUCTION

Liver disease is a common cause of clotting abnormalities such as decreased synthesis of normally structured clotting factors, increased utilisation of consumable factors, or combinations of all these abnormalities[1]. The wide pattern of clotting abnormalities found in liver diseases endorses the importance of the normal liver in the maintenance of normal haemostatic balance. Haemostasis is in the strict sense of the word the arrest of bleeding and involves a well balanced interaction between vessel walls, blood flow, platelets, clotting factors, fibrinolysis and the reticulo-endothelial system. Normally there appears to be a dynamic equilibrium between these factors, characterised by the absence of bleeding and efficient vascular function[2]. Physiologically this explains the marked changes in haemostasis sometimes seen in liver disease which exerts its effect mainly on the coagulation or clotting mechanism.

Owren[3] compared the ability to synthesise coagulation factors with the functional capacity of the hepatocyte. Therefore clotting tests, designed to measure factors produced by the liver, are in effect liver function tests. Unfortunately, such clotting tests, including assays, are not of great help in differential diagnosis of liver disease, as they often provide inadequate information on the complex mechanisms found in such disease processes.

Before studying the effect that normal and abnormal liver function has on the coagulation mechanism, it is proposed to delineate the factors concerned in the blood

clotting processes and to briefly outline the reactions involved.

1.2 BLOOD COAGULATION

A serious problem in understanding blood coagulation has been and still is the conflicting nomenclature used for the description of various coagulation constituents. This has rendered communication difficult, indeed the uninitiated investigator who starts working in this area is handicapped by this state of semantic confusion. An International Committee on Blood Coagulation Factors was formed in 1954 in order to design a common terminology for the unequivocal identification of blood coagulation factors.

The Committee has met almost annually and Roman numerals have been assigned to activities, the "absence" of which results in clinical abnormalities of blood coagulation. In the symbolic description established by the nomenclature committee, a Roman numeral refers to the inactive precursor state of the clotting factor in question; the corresponding active form of the clotting factor is denoted by the Roman numeral followed by the letter "a". Since numerals in themselves are principally meaningless, a table is composed (Table 1) in which the Roman numerals are correlated with the most common synonyms that are or were used in the literature.

The physical and chemical changes which result in the formation of a blood clot have been the subject of much investigation. One of the earliest blood coagulation theories was proposed by Morowitz[4,5] in which he postulated that prothrombin was converted to thrombin by tissue thrombokinase (thromboplastin) and calcium ions. Thus four factors were regarded as necessary components of the classical Morowitz theory of coagulation, i.e. calcium, thromboplastin, prothrombin and

TABLE 1

Roman Numerals	Synonyms
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue Thromboplastin
Factor IV	Calcium Ions
Factor V	Labile Factor, Pro-Accelerin
Factor VI	Not assigned
Factor VII	Stable Factor, Pro-Convertin
Factor VIII	Anti-Haemophilic Factor (AHF) and Anti-Haemophilic Globulin (AHG)
Factor IX	Plasma Thromboplastin Component Christmas Factor
Factor X	Stuart Factor, Prower Factor, Stuart-Prower Factor
Factor XI	Plasma Thromboplastin Antecedent (PTA)
Factor XII	Hageman Factor
Factor XIII	Fibrin Stabilising Factor (FSF)

fibrinogen. The essential feature of this theory was the existence of separate inactive components which could be activated into a two stage reaction by cellular damage as depicted in figure 1.

Though there had been disagreement with the classical theory at the beginning of this century it was generally accepted to be the simplest and most workable explanation of the facts of coagulation as then known. However, it became clear that more than four factors were concerned with coagulation[6,7,8]. Indeed by 1953 the theoretical position of the classical theory of Morowitz had to be modified to allow two new factors, namely factor V and factor VII to be included[9]. These factors were defined as accelerators of one of the basic reactions as shown in figure 2.

In recent years it has been established that the process of clotting involves a series of stages in a chain reaction[10,11]. Many of these are enzyme-substrate reactions initiated by contact activation and ending with the formation of a stabilised fibrin clot. Such a scheme is shown in figure 3 and is the "cascade" hypothesis of prothrombin activation[12,13]. This hypothesis states that all clotting factors are distinct entities in plasma, synthesized independently, and present in plasma as inactive precursors. Each coagulation factor is activated by the preceding one in a chain of events that ultimately leads to the conversion of prothrombin to thrombin.

Thus the formation of thrombin from prothrombin is seen as a complex mechanism in which plasma coagulation factors, phospholipids, and/or extracts from tissue cells participate. However, the initial hypothesis only explained the pathway by which intrinsic thromboplastin

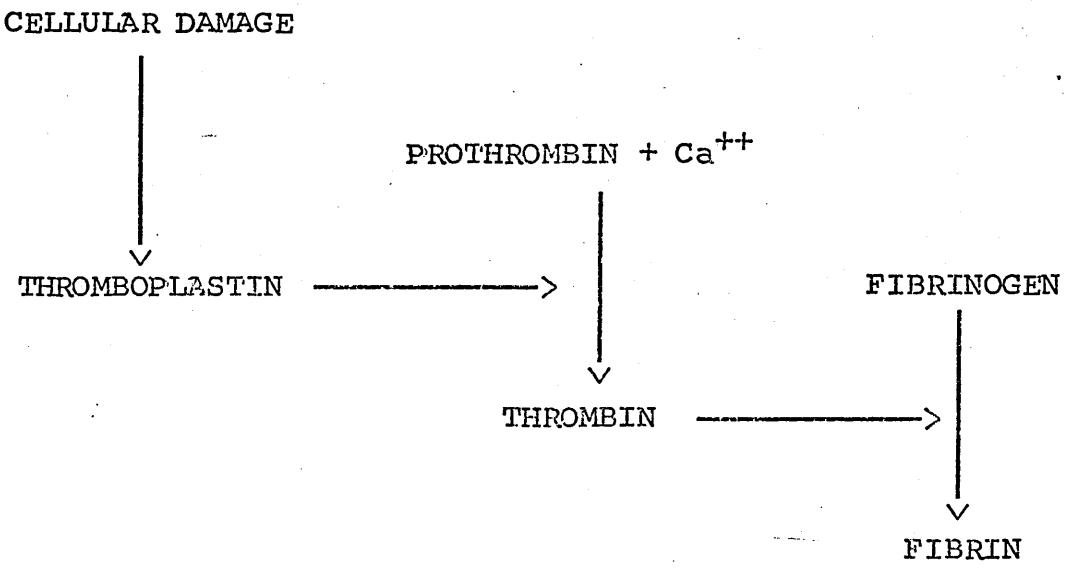


Figure 1

The four-factor theory of blood clotting

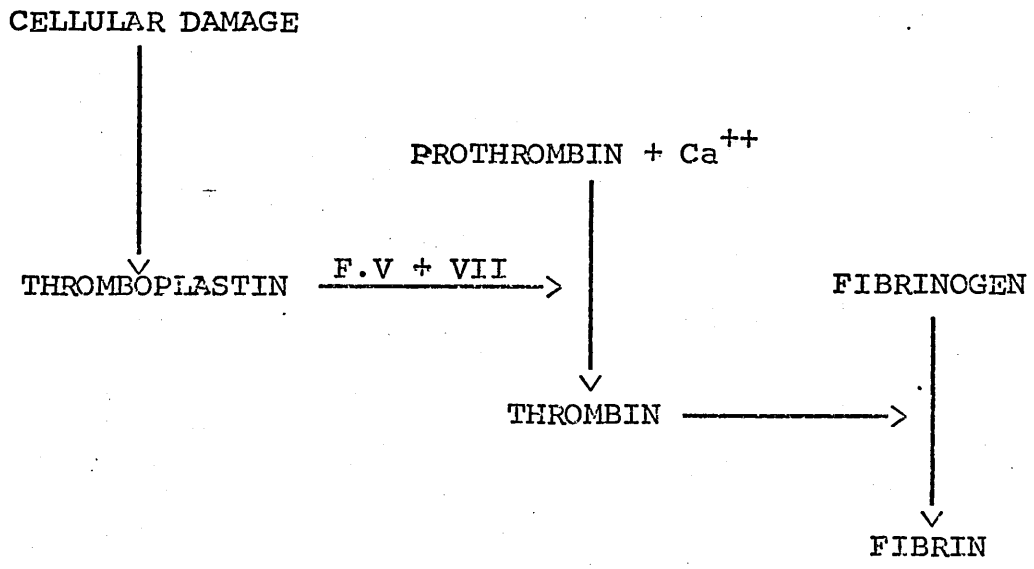


Figure 2

The four-factor theory expanded to include factors V and VII

INTRINSIC SYSTEM

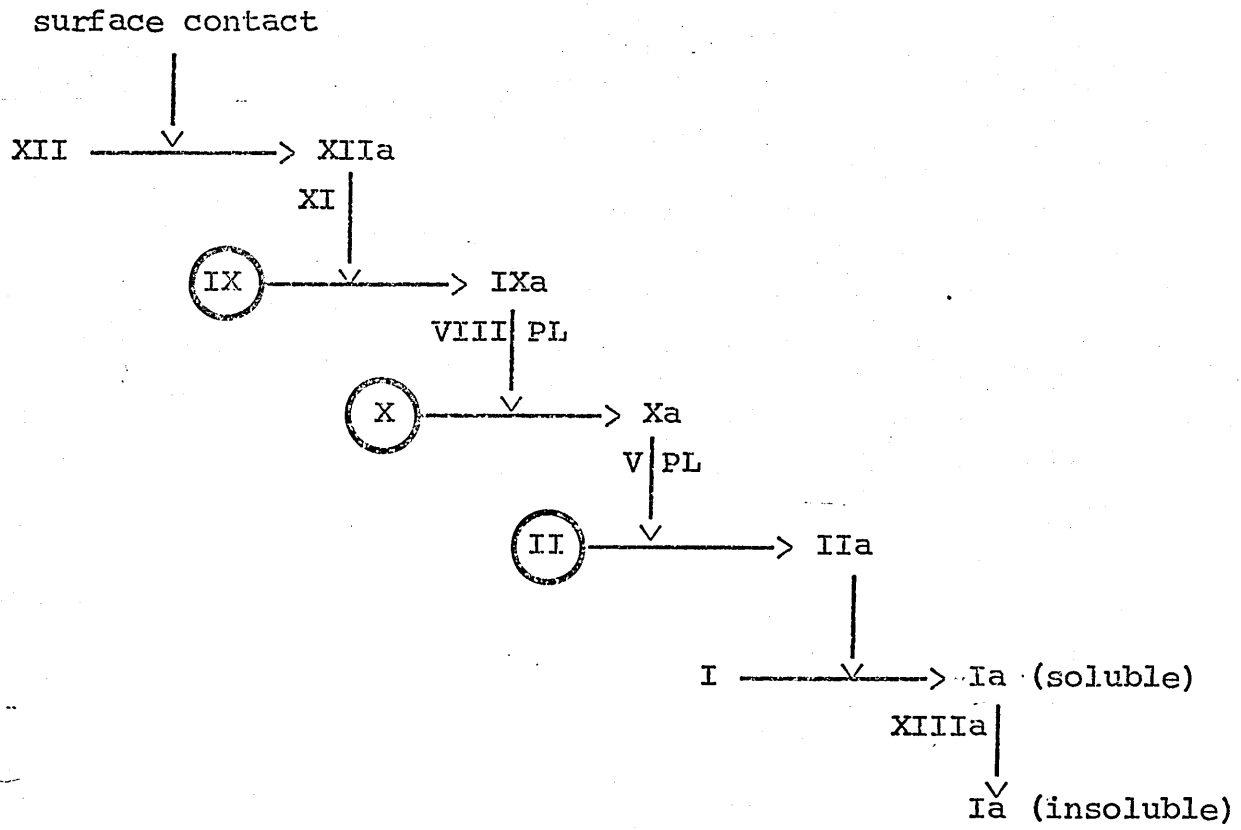


Figure 3

Cascade or waterfall concept of prothrombin activation (intrinsic pathway)

The encircled factors are all Vitamin K dependent and coumarin sensitive.

is produced from contact activation involving plasma factors. An alternative pathway for the production of extrinsic thromboplastin initiated by tissue substances not normally present in the circulating blood, was discovered. More specifically it implies the participation of tissue factors in the generation of thrombin as shown in figure 4.

The original cascade model of blood coagulation pathways as proposed by MacFarlane has further been modified following the recognition of the formation of complexes between certain activated factors. A complex between factor XIIa and XI has been shown and evidence has been provided that this activates factor IX[14]. Likewise it has been postulated that factor IXa and factor VIII form a complex on the surface of phospholipid in the presence of calcium ions[15]. This complex may act as a factor X converting enzyme. The formation of a complex of factor V and factor X with phospholipid and calcium ions has been established and this converts prothrombin into thrombin[16]. A complex of tissue thromboplastin factor VII phospholipid and calcium ions has been proposed which also activates factor X[17].

Although the division of the coagulation mechanism into intrinsic and extrinsic pathways is a useful model no strict separation does exist. Interactions between intermediate reaction products have been demonstrated, for example, the combined effect of factor XII and IX on factor VII[18]. Figure 5 incorporates these most recent findings promoting the idea that several complexes formed between phospholipids, certain coagulation factors and calcium ions are involved in the formation of thrombin from prothrombin.

It is in the formation of these complexes that the vitamin K dependent factors are believed to play an important role.

EXTRINSIC SYSTEM

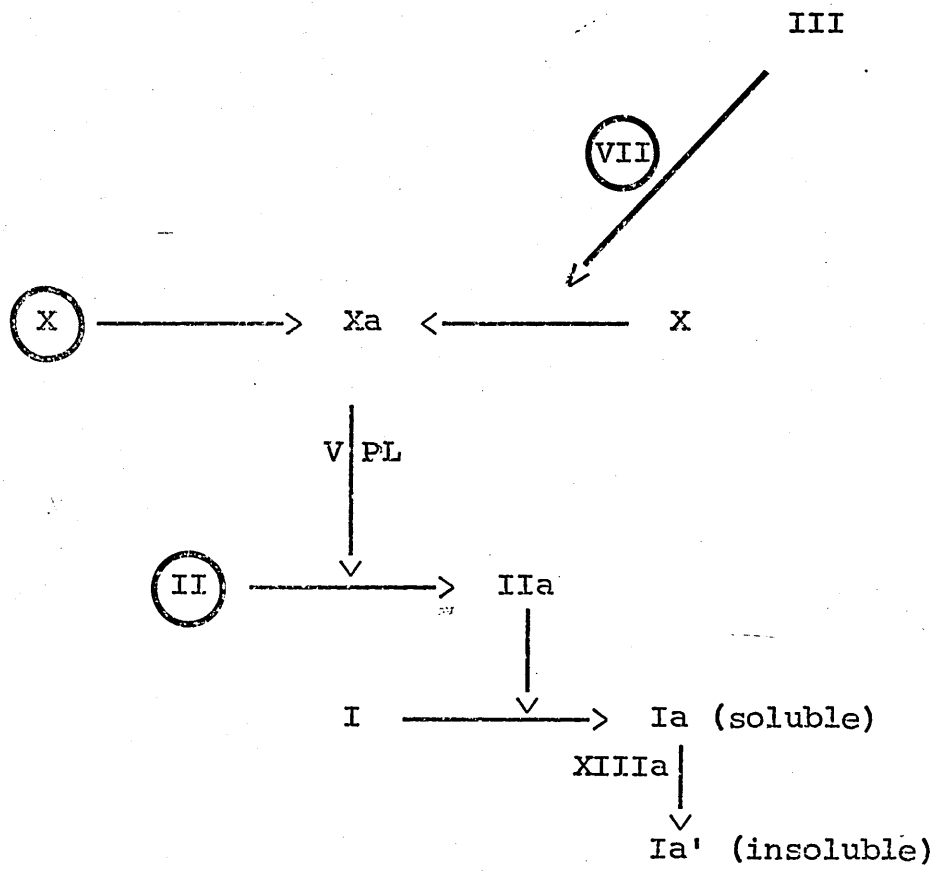


Figure 4

Extrinsic pathway of prothrombin activation

The encircled factors are all vitamin K dependent and coumarin sensitive.

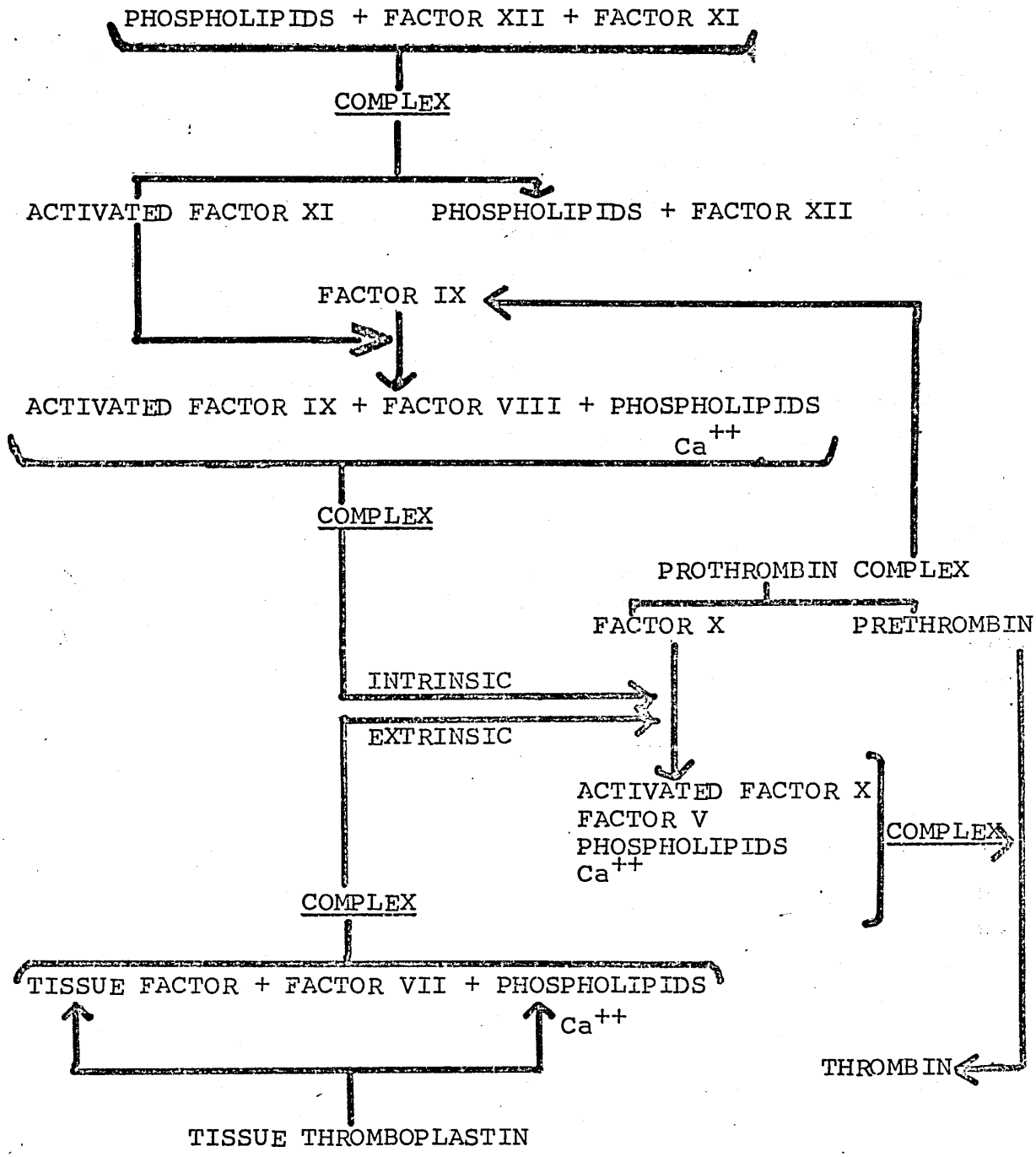


Figure 5

Activation of prothrombin to thrombin via the intrinsic and extrinsic pathways

1.3 PHYSIOLOGICAL ROLE OF THE LIVER IN BLOOD COAGULATION

The liver, the largest secreting organ in the body is known to be the main site of synthesis of plasma proteins[19]. It is believed to be the major site of synthesis of most of the plasma clotting factors. Indeed there is strong evidence that clotting factors I, II, V, VII, IX and X are produced primarily, some of them exclusively by the liver. In addition to being synthesised in the liver, the clotting factors have a short survival in the circulation and their plasma concentration can be assayed by reliable methods. Because the synthesis is reflected by blood plasma levels, which depend on production and consumption, the determination of the level and type of clotting factors in circulation is found to be useful in assessing the course of liver disease. Four of the plasma clotting factors, II, VII, IX, X, are dependent on Vitamin K for activity. Therefore the plasma levels of the Vitamin K dependent factors which are specifically synthesised by the hepatocyte cells in the liver, reflect the synthetic capacity of the liver, providing there is no Vitamin K deficiency.

1.4 VITAMIN K

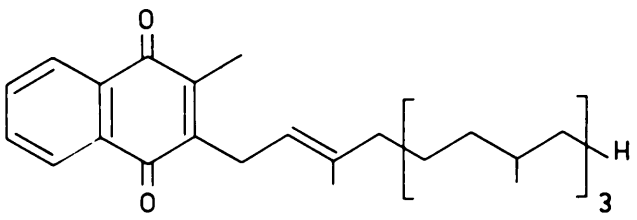
In 1935 Dam[20] discovered a factor in food which prevented haemorrhage in newborn chicks. He called this factor Vitamin K and postulated that it was necessary for the hepatic synthesis of clotting factors[21]. In 1939 there was the first indication that the bleeding disorder connected with Vitamin K deficiency is related to a lack of prothrombin[22] (Factor II).

1.4.1 Chemistry absorption and kinetics of Vitamin K

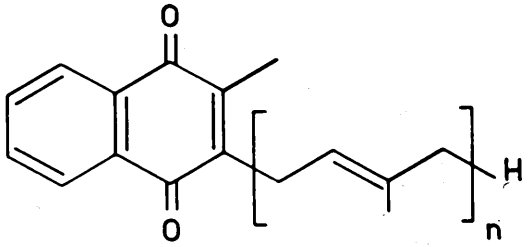
The isolation and elucidation of the chemical structure of Vitamin K was carried out by several groups of workers independently. Natural forms of Vitamin K, designated K_1 and K_2[23] are derivatives of 2-methyl-3 4 naphthoquinone (Fig.6).

Vitamin K_2 represents a series of compounds, the menaquinones, in which the phytyl group at position 3 of phylloquinone (K_1) is replaced by a side chain built up of two to nine isoprenyl groups. Phylloquinone is found in plants, and is the only natural Vitamin K available for therapeutic use. The menaquinones are synthesised, in particular, by gram positive bacteria. There are also a number of synthetic quinone derivatives which have been tested for Vitamin K activity[24]. Amongst those with activity approaching that of the natural vitamin is 2-methyl-1-4 naphthoquinone, known as menadione (Fig.7),

The essential difference between the natural and synthetic forms of Vitamin K is the length of the side chains. Evidence is available which suggests that the liver converts synthetic forms of Vitamin K to active forms[25]. Bile is required for the absorption of natural Vitamin K in the upper part of the small bowel[26]. Vitamin K_1 and K_2 are devoid of pharmacodynamic action: however, menandione and its water soluble derivatives cause respiratory depression and death in animals in the dose range of 200-500 mgms per kilo[27]. These doses are far beyond the range employed in therapy. Vitamin K can be obtained from two sources - (1) exogenous Vitamin K available from vegetables, in particular those with dark green leaves; (2) endogenous Vitamin K produced by normal bacterial flora in the bowel. Once absorbed the



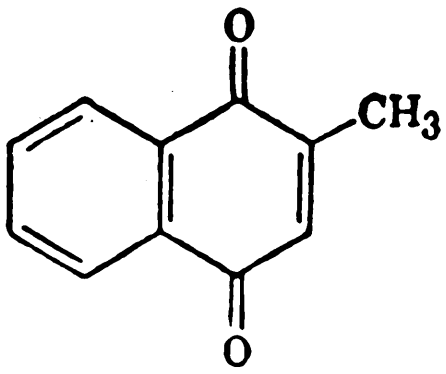
Vitamin K_1 = phylloquinone



Menaquinones
n = 4 MK-4

Figure 6

Various forms of natural Vitamin K



Menadione
(2-methyl-1,4-naphthoquinone)

MENADIONE

Figure 7

Synthetic Vitamin K

Vitamin K appears to complex with lipoporteins and chylomicrons in circulation before being transported to the liver. Animal studies indicate that the liver is the main storage depot of Vitamin K and the liver is also the principal storage site of Vitamin K in man[28]. Just how much Vitamin K is required for physiological needs is difficult to assess. Human requirement appears to be satisfied by the average diet and in addition the vitamin synthesised by intestinal bacteria is available. Work carried out on dogs by Quick and his group[29] and later confirmed by Quick[30] established the daily requirement of natural Vitamin K for adult dogs. They found that 0.5 micrograms per kilo of body weight was the minimum daily dose which would maintain prothrombin at the normal level. When the dose was reduced to 0.25 micrograms prothrombin dropped to the 50 per cent level. While adult dogs require an extremely small maintenance dose of Vitamin K, growing puppies in marked contrast need daily as much as 10 micrograms per kilo of body weight. These findings appear to suggest that requirements for Vitamin K are significantly influenced by growth[31].

1.4.2 Role of Vitamin K in the Synthesis of Clotting Factors

The principal biological activity of Vitamin K is related to the production of four plasma proteins, i.e. clotting factors II, VII, IX and X. The biological activity of various forms of Vitamin K has been reviewed by Griminger[32]. Exactly what the active form of Vitamin K is and whether this form is the same in different species is not known. It seems very probable that the composition of Vitamin K in liver is determined by the type of Vitamin K absorbed from the gut and not primarily by metabolic events in the liver. The exact subcellular localisation of Vitamin K and of coagulation factor

biosynthesis is not yet precisely known. Recently, however, Bell and Matchiner[33] showed that injection of physiological amounts of 3H-labelled phylloquinone into Vitamin K deficient rats resulted in the deposition of radioactive label in the microsomefraction of rat liver. If the vitamin or principal active metabolite is bound to its site of action, this finding indicates that Vitamin K acts at the microsomal level. Martius[34] postulated that Vitamin K might have a function in electron transport in mammalian tissues, as it does in bacterial systems where a deficiency results in a defect in oxidative phosphorylation. The rapid turnover of the Vitamin K dependent clotting factors would make them particularly sensitive to a decreased energy supply in the cell. Arguments in support of this hypothesis and objections against it have been reviewed by Pennock[35].

Research has also shown that Vitamin K does not constitute part of the clotting factor molecule and recent research indicates that the naphthaquinone nucleus of the Vitamin K could be the prosthetic group of an enzyme system which produces the Vitamin K dependent factors II, VII, IX and X[36]. In addition, Vitamin K does not maintain the circulating level of these factors by preventing their degradation, nor does it affect the release of completed clotting factors from liver hepatocytes[37,38]. The current hypothesis on the role of Vitamin K in blood coagulation factor biosynthesis is that it activates an intracellular protein without coagulant activity and that Vitamin K is involved in the attachment of different prosthetic carbohydrates moieties with the resulting formation of factors II, VII, IX and X at the microsomal level[39].

Support for this hypothesis comes in examining the steps in protein synthesis and the positions at which several inhibitors interfere in the biosynthesis process.

Figure 8 summarises the basic steps of protein synthesis.

The administration of actinomycin D which instantaneously inhibited DNA-transcription in Vitamin K deficient rats was unable to block the rise in the plasma factor II following administration of Vitamin K. This makes the existence of a controlling function at the level of DNA-transcription, as a hypothesis, unattractive[40]. The question of a link between de novo Vitamin K action and de novo protein synthesis has been studied with inhibitors of general protein synthesis, i.e. puromycin and cyclohexamide. When cyclohexamide is given to Vitamin K deficient rats 30 minutes prior to the administration of Vitamin K, 70% of expected biologically active factor II is produced[41]. This indicates inhibition of factor II synthesis of only 30%. On the other hand cyclohexamide will inhibit general protein synthesis by 80-90%. In similar experiments newly-formed factor II contains no radioactive amino-acids if these amino-acids are administered after cyclohexamide suggesting that the newly formed factor II came from a pre-existing pool of protein[42]. Prydz and Gaudermack[43] observed that puromycin did not inhibit the release of factor VII from a microsomal subfraction of rat liver after a period of time. Olson[44] could still produce factor VII and X activity in rat liver of coumarised rats with Vitamin K given 2½ hours after puromycin.

These studies suggest that under conditions inhibiting the formation of coreprotein Vitamin K has been able to effect the production of factors II, VII and X from an intracellular pool. Thus the precursor or precursors of factors II, VII, IX and X are produced in the hepatocyte and then Vitamin K is required for the complete formation of these factors from the precursors. It is possible that the Vitamin K-dependent step is the attachment of the carbohydrate moiety to the clotting protein.

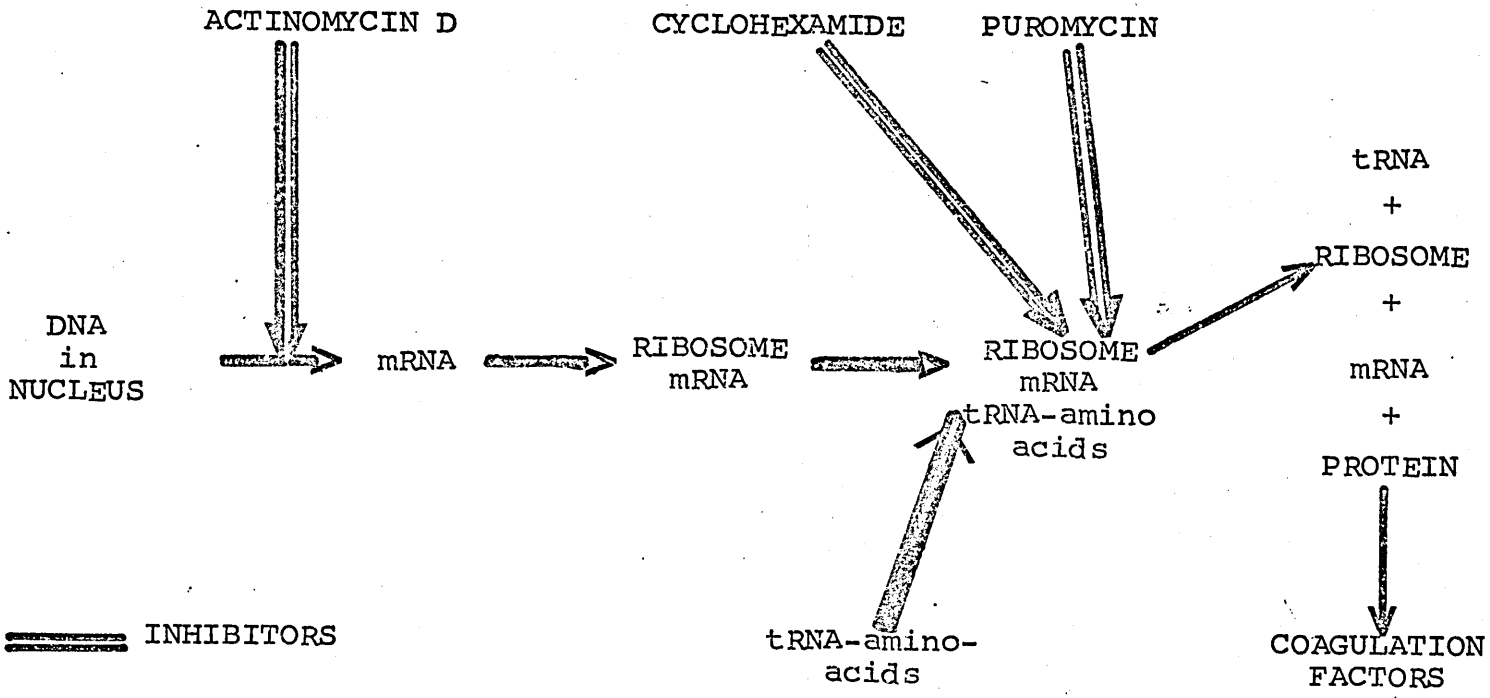


Figure 8

Basic steps of protein synthesis

It was reported that coumarin treatment of rats reduced the plasma level of factor II and inhibited the incorporation of glucosamine into factor II[45]. Vitamin K given to coumarin treated rats caused an increased rate of glucosamine incorporation. Conclusions from these studies were that the site of action of both dicoumarol and Vitamin K with respect to factor II synthesis is after the incorporation of amino-acids into the core protein. More precisely at, or prior to, the attachment of glucosamine to the core protein by glucosamine transferase. Reporting on Vitamin K dependence of glucosamine and mannose incorporation into the factor II molecule, Johnson[46], using labelled glucose and mannose, indicated that the site of function of Vitamin K is in the step involving the addition of the first carbohydrate to the polypeptide chain. However, later investigations on the carbohydrate content of purified factor II from both control and dicoumarol treated rats show that the decrease in coagulant activity of factor II was not due to altered carbohydrate content[47]. The investigation concluded that the site of action of dicoumarol inhibition of factor II biosynthesis follows the synthesis of core protein. Furthermore, it precedes glycolation. Figure 9 shows hypothetical mechanism of the action of Vitamin K on the synthesis of the Vitamin K dependent factors.

More recently studies on Factor II[48,49,50] revealed that it had the same antigenic determinants as abnormal factor II. The main difference between the two molecules was an inability to bind calcium and be adsorbed on to barium sulphate. Normal prothrombin can do both, whereas the abnormal protein cannot. From this type of analysis involving factor II it was suggested that the Vitamin K dependent step involves modification of amino acid residues to form calcium binding sites[51,52].

The use of N.M.R. and mass spectometry indicated that the peptide obtained from normal factor II contained

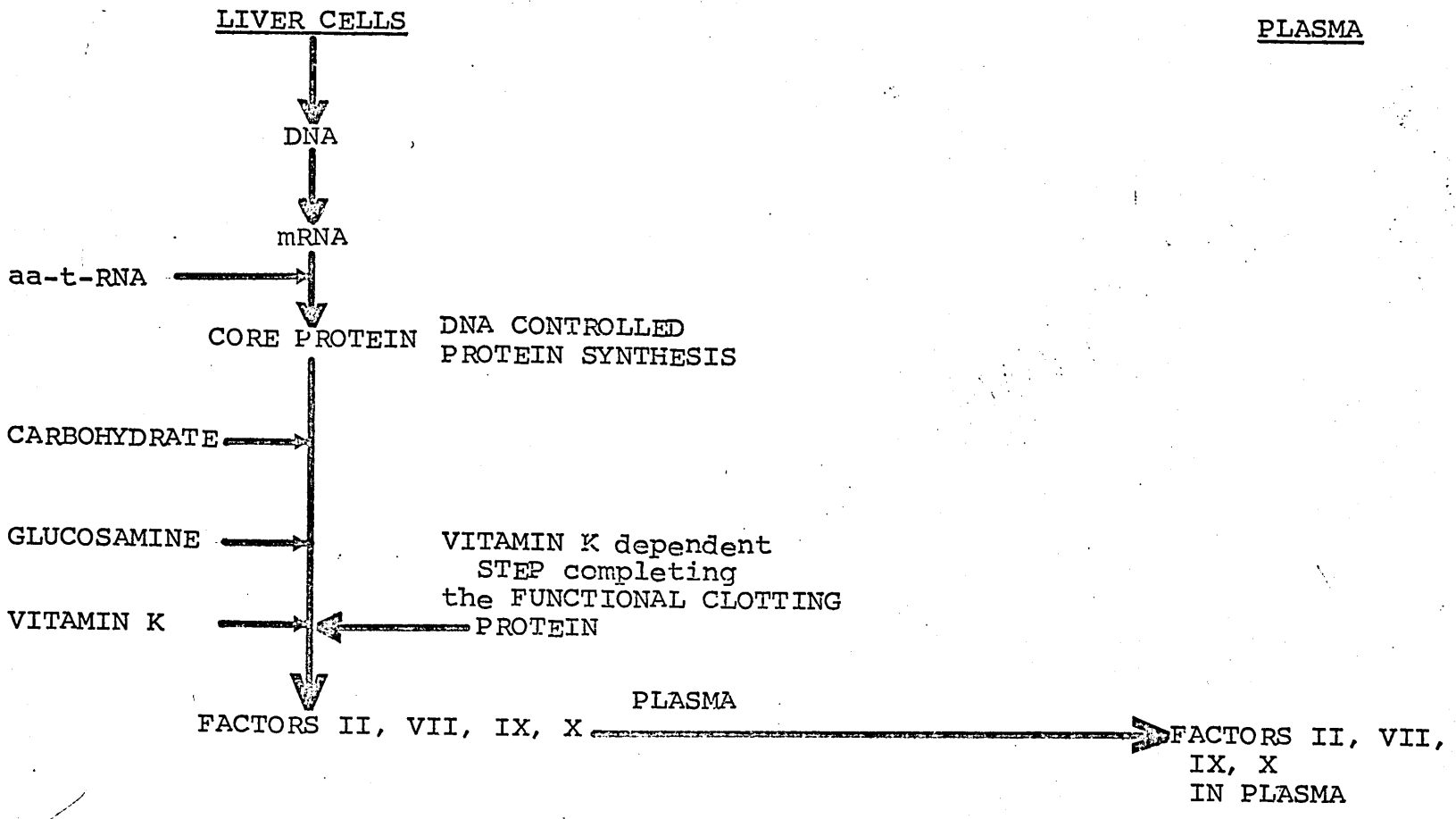


Figure 9

Hypothetical mechanism of the action of Vitamin K

an extra COOH group on each of two glutamic acid residues turning it into γ-carboxyglutamic acid (Fig.10) and these were missing on abnormal forms of factor II[53,54]. It would appear therefore that Vitamin K dependent structures are γ glutamic acid residues and that Vitamin K is involved in the carboxylation of glutamic acid.

1.4.3 Proteins induced by Vitamin K Absence

The work on Vitamin K and hepatic synthesis of factors II, VII, IX and X by Hemker et al[55] showed that patients treated with Vitamin K antagonists develop a circulating competitive inhibitor and postulated that it was a precursor molecule, going into the circulation because of the inhibition of a late Vitamin K dependent step. These postulated precursor molecules were called "proteins induced by Vitamin K absence" (PIVKA). As each of the four Vitamin K dependent factors may have its own precursor, it may be possible to have PIVKA II PIVKA VII PIVKA IX PIVKA X in circulation in various stages of liver disease. Figure 11 outlines Hemker's hypothesis on PIVKA protein formation.

1.4.4 The influence of drugs on Vitamin K

Oral anticoagulants such as the coumarin and indanedione drugs interfere with the action of Vitamin K within the hepatocyte[56]. These drugs do not directly inhibit factors II, VII, IX and X, but lower the plasma levels of these coagulation factors by decreasing production. Because of the similar structure of coumarin derivatives and Vitamin K (fig.12) it was suggested that the antagonism between the compounds is of a competitive type.

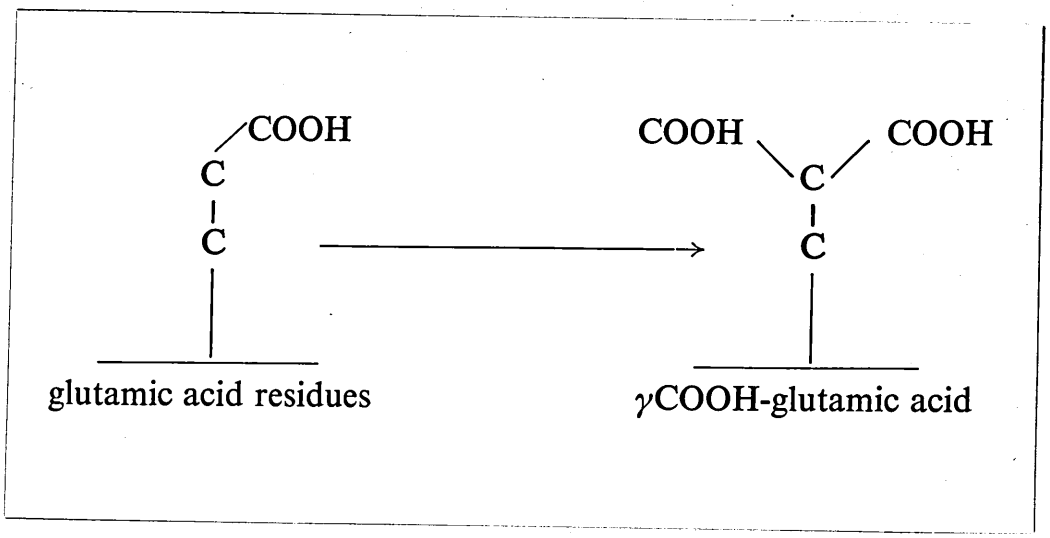


Figure 10

Carboxylation of glutamic acid residues

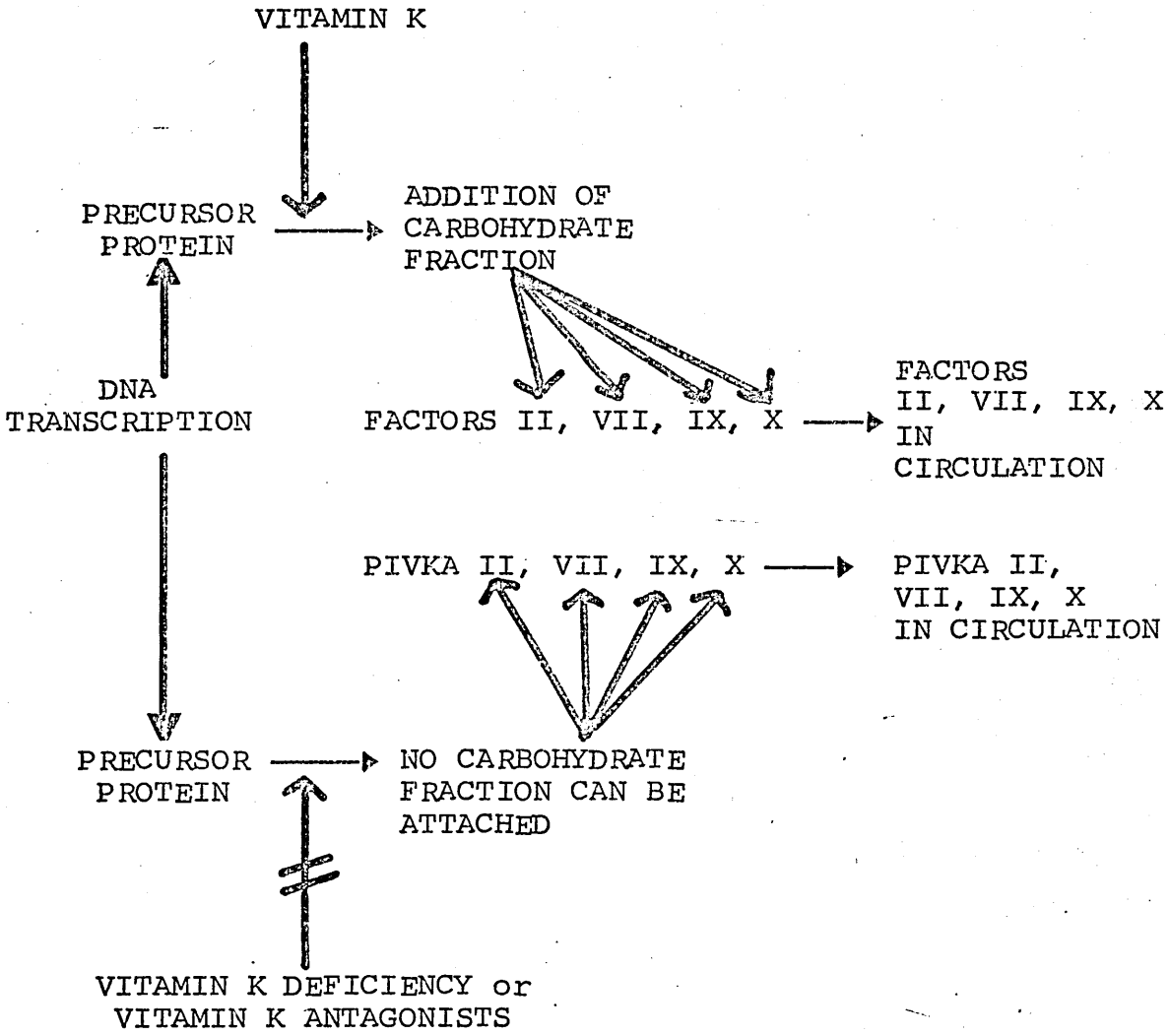


Figure 11

P.I.V.K.A. Protein Formation

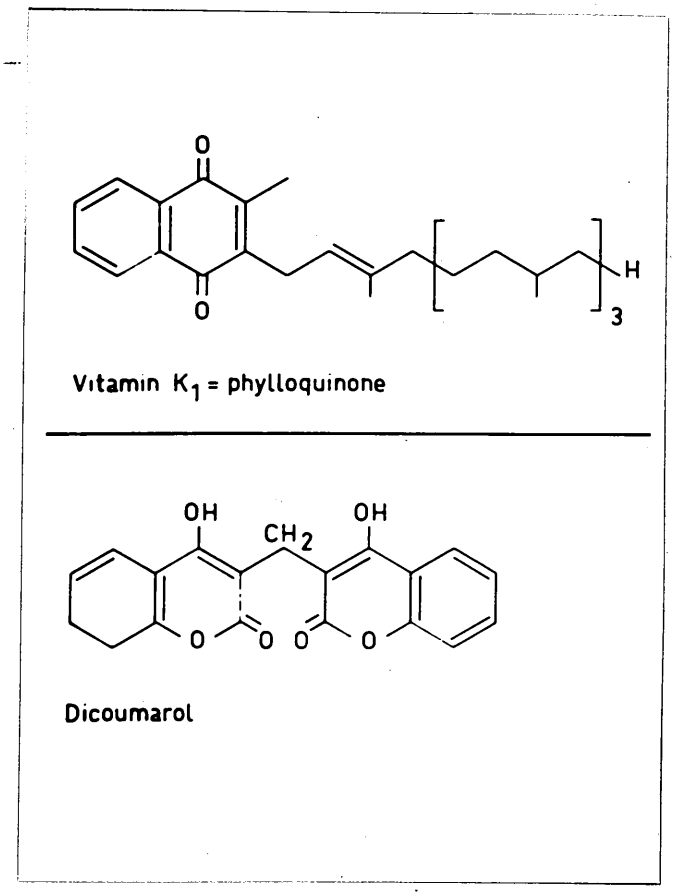


Figure 12

Structural relationship between dicoumarol and vitamin K_1

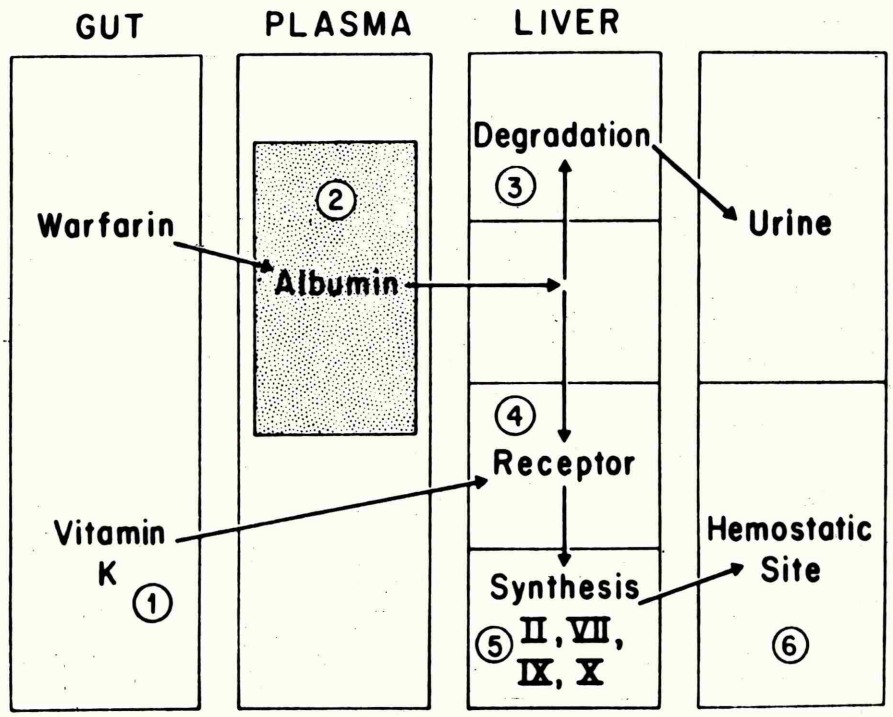
More recent research suggests that the oral anticoagulants interfere with the vitamin K binding site and that this site is important in the synthesis of factors II, VII, IX and X[57]. (Fig.13).

The structural relationship between Vitamin K and bishydroxycoumarin (dicoumarol) and the antagonistic actions of the two compounds in vivo provide the basis for the accepted theory that bishydroxycoumarin acts as an anti-vitamin by displacing Vitamin K in some essential enzyme system.

The relationship between Vitamin K and bishydroxycoumarin is of more than theoretical interest because of the wider application of the latter compound in the field of anticaogulant therapy. If the Vitamin K dependent factors are reduced to a dangerous level by the effect of anticoagulant therapy it can be counteracted within a period of hours by large doses of Vitamin K. Natural Vitamin K appears to be more effective than the synthetic soluble forms of Vitamin K. When large doses of Vitamin K are administered it is difficult to reduce the Vitamin K dependent factors when bishydroxycoumarin is readministered[58].

1.4.5 Warfarin Resistance

Warfarin sodium is almost completely absorbed following oral administration and 97% of the drug is bound to plasma proteins. The plasma half life following an oral dose of 1.5 mgm/kg is 44 hours $\pm$ 13 hours[59]. The average patient needs a daily dose of about 8 mgm, but requirements may vary from 1 - 25 mgm. Occasionally patients have been described who require excessively large doses of warfarin of the order of 80 - 150 mgm a day[60,61] to maintain anticoagulant effect. Plasma warfarin levels and half-life times



Metabolic Pathways of Warfarin and Vitamin K.

Figure 13

Metabolic Pathways of Warfarin and Vitamin K showing Interaction at the Liver Receptor Site

in these patients are similar to other individuals after a standard oral dose of warfarin. This resistance is put down to a mutation of the receptor site in the liver for warfarin. These patients are also insensitive to doses of Vitamin K. Studies involving identical and non-identical twins have indicated genetic control mechanisms for dicoumarol metabolism[62]. Other studies have shown that genetic resistance to warfarin is inherited as an autosomal dominant characteristic[63]. Drugs can also cause a decreased warfarin effect probably by induction of microsomal enzymes of the liver, resulting in increased metabolism of the anticoagulant[64].

1.4.6 Vitamin K Dependent Clotting Factors II, VII, IX and X

Although Vitamin K and an intact liver are shown to be necessary for the production of factors II, VII, IX and X, direct evidence that the factors are produced in the hepatic parenchymal cell came in 1964[65]. By immunofluorescent techniques it was shown that factor II existed within hepatocytes and not in cells of the bile duct, bone marrow or lymph nodes. Additional studies by perfusion of rat livers demonstrated the release of factors II, VII, IX and X. Characteristics of Vitamin K dependent factors are shown in Table 2.

1.5 LIVER DISEASE AND COAGULATION ABNORMALITIES

Clotting abnormalities may accompany any type of liver disease. The degree of severity of acquired clotting abnormalities due to the effects of liver disease depends to what extent the reserve functional capacity and the power of regeneration of the liver have been

TABLE 2

Properties of Vitamin K dependent factors

Factor	Cell/Origin	Mol.Wt.	Biological Half-Life	Physiological Role
II	Hepatocyte	62,000	50 - 80 hr	Precursor thrombin
VII	Hepatocyte	63,000	3 - 5 hr	Activates Factor X
IX	Hepatocyte	50,000	24 - 36 hr	Activated by Factor XI, forms complex with Factor VIII and calcium and phospholipid
X	Hepatocyte	86,000	42 hr	Factor X when activated reacts with calcium, phospholipid and Factor V to activate Factor II

impaired. In disease, one or more of the liver functions may be interfered with, but rarely all. Therefore no single test will reveal all dysfunction. Tests must be selected to suit the needs of individual patients, and even under well controlled conditions, there may be a diversity of results due to the complex interrelationships of liver function and structure.

Simple classification of liver diseases processes is difficult. One reason is the lack of clear cut delineation between many diseases; furthermore, transition from one type of disease to another is frequent. The simple outline as shown in figure 14 shows some cause of these disease processes and their interrelationships.

1.5.1 Hepatocellular Diseases

Coagulation abnormalities that are found in hepatocellular disease are complex, possibly involving one or more of the following mechanisms: (1) decreased synthesis of clotting factors; (2) production of abnormal clotting factors; (3) increased utilisation of clotting factors. In some instances all three mechanisms can be involved, contributing to severe clotting abnormalities.

Decreased synthesis of factors II, VII, IX and X is frequently seen in parenchymal liver disease and they may become diminished before there is other evidence of liver disease[66]. Abnormal clotting factors can also be produced in hepatocellular disease. The resulting factors can have acquired molecular abnormalities, e.g. abnormal fibrinogen molecules (factor I variants) have been found in types of hepatocellular liver disease[67] and analysis of these showed a protein which lacks a glucosidic moiety for normal fibrin polymerisation. It is possible that other abnormal clotting proteins can

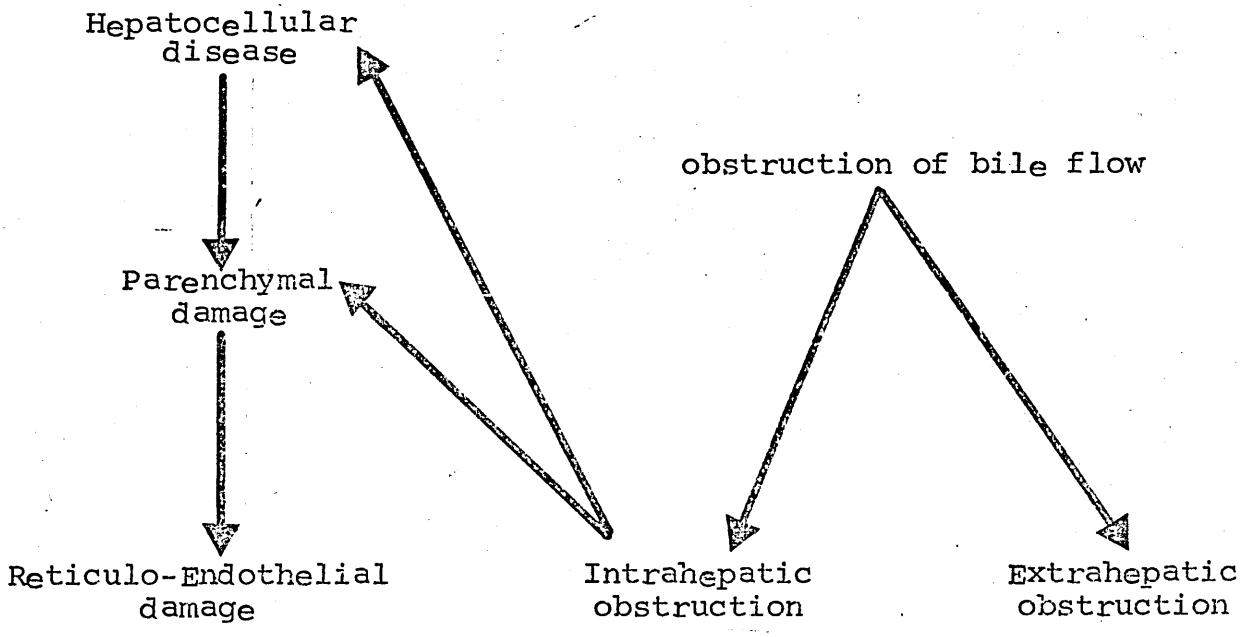


Figure 14

Liver disease processes

also be produced in hepatocellular disease.

Increased utilisation of coagulation factors is a frequent complication of liver disease[68,69]. It involves other mechanisms brought into play in liver disease, e.g. excessive fibrinolysis, proteolysis of clotting factors or disseminated intravascular coagulation (DIC). These co-existent disorders are often more life-threatening than the liver disease processes. Mechanisms such as (1) cellular damage resulting in the release of thromboplastic-type substances into the circulation and (2) impaired hepatic clearance mechanisms, found frequently in liver disease, can trigger off these dangerous co-existent disorders. The diseased liver, with its decreased ability to remove clot-promoting substances from the circulation, such as thrombin, is under stress. This can lead to "consumption" of many clotting factors (consumptive coagulopathy) particularly those of the Vitamin K dependent group II, VII, IX and X. Thus in any investigation into liver disease these mechanisms must be taken into account, as their profound effect on the coagulation mechanism can distort the analysis of laboratory findings.

1.5.2 Bile Salt Deficiency

Vitamin K deficiency has long been recognised as a complication of diseases associated with biliary obstruction[70]. Experimental conditions when dogs were deprived of bile showed that the dogs developed depressed levels of factor II. Patients with biliary obstruction on being supplied with bile, showed a slow return of Vitamin K dependent factors to normal[71]. This shows how important bile is to the natural lipid-soluble Vitamin K, as bile is absent in conditions of biliary obstruction. However, if Vitamin K and bile were given together the values showed a rapid return to normal. Defects in the intestinal absorption of fats

can also affect Vitamin K absorption.

1.5.3 Haemorrhagic disease of the newborn

The tendency of the newborn infant to bleed during the first few days of life was recognized early in man's history, and it hardly seems accidental that the rite of circumcision was postponed until the eighth day by the Mosaic law. The recognition of the haemorrhagic state in the newborn as a distinct clinical entity should be credited to Townsend[72], who, in 1894, concluded that it was distinct from haemophilia and was self-limited. Nearly all newborn babies, particularly after one to three days of life, have reduced levels of Vitamin K-dependent factors II, VII, IX and X[73]. The precise cause of this is not known. It could be due to (a) lack of Vitamin K intake; (b) poor utilisation of Vitamin K by the immature liver of babies; or perhaps (c) by the lack of bacterial flora in the intestine. Another explanation could be that the human foetus, like rapidly growing puppies, requires large daily amounts of Vitamin K. As the maternal organism is incapable of supplying the baby with more than the minimum requirement, little can be stored and an adequate reserve cannot be built up. The newborn infant, having neither a store of Vitamin K-food or bacteria in the intestine, is unable to meet the rapid metabolism of the Vitamin K dependent factors, thus the result is a precipitous fall of the factors concerned. In the course of one to three days the Vitamin K becomes available probably from bacterial flora in the intestine, as the milk supply does not appear to contain Vitamin K[74] and the levels of factors II, VII, IX and X rise. Normal adult levels of Vitamin K dependent factors in children are reached during the first year of life[75].

1.5.4 Vitamin K Deficiency

This deficiency may be relative or absolute. The effect of lack of absorption of Vitamin K is that it results in absolute deficiency. On the other hand, administration of antivitamin drugs, e.g. coumarins, results in a relative deficiency of Vitamin K. In each case synthesis of factors dependent on Vitamin K, i.e. factors II, VII, IX and X is affected.

1.5.5 Diet and Vitamin K Deficiency

This has been found to be rare in man, possibly due to alternative sources available to man (food and intestinal bacteria). Small diet changes involving omitting Vitamin K, have resulted in small changes in Vitamin K dependent factor levels[76]. Sometimes the dietary Vitamin K deficiency does not occur under usual circumstances, but requires that the Vitamin K-producing-bacteria level within the intestine be altered before Vitamin K deficiency becomes noticeable. Such conditions occur in the "sterilisation syndrome"[77] when broad spectrum antibiotics are used. The use of the antibiotics affects the daily intake of Vitamin K. This is particularly evident when chronically ill patients with restricted oral intake, are placed on broad spectrum antibiotics.

1.6 ACQUIRED DISORDERS OF THE VITAMIN K DEPENDENT FACTOR

These disorders involving single Vitamin K deficient factors have been described[78]. These disorders are rare and have various causes ascribed to them. Isolated Factor X deficiencies have been described and possible causes suggested have been :

(1) selective fungicidal effect; (2) block of the factor X synthesis independent of the vitamin K step; (3) amyloidosis; (4) malignancy of the liver[79]. Factor II deficiency was reported in a case receiving multi-drug therapy, one of which is thought to have interfered with cell synthesis[80]. In the case of acquired factor VII deficiency this was thought to have been the effect of a related disorder, fibrosis[81]. The majority of acquired disorders involving single deficiencies apparently stem from defects of sub-cellular or molecular components of the liver.

1.7 HEREDITARY DISORDERS OF THE VITAMIN K DEPENDENT FACTORS

Hereditary disorders involving the vitamin K dependent factors are well documented[82,83]. Some of the earliest findings indicated a true deficiency of the particular factor. Now, however, using data from current research into this type of disorder, including the use of immunological and coagulation techniques, hypothetical models of these clotting factor deficiencies can be constructed. Genetic variants of factor VII following antibody neutralisation with antibody to factor VII seem to indicate that the defect is not due to lack of synthesis of factor VII[84]. The group of patients with this defect of factor VII also showed that they had inherited different genetic variants of the normal factor VII molecule with varying amounts of biological activity[85]. Studies using antibodies to factor X were used to analyse hereditary disorders of factor X[86]. The results indicated that the congenital factor X defect was due to different structurally defective forms of factor X, presumably due to different genetic mutations. Congenital deficiencies of factor II have been described showing both structural and activation defects[87]. Variants of factor IX have been described

with two groups being classified[88]. One group appear to have an antigenic equivalent to factor IX and the other group appear to have a factor IX molecule which cannot be detected immunologically. It would seem from the increasing research into hereditary disorders of the Vitamin K dependent factors, that the defects are not simply deficiencies of the particular factors but structurally abnormal forms entering into circulation.

1.8 AIMS OF THE THESIS

(a) To study the effect of liver disease on the synthesis of Vitamin K dependent factors.

(b) To assess the effect of treatment with Vitamin K.

(c) To raise antibodies to the Vitamin K dependent factors and assess the production of abnormal clotting factors in liver disease.

It is hoped that these studies will show whether functional abnormalities in individual patients with liver disease as assessed by clinical and histopathological criteria are reflected by corresponding functional abnormalities in clotting proteins.

CHAPTER II

THE EFFECT OF LIVER DISEASE ON THE SYNTHESIS OF VITAMIN K DEPENDENT CLOTTING FACTORS II VII IX X

2.1 INTRODUCTION

In order to assess the overall effect of liver disease on the Vitamin K dependent factors II, VII, IX and X, estimation of the levels of these factors were carried out in a group of hospital patients with liver disease, using specific one-stage clotting factor assays and the estimation of the prothrombin ratio.

2.2 METHODS AND MATERIALS

2.2.1 Specimen collection and the preparation of plasma

Blood samples were collected by clean venepuncture using a needle of 21 gauge or larger and disposable plastic syringes. For most coagulation assays 9 volumes of blood were gently mixed with 1 volume of 3.8% (W/V) Trisodium Citrate dehydrate unless otherwise stated. Blood samples were centrifuged without delay for 15 minutes at 3000 rev/min. (1400 g) and the platelet "poor" plasma transferred into plastic containers (1 ml volumes) using plastic pipettes. Plasma samples were stored in the plastic containers at -20°C unless assays were being performed immediately. To ensure an equal handling of all samples coagulation assays were performed on either fresh samples or, if this was not possible, freshly thawed samples which were discarded following analysis.

2.2.2 Pipetting techniques

Automatic pipettes (Oxford Laboratories) were used throughout the assay systems in an attempt to control systematic technical errors. During the course of the analysis these were checked for accuracy under working conditions. Fresh plastic tips were used to avoid carry-over and contamination in the assay system.

2.2.3 Dilutions

In all the assay systems, various dilutions of plasma are prepared using consistent techniques. When these were prepared the assay was immediately performed and the dilutions discarded.

2.2.4 Storage of bench reagents

Reagents used in the assay techniques were kept at 4°C until required. Those reagents which were common to many techniques, e.g. calcium chloride and which were stable at 37°C were kept at that temperature during the period of analysis and then discarded.

2.2.5 Reagents

Calcium chloride

Working solutions of calcium chloride were prepared by dilution of a stock M/I solution as required. Fresh dilutions were used with each analysis.

Chloroform extract of brain

(Bell & Alton Platelet Substitute 1954)
This was purchased commercially from Diagnostic Reagents, Thame, Oxford. In the assay systems the optimum dilution used was determined according to the assay system being used.

Commercial kits

were used in the factor II and factor X assays. These were obtained from Diagnostic Reagents Ltd., Thame, Oxon.

Factor VII and IX deficient substrate plasmas

Blood was obtained from patients congenitally deficient in these factors.

Factor X deficient substrate plasmas for use in X assay method

A commercial preparation of charcoal filtered ox plasma was obtained from Diagnostic Reagents Ltd, Thame, Oxon.

Glyoxaline (Imidazole) Buffer (pH 7.3 - 7.4)

3.4 gm Imidazole and 5.8 gm sodium chloride were dissolved in 500 ml distilled water.
18.6 mls N/I hydrochloric acid were added and the volume made up to 1 litre. The reagent was stored at 4°C.

Kaolin suspension

was obtained from British Drug Houses Ltd.
A suspension of 5 mgms/ml in Owren's Buffer made in small volumes (100 ml) and stored at 4°C.

Owren's Buffer (pH 7.35)

11.75 gms of sodium diethylbarbiturate and 14.67 gms of sodium chloride dissolved in a mixture of 1570 mls of distilled water and 430 mls of 0.1N hydrochloric acid.

RVV/Cephalin

Obtained commercially from Diagnostic Reagents Ltd., Thame, Oxon.

Saline extract of brain

Made by the method of Quick[89] using acetone dried human brain.

2.3 TECHNIQUES

2.3.1 One Stage Prothrombin

Samples : Control pooled citrated plasma
Patient citrated plasma

Reagents : Saline extract of brain
Calcium Chloride - 0.025 M solution

Technique : Mix in a clotting tube 0.2 mls plasma, 0.2 mls brain extract. Warm at 37°C for 90 seconds. Add 0.2 mls calcium chloride and record clotting times.

Test is repeated in triplicate. Normal plasma with this technique will clot between 11 and 15 seconds. Express results either in seconds or as a ratio of :

$$\frac{\text{Clotting time of patient in secs.}}{\text{Clotting time of control in secs.}}$$

2.3.2 Factor II assay (Using Taipan Venom)

Factor II is converted directly by the action of Taipan snake venom in the absence of any known clotting factor into thrombin. The resulting thrombin converts extra source of fibrinogen added to the reaction mixture. The clotting time of the fibrinogen is proportional to the concentration of prothrombin in the mixture.

Samples : Test citrated plasma
10 pooled normal citrated plasma (standard)

Materials : (Diagnostic Reagents Ltd., Thame, Oxon)
Taipan snake venom + calcium chloride
Adsorbed oxalated bovine plasma
Platelet substitute.

Technique : Prepare dilutions of plasma sample in glyoxaline buffer
Dilutions of 1/10, 1/50, 1/100 are standard preparations

Into clotting tubes add :
- 0.1 cephalin
- 0.1 ml fibrinogen
- 0.1 ml plasma dilution

Warm to 37°C
Add 0.2 mls of the mixture of taipan venom and calcium chloride solution.
Record clotting times.
Test all dilutions in triplicate and obtain mean clotting times in seconds.

Analysis of results : Clotting times of the standard normal pooled plasma are plotted against the reciprocal of plasma concentrations on linear co-ordinate paper and should give a straight line.
Value of the test is obtained by interpolation from the standard curve.

2.3.3. Factor VII assay (Using saline extract of brain)

Samples : Fresh citrated plasma
10 pooled normal citrated plasmas (standard)

Materials : Saline extract of acetone dried brain
0.025 m calcium chloride
Specific factor VII substrate plasma (congenital)

Technique : Dilutions 1/10, 1/30, 1/100 of the standard and patient samples are prepared in glyoxaline.

The three dilutions are tested together in the following manner :

Mix in glass clotting tubes -
0.1 ml substrate plasma,
0.1 ml brain extract,
0.1 mls plasma dilution.
Warm to 37°C for 1 minute.
Add 0.1 ml calcium chloride, note clotting times.
Test in duplicate.

Repeat procedure with the three patient dilutions.

Analysis : Standard clotting times are plotted against dilutions on double log paper and the patient's results derived from the interpolation from the standard curve.

2.3.4 Factor IX assay (One stage assay)

Samples : Fresh non-contacted citrated plasma kept in plastic tubes until assayed.
Pool of 10 non-contacted normal citrated plasma.

Reagents : Kaolin
Chloroform extract of brain
Glyoxaline
Substrate plasma (congenital 0% F.IX deficient plasma)
Calcium chloride 0.025 M solution.

Techniques : Standard and test plasma are diluted in glyoxaline buffer 1/10, 1/30, 1/100.
The three dilutions of each are tested at the same time, as follows :

Add 0.1 mls substrate;
0.1 ml plasma dilute,
0.1 ml cephalin,
together in a glass tube.
Warm to 37°C.
Add 0.1 ml Kaolin suspension, mix and leave undisturbed for five minutes.
Add 0.1 mls calcium chloride solution.
Record clotting times.

Repeat this technique to give duplicate results.

Analysis : Clotting times are plotted against dilutions on double log paper and the patient's results are obtained from the standard graph.

2.3.5 Factor X assay (one stage assay)

This assay involves using standard and test plasma at various dilutions in a charcoal filtered substrate ox plasma. The substrate is devoid of Factors VII or X, but has excess factors V, II and I. The clotting times obtained with this substrate are proportional to the factor X concentration in the plasma dilutions tested.

Samples : Test citrated plasma
10 pooled normal citrated plasmas (standard)

Materials : (Diagnostic Reagents Ltd, Thame, Oxon)
Glyoxaline
R.V.V./Cephalin
Calcium chloride 0.025 M solution.
Substrate - Factor X deficient plasma.

Technique : Both standard and patient plasmas are diluted 1/10, 1/30, 1/100 in glyoxaline buffer. Three dilutions of each are tested simultaneously by mixing :

0.1 mls substrate plasma
0.1 mls plasma dilution

in a glass tube and warm to 37°C. Add 0.1 mls R.V.V./cephalin mixture and exactly 30 seconds later add 0.1 mls calcium chloride solution.

Repeat to obtain duplicate results.

Analysis : Clotting times are plotted against dilutions on double log graph paper and patient's results obtained from the standard curve.

2.4 SUBJECTS STUDIED

2.4.1 Control Subjects

Twenty-one control subjects were investigated. They were all volunteers between the ages of eighteen and fifty-five years. They included twelve males and nine females. There was no history of liver disease and no significant history of drugs or alcohol intake was recorded at the time of the investigations.

A control series was gathered together to provide data with which to compare any changes found in patients with clinical liver disease. The large quoted variations of normal ranges, in the literature, for assay values of the Vitamin K dependent factors II, VII, IX, X are possibly due to (a) different components used in assay systems, or (b) variations in techniques from laboratory to laboratory.

TABLE 3

The Results of Factors II, VII, IX, X Assays and Prothrombin Ratio obtained in Control Subjects

	Proth. Ratio	F.II %	F.VII %	F.II %	F.X %
Control 1	1.00	70	97	65	78
" 2	1.15	75	116	60	81
" 3	1.20	95	119	75	69
" 4	1.00	130	125	69	75
" 5	1.10	119	93	75	90
" 6	1.13	75	75	95	61
" 7	1.00	97	115	100	63
" 8	1.00	155	120	112	90
" 9	1.00	129	114	106	81
" 10	1.23	77	79	83	72
" 11	1.20	115	126	145	114
" 12	1.00	148	159	140	110
" 13	1.00	136	100	130	95
" 14	1.05	79	75	114	98
" 15	1.09	102	70	120	99
" 16	1.00	116	75	114	116
" 17	1.10	159	129	118	103
" 18	1.00	125	79	136	104
" 19	1.00	132	150	119	96
" 20	1.08	124	80	122	110
" 21	1.00	126	110	105	70

TABLE 4

Mean Standard Deviation and Normal Range for Factors II, VII, IX, X and Prothrombin Ratio obtained in Control Subjects

	Mean Values	Standard Deviation (S.D.)	Normal Range
Proth. Ratio	1.06	0.08	1.0 - 1.22
F.II	113%	27%	60 - 167%
F.VII	110%	26%	58 - 162%
F.IX	106%	25%	56 - 155%
F.X	90%	17%	58 - 124%

TABLE 5

Types of Patients Investigated
(See Appendix)

Group	No. Studied
Biliary Cirrhosis	3
Alcoholic Cirrhosis	6
Cirrhosis	1
Obstructive Jaundice	6
Hepato-cellular Jaundice	2
Liver Failure	1
Schistosomiasis	2
Liver Disease/Disseminated I.C.	2
Carcinoma, Secondary to Intra-hepatic Carcinoma	2
Pancreatic Failure involving the Liver	1
Chronic Active Hepatitis	4
Prolonged Viral Hepatitis	1
Chronic Liver Disease	2

TABLE 6

The Results of Factors II, VII, IX, X Assays and Prothrombin Ratio in Liver Disease Patients

Patient No.	Sex	Clinical Diagnosis	Proth. Ratio	F.II %	F.VII %	F.IX %	F. %
1	F	Biliary Cirrhosis	1.9	40	37.5	48	34
2	F	" "	1.2	82	62	80	74
3	F	" "	1.4	50	45	50	58
4	F	Alcoholic Cirrhosis	1.5	37	47	22	37
5	F	" "	1.3	58	50	64	52
6	F	" "	2.5	4.5	26	48	47
7	F	" "	1.3	90	80	100	90
8	M	" "	1.08	90	64	110	115
9	M	" "	1.2	41	35	34	48
10	M	Cirrhosis	1.0	72	60	82	70
11	M	Obst. Jaundice	2.0	37	39	33	21
12	M	" "	4.0	12	2.5	10	12
13	M	" "	1.8	28	15	23	18
14	F	" "	1.7	30	22	20	34
15	F	" "	2.1	18	14	24	10
16	M	" "	2.0	28	15	23	18
17	M	Hepato-Cellular Jaundice	2.0	24	19	18	41
18	F	" " "	1.58	21	16	30	46
19	F	Liver Failure	8.6	5.5	2	19	5
20	M	Schistosomiasis	1.3	56	48	54	49

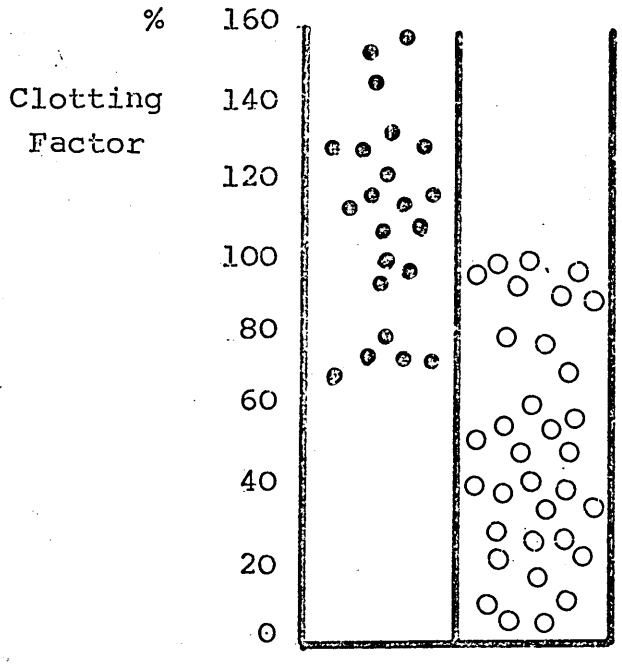
TABLE 6 (continued)

Patient No.	Sex	Clinical Diagnosis	Proth. Ratio	F.II %	F.VII %	F.IX %	F.X %
21	M	Schistosomiasis	1.6	49	49	51	48
22	M	DIC/Liver Disease	4.6	8.5	4	7.5	45
23	M	" " "	2.3	54	24	64	38
24	F	Carcinoma	1.2	42	26	52	45
25	F	"	1.3	100	41	45	76
26	F	Pancreatic Failure/ Liver	1.0	90	80	100	90
27	M	Chronic Active Hep.	1.3	100	110	86	120
28	F	" " "	1.16	98	70	84	80
29	F	" " "	1.15	90	70	102	80
30	M	Chronic Hep.	1.3	59	56	56	46
31	F	Prolonged Viral Hep.	1.1	40	39	40	44
32	M	Chronic Liver Disease	1.3	59	87	90	80
33	F	" " "	1.2	89	78	84	81
		Mean values	1.86	51.53	43.42	50.11	53.09
		Standard Deviation	1.44	29.87	26.98	28.60	28.72

2.5 ANALYSIS OF INDIVIDUAL ASSAY AND PROTHROMBIN RATIO RESULTS IN PATIENTS WITH LIVER DISEASE

2.5.1 Factor II assays

The factor II assay levels obtained were plotted and are shown in figure 15. On analysis they showed that of thirty-three patients investigated, twenty-three (79%) had assay levels outside the normal control range (Normal range 60-170%).



A. Patients' Factor II Assays O

B. Controls' Factor II Assays ●

FACTOR II ASSAYS

Figure 15

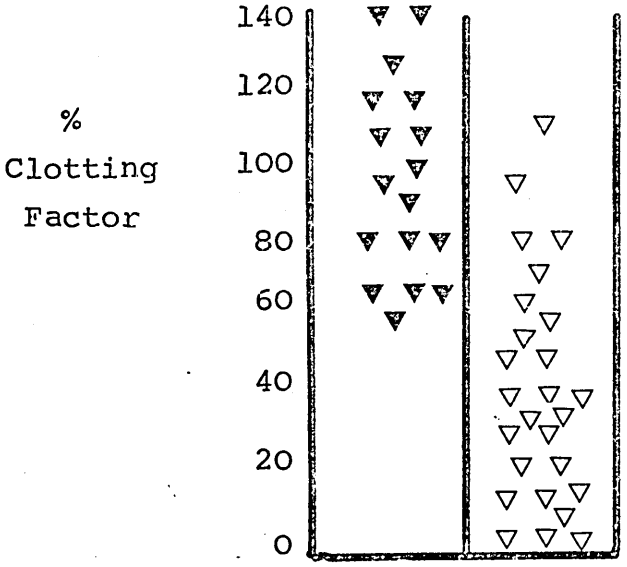
Further analysis of these showed that different degrees of reduced levels are present.

Assay Levels (%)	No. of Patients
50 - 59	6
40 - 49	5
30 - 39	3
20 - 29	4
10 - 19	2
0 - 9	3

The control factor II assay and patients' factor II assay were compared using the t-test. A value for t of 7.66N = 52 ($p = 0.001$) indicated a highly significant difference between the two sets of results.

2.5.2 Factor VII assays

The factor VII determinations on all of the liver disease patients were analysed (Fig.16). Of thirty-three patients' assays, twenty-two (66%) had lowered levels (control normal range = 58-162%).



A. Patients' factor VII Assays ▽

B. Controls' factor VII Assays ▼

FACTOR VII ASSAYS

Figure 16

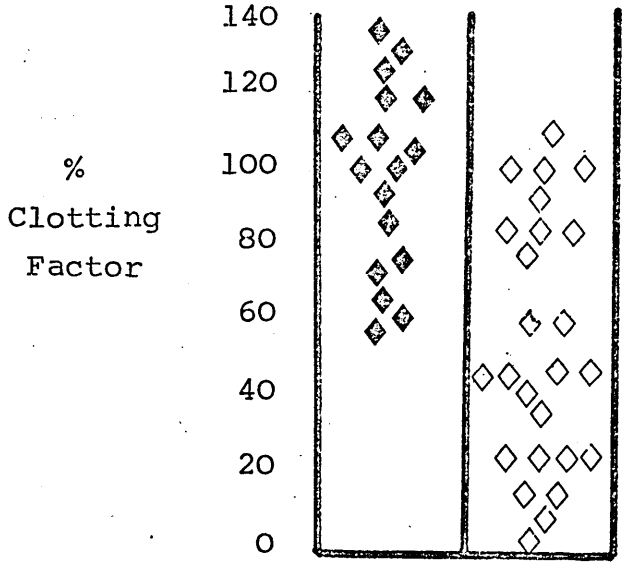
Further analysis of these showed that different degrees of reduced levels are present.

Levels	No. of Patients
50 - 59%	1
40 - 49%	5
30 - 39%	4
20 - 29%	3
10 - 19%	5
1 - 9%	4

The control and patients' factor VII assays were compared using the t-test. The value of 5 obtained was 8.22. Comparing this value with value from tables for 52 degrees of freedom, $p = 0.001$, indicating a highly significant difference between the two sets of results.

2.5.3 Factor IX assays

Results of factor IX assays (Fig.17) showed only twenty patients (60%) of the thirty-three investigated had assays outside the established normal range (Normal range = 56 - 155%).



A. Patients' Factor IX Assays ◇
B. Controls' Factor IX Assays ◆

FACTOR IX ASSAYS

Figure 17

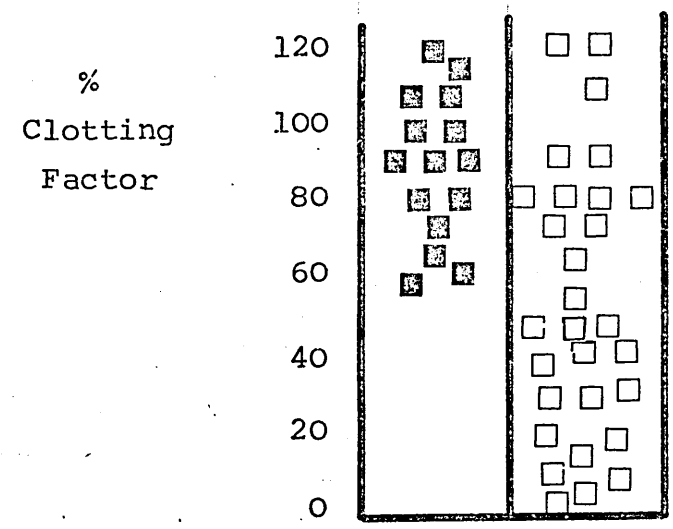
Again, further analysis showed that varied and abnormal assay levels of factor IX were present amongst the liver disease patients (N.R. = 56% - 155%).

Levels	No. of Patients
50 - 56%	4
40 - 49%	4
30 - 39%	3
20 - 29%	5
10 - 19%	3
1 - 9%	1

The control and patients' factor IX results were compared using the t-test. The value of t obtained was t = 6.55. Comparing this value with values from tables for 52 degrees of freedom, p = 0.001, indicates a highly significant difference between the two sets of results.

2.5.4 Factor X assays

The results of factor X assays are given in Fig.18. Analysis of these assays results shows that of the thirty-three patients, twenty-one (63%) had lowered factor X assays (N.R. = 58% - 124%).



A. Patients' Factor X Assays □

B. Controls' Factor X Assays ■

FACTOR X ASSAYS

Figure 18

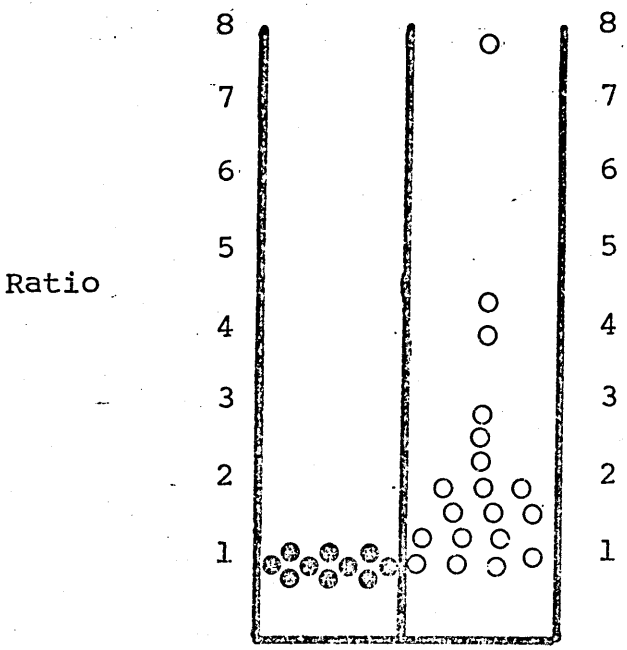
Analysis of individual assay results, as in the other vitamin K dependent assays, also shows that varying levels of factor X are present.

Levels	No. of Patients
50 - 58%	1
40 - 49%	10
30 - 39%	4
20 - 29%	1
10 - 19%	4
1 - 9%	1

Again, the t-test was used to compare the control and patient factor X assays. The value of t obtained was $t = 5.20$. Comparing this value with values obtained from tables for 52 degrees of freedom $p = 0.001$, indicating a highly significant difference between the two sets of results.

2.5.5 Prothrombin Ratio

The one stage prothrombin times obtained in the liver disease patients were expressed as a ratio (Table 6). Analysis of these showed that abnormal prothrombin ratios were encountered in twenty-seven patients (81%); of these twenty had a slight disturbance of the prothrombin ratio (1.2 - 1.9), six had a moderate disturbance (2.0 - 3.0) and three formed a group with a more severe type of disturbance (4.0; 4.6; 8.6). Thus the majority of patients investigated had disturbances of the prothrombin ratio ranging from mild, 1.2, to severe, 8.6 (Fig.19). These findings will be discussed.



A. Patient Prothrombin Ratio ○

B. Control Prothrombin Ratio ◉

PROTHROMBIN RATIO

Figure 19

2.6 ANALYSIS OF THE INTERRELATIONSHIP BETWEEN THE VITAMIN K DEPENDENT CLOTTING FACTORS II, VII, IX, X IN LIVER DISEASE

2.6.1 The relationship between specific factor assays in liver disease

Analysis of the results in the study showed that good correlation existed between the levels of the individual factors (Table 7), which would appear to support the possibility that in liver disease states synthesis of individual clotting factors can be affected to the same extent by the disease process.

TABLE 7

Correlation between the Vitamin K Dependent Factors II, VII, IX, X in Liver Disease

Factors	Correlation Coefficient (r)	Significance of the Correlation (P)	Significance
II and VII	0.73	< 0.001	High
II and IX	0.74	< 0.001	High
II and X	0.80	< 0.001	High
VII and IX	0.78	< 0.001	High
VII and X	0.65	< 0.001	High
IX and X	0.75	< 0.001	High

2.6.2 The relationship between individual clotting factor levels in liver disease

It has been suggested that factor VII may be sensitive to the effects of liver disease[89] and in order to corroborate this hypothesis an analysis of the ratios between clotting factors II and VII, II and IX, II and X, VII and IX, VII and X and IX and X was examined, firstly for the control series and then for the individual patients in order to observe if any of the clotting factors were demonstrating preferential reduction in liver disease. Statistically significant deviation of any ratio from that obtained in normal individuals indicates preferential reduction of one of the factors examined.

2.6.2(i) Results - Control Ratios

The results of the ratios obtained between individuals of the control series are shown in Table 8. In the control subjects examined the ratios obtained between the Vitamin K dependent clotting factors II, VII, IX and X showed a remarkable degree of constancy, (Table 8, means and standard deviations). From the results it would therefore appear that in health no preferential level of any individual clotting factor is found in the circulation.

2.6.2(ii) Results - Patient Ratios

Table 9 shows the ratios between clotting factors II and VII, II and IX, II and X, VII and IX, VII and X, IX and X, obtained in patients with liver disease.

2.6.2(iii) The relationship between factor VII and factors II, IX and X in liver disease

The ratios between factor VII and the other Vitamin K dependent factors were analysed and compared with those ratios obtained in the control series (Table 10).

TABLE 8

Ratios obtained between Factors II/VII, II/IX, II/X, VII/IX, VII/X, IX/X in the Control Series

Control	Ratio					
	II/VII	II/IX	II/X	VII/IX	VII/X	IX/X
1	0.72	1.07	0.89	1.4	1.2	0.83
2	0.65	1.25	0.92	1.9	1.4	0.74
3	0.79	1.26	1.3	1.5	1.7	1.08
4	1.04	1.8	1.7	1.7	1.6	0.92
5	1.27	1.5	1.3	1.24	1.03	0.83
6	1.0	0.78	1.2	0.78	1.25	1.55
7	0.85	0.97	1.5	1.15	1.7	1.5
8	1.29	1.3	1.7	1.07	1.3	1.2
9	1.13	1.2	1.5	1.07	1.4	1.3
10	0.97	0.92	1.06	0.95	1.09	1.15
11	0.92	0.79	1.0	0.86	1.1	1.27
12	0.93	1.05	1.5	1.13	1.4	1.27
13	1.36	1.04	1.43	0.76	1.05	1.36
14	1.05	0.69	0.86	0.64	0.76	1.16
15	1.45	0.86	1.03	0.6	0.7	1.2
16	1.52	1.01	1.0	0.66	0.65	0.98
17	1.22	1.33	1.5	1.09	1.24	1.13
18	1.58	0.91	1.2	0.58	0.76	1.3
19	0.88	1.1	1.3	1.26	1.5	1.23
20	1.50	1.01	1.12	0.65	0.73	1.1
21	1.14	1.2	1.8	1.04	1.57	1.5
Mean Ratios	1.09	1.1	1.2	1.06	1.19	1.16
Std.Dev.	0.25	0.27	0.28	0.3	0.33	0.24

TABLE 9

Ratios obtained between Factors II/VII, II/IX, II/X, VII/IX, VII/X, IX/X in the Patient Series

Patient No.	Ratio of	Ratio					
		II/VII	II/IX	II/X	VII/IX	VII/X	IX/X
1	Biliary Cirrhosis	1.06	0.83	1.17	0.78	1.10	1.41
2	" "	1.31	1.02	1.1	0.78	0.83	1.08
3	" "	1.11	1.0	0.86	0.9	0.71	0.86
4	Alcoholic Cirrhosis	0.74	1.5	0.94	2.1	1.21	0.59
5	" "	1.16	0.9	1.15	0.78	0.96	1.23
6	" "	1.73	0.9	0.95	0.54	0.55	1.02
7	" "	1.12	0.9	1.0	0.8	0.88	1.11
8	" "	1.4	0.81	0.78	0.58	0.55	0.95
9	" "	1.17	1.2	0.85	1.02	0.73	0.70
10	Cirrhosis	1.2	0.81	1.02	0.93	0.85	1.17
11	Obst. Jaundice	0.94	1.12	1.76	1.18	1.85	1.57
12	" "	4.8	1.2	1.8	0.25	0.2	0.83
13	" "	1.8	1.2	1.55	0.6	0.83	1.27
14	" "	1.36	1.5	0.8	1.1	0.64	0.58
15	" "	1.2	0.75	1.8	0.58	1.4	2.4
16	" "	1.86	1.21	1.55	0.65	0.83	1.2
17	Hepato-Cell. Jaundice	1.2	1.3	0.58	1.05	0.46	0.43
18	" " "	1.3	0.7	0.45	0.53	0.34	0.64

TABLE 9 (continued)

Patient No.	Ratio of	Ratio					
		II/VII	II/IX	II/X	VII/IX	VII/X	IX/X
19	Liver Failure	2.75	0.29	1.1	0.1	0.4	3.8
20	Schistosomiasis	1.16	1.03	1.14	0.88	0.97	1.10
21	"	1.0	0.96	1.02	0.96	1.02	1.06
22	L. Disease/DIC	2.1	1.13	1.88	0.53	0.88	1.6
23	" "	2.24	0.84	1.4	0.37	0.63	1.68
24	Carcinoma	1.6	0.8	0.93	0.5	0.57	1.15
25	"	2.4	2.2	1.3	0.9	0.53	0.59
26	Pancreatitis/L.D.	1.12	0.9	1.0	0.8	0.88	1.11
27	Chronic A. Hep.	0.9	1.16	0.83	1.2	0.91	0.78
28	" " "	1.4	1.16	1.22	0.83	0.87	1.05
29	" " "	1.2	0.88	1.12	0.68	0.88	1.28
30	" " "	1.05	1.05	1.28	1.0	1.2	1.2
31	Viral Hepatitis	1.02	1.0	0.9	0.97	0.88	0.9
32	Chronic L. Disease	0.68	0.65	0.73	0.96	1.08	1.12
33	" " "	1.14	1.05	1.04	0.92	0.96	1.03
	Mean Ratio	1.5	1.02	1.1	0.8	0.83	1.17
	Std. Dev.	0.76	0.31	0.35	0.34	0.31	0.61

Ratios involving Factor VII showed statistically highly significant deviation from the control ratios ($p = 0.025$, $p = 0.0125$, $p = 0.0005$) indicating preferential reduction of factor VII in relation to factor II, IX or X. In practical terms as the factor VII falls it does so at an increased rate when compared with factors II, IX or X.

2.6.2(iv) The relationship between factor II and factors IX and X in liver disease

The ratios involving factors II, IX and X were analysed and compared with the ratios obtained in the control series (Table 11). There was no statistically significant difference between the ratios II/IX obtained with patients from those of the control group. Therefore progressive reduction of factor II in liver disease is accompanied by a similar reduction in the levels of factor IX and no preferential effect is observed. The ratio factor II/X, however, did show a significant difference, $p = 0.05$, despite the similarity in mean values, indicating a slightly preferential reduction of factor X in relation to factor II in liver disease.

2.6.2(v) The relationship between factor IX and X in liver disease

Ratios between factor IX and X were analysed and compared with those obtained in the control series (Table 12).

There was no statistically significant difference between the ratios obtained ($p = 0.9$) and it was concluded that in liver disease factors IX and X are reduced in parallel to a similar degree.

TABLE 10

The relationship between Factor VII and Factors II, IX, X in Liver Disease

Ratio	Control		Patients		t value	Degrees Freedom	P
	Mean	S.D.	Mean	S.D.			
II/VII	1.09	0.25	1.5	0.76	2.04	52	< 0.025
VII/IX	1.06	0.3	0.8	0.34	2.28	52	< 0.0125
VII/X	1.19	0.33	0.83	0.31	3.80	52	< 0.005

TABLE 11

The relationship between Factor II and Factors IX and X in Liver Disease

Ratio	Control		Patients		t value	Degrees Freedom	P
	Mean	S.D.	Mean	S.D.			
II/IX	1.1	0.27	1.02	0.31	0.82	52	0.2
II/X	1.2	0.28	1.1	0.35	1.70	52	0.05

TABLE 12

The relationship between Factors IX and X in Liver Disease

Ratio	Control		Patients		t value	Degrees Freedom	P
	Mean	S.D.	Mean	S.D.			
IX/X	1.16	0.24	1.17	0.61	0.03	52	0.9

2.6.2(vi) The relationship between the prothrombin ratio and factors II, VII, X in liver disease

Previous analysis (section 2.5.5) had revealed that twenty-seven patients with liver disease out of a total of thirty-three had abnormal ratios. Statistically significant differences ($p = 0.01$) were observed between the prothrombin ratios of the control series and the patients with liver disease. In this analysis using correlation coefficients, an attempt was made to observe to what extent the prothrombin ratio is sensitive to levels of the undivided vitamin K dependent factors in liver disease (Table 13).

Good correlation was obtained between the prothrombin ratio and factors VII or X, as the variates change in opposite directions. Therefore the prothrombin ratio appears equally sensitive to both factors. Only medium correlation, however, existed between the prothrombin ratios and factor II ($r = 0.53$; $p = 0.01$) indicating that the prothrombin ratio is less sensitive to changes in factor II levels than it is to changes in the levels of factors VII or X in liver disease. The study appears to confirm the usefulness of the prothrombin ratio as a sensitive screening test in liver disease.

TABLE 13

Correlation between Prothrombin Ratios and Factor II, VII and X Levels in Liver Disease

	N	Value of r	P	Significance
Factor II	33	0.53	0.01	Medium
Factor VII	33	0.56	< 0.001	High
Factor X	33	0.57	< 0.001	High

2.7 DISCUSSION

2.7.1 Effects of Liver Disease on the Levels of the Vitamin K Dependent Clotting Factors II, VII, IX, X

The emphasis of this study has been to assess the effect of liver disease on the synthesis of the vitamin K dependent clotting factors II, VII, IX, X. Routine diagnosis in hepatology in general is dependent upon clinical and histopathological criteria, although in recent years clinical chemistry has played an increasing role in aiding diagnosis. The specific attention paid to the relationship between the disease process and its effect on the synthesis of clotting factors in this study affords a simple pathway to aid diagnosis and to differentiate between various forms of liver disease. If clotting factors fulfil certain criteria, e.g. being produced solely by the liver and being minimally influenced by extra-hepatic factors, any changes in its blood concentration, correlating or not with established parameters, should be considered as expression of liver function changes.

A small population of thirty-three hospital patients with thirteen different clinical types of liver disease were investigated. The analysis revealed that an overall reduction of factors II, VII, IX, X is a common finding in liver disease, whereas single factor deficiencies were not observed. As shown in Table 9, twenty-three (69%) of the liver disease patients had abnormal clotting factor assays affecting some or all of the vitamin K dependent factors. Furthermore, these findings were observed in eleven of the thirteen different clinical states of liver disease. Since varying degrees of clotting factor abnormality were demonstrated it is obvious that the clotting factor assays were reflecting the extent of the damage caused by the disease process and were therefore of diagnostic and prognostic value.

In hepatitis, all the five coagulation factor levels analysed (II, VII, IX, X, prothrombin ratio) were less frequently found to be abnormal (Table 6). The exception to this finding were two patients, one of whose assay levels were observed to be just sub-normal, and the other with prolonged viral hepatitis in whom recorded assay levels were very abnormal. It would appear therefore that generally the total synthetic capacity is not impaired and that the majority of the patients had a well compensated disease process.

Amongst the cirrhotic patients coagulation tests were frequently found to be abnormal (Table 6) indicating synthesis impairment. This coagulation abnormality is therefore a useful index of the degree of decompensated liver disease in these patients.

Obstructive jaundice patients were shown to have abnormal assays of varying severity (Table 6), thus illustrating the effect that an obstructive element has on the synthesis of the vitamin K dependent clotting factors. Whether this type of liver disease is accompanied by the appearance of protein induced by vitamin K absence (P.I.V.K.A.) is under investigation and will be discussed elsewhere in this thesis.

Liver disease associated with hepato-cellular jaundice has a severe effect on the synthesis of coagulation protein (Table 6). By what mechanism this is achieved is not clear and will be further commented upon. Similarly, carcinoma, secondary to intra-hepatic carcinoma, is seen to have a variable effect on synthesis, obviously reflecting the degree of involvement with the normal liver structure.

Liver failure (Table 6) is seen to have the most catastrophic effect on the protein synthesis pathways, the lowest factor levels being recorded in this disease process. The levels of the factors

VII and X are seen to be extremely low in the case investigated (Table 6) giving rise to the markedly abnormal prothrombin ratio of 8.6 (N range 1.0 - 1.1).

Disseminated intravascular coagulation involved with the liver, is an extremely complex situation with no one simple explanation. If the liver in liver disease is unable to diffuse potentially dangerous complexes involving thromboplastic elements, which it apparently manages to do in health, a profound effect on both the circulating clotting factor levels and the pathway of synthesis, can result. The pathway to be elucidated appears to be one of trigger mechanisms (i.e. thromboplastic complexes) and their effect on (a) consumption; (b) synthesis pathways in liver disease.

Schistosomiasis can have an effect on the synthesis of coagulation factors. The infestation of the liver by the worms affects normal function. Therefore coagulation studies can be of prognostic value in assessing disease processes.

2.7.2 The value of individual tests

2.7.2(i) Specific factor assays

Specific assays are costly and time consuming to carry out. In addition they are routinely only available in the larger laboratories which is a limiting consideration in liver disease investigations. Since specific assays, if well controlled, determine the level of an individual clotting factor they can be used to indicate possible malfunction in a section of the chain of clotting reactions. This improves on general screening tests which must be regarded as having limitations which could perhaps cause unjustified conclusions to be reached. In addition general screening

tests only give quantitative answers, i.e. comparing clotting times of patient's plasma with the clotting time of the control normal plasma. The value of the screening test can be improved by repeating the test every few hours and comparing the results with the original findings.

As discussed previously (section 2.7.1) the individual factor levels reflect the effect of the liver disease processes and in addition good correlation is apparent between the individual factor levels in liver disease (Table 7). This finding suggested that the factors decreased to the same level in the disease process and that the levels reflect the severity of the condition, with no individual factor being preferentially affected. However, straightforward correlation coefficient studies did not take into account the variation in normal ranges between the individual vitamin K dependent clotting factors. These varied between the factors (Table 4) thus the simple correlation (Table 7) would be misleading. Therefore analysis of the ratios between factors II, VII, IX and X was carried out (Tables 10-12) in order to see if any factor was depressed by liver disease to a greater extent than the other K dependent factors. The ratios between F.II/VII, F.II/IX, F.II/X, F.VII/IX, F.VII/X, F.IX/X were examined critically on the basis that for any selective reduction of any factor, the ratio containing that factor would be increased or decreased according to its position in the ratio calculation. As the normal control ratios were established as having values of unity (Table 8) it was concluded that in health no preferential level of any of the Vitamin K dependent factors had been observed. Analysis of the ratios obtained in liver disease patients using factor VII compared with the control ratios (Table 10) however showed that factor VII is depressed preferentially in liver disease. Further analysis showed that factor VII level appears to be more sensitive than factor X,

factor IX and factor II levels in that order, to liver disease processes. Factor X also showed preferential reduction in liver disease, but only against factor II.

Comparing circulating levels of factor II with factor IX, and factor X with factor IX, however, showed that in liver disease factor II /factor IX and factor IX/ factor X were reduced in parallel and to a similar degree (Tables 11 and 12).

In conclusion, factor VII appears to be the factor most preferentially reduced in liver disease against factor X, factor IX and factor II in that order. The mechanism by which this is achieved is that of increasing liver damage. Thus, factor VII falls rapidly in comparison with factor II and again falls to a lesser extent when compared with levels of factor IX and factor X, with the onset of liver disease.

On comparing these ratio findings with the half life of the individual clotting factors in circulation, similarity was noticed. Quoted $T\frac{1}{2}$ of the vitamin K dependent factors are - factor VII: 3 hours; factor IX: 24-36 hours; factor X: 30-40 hours; factor II: 50-80 hours (Introduction). The finding in this study that factor VII is reduced preferentially against factors X, IX and II in that order in liver disease could be theoretically explained, that this observation merely reflects the half life in circulation of the individual factors and factor VII appears to be preferentially and selectively sensitive to liver disease because of its much lower half life. Therefore any estimation of this important factor in liver disease can be used and regarded as a reliable index of the synthetic ability of the hepatocyte. This sensitivity to synthesis failure can be used as an important clinical indicator of liver function and its rate of synthesis of clotting factors. However, this factor is difficult to assay due to the scarcity of both congenital and acquired factor VII deficient plasma, despite this study

confirming its particular value in liver disease and its prognosis[90].

2.7.2(ii) Prothrombin ratio

The value of the prothrombin ratio has been described as its use in broad screening procedures in liver disease[91]. This has been confirmed in this study. Despite being a relatively simple test to standardise and carry out, one problem that can affect its sensitivity to the depression of the individual clotting factors, especially factors VII and X, is thought to be the type of tissue thromboplastin being used[92]. Sensitivity can also be affected by batch to batch variation of thromboplastins as well as different animal species. These can be minimised by using Manchester reagent (B.C.T.). With the good correlation obtained in this study (Table 13) between prothrombin ratio and factors VII and X, the ratio appears to be very sensitive to circulating levels of these factors. Similarly, the moderate correlation between the prothrombin ratio and factor II underlines the indirect effect lowered levels of factor II has on the ratio. Many authors have commented upon the lack of relationship between factor IX levels and the prothrombin ratio[93], therefore it would appear reasonable to include other screening tests in addition to the prothrombin ratio, which are sensitive to factor IX levels. This would involve using screening procedures of the intrinsic pathway and extrinsic pathways in assessing liver disease effect.

Analysis of the mean prothrombin ratio (mean = 1.9, standard deviation = 1.44) in liver disease revealed that the liver disease patients were significantly abnormal ($t = 2.58$; $p = 0.01$) when compared with the control group (mean = 1.1, standard deviation = 0.12). The effects of obstructive jaundice on the prothrombin ratio were particularly noticeable (mean = 2.3, standard deviation = 0.86), whereas in hepatitis no such effects were observed (mean = 1.2, standard deviation = 0.09) indicating sensitivity to the fall

in the levels of the vitamin K dependent clotting factors (Table 6) and the severity of the different disease processes. In addition when levels of factor VII, factor X and factor II fell below 10% of normal abnormally high ratios were obtained (Table 6; ratio = 8.6). These findings support the value of using the prothrombin ratio as a useful index of the effect of liver disease.

In conclusion, this study in evaluating the effect of liver disease on the vitamin K dependent factors, using specific assay and prothrombin ratio, shows that there is a correlation between the synthetic ability of the liver in health and circulating levels of the factors. This synthetic ability is disturbed in liver disease in varying degrees of severity according to the disease process. Therefore coagulation factor studies can, by giving accurate assessment of circulating levels, be of value in clinically assessing the effect and extent of the disease process.

CHAPTER III

SERIAL CHANGES IN THE VITAMIN K DEPENDENT CLOTTING FACTORS FOLLOWING THE ADMINISTRATION OF VITAMIN K

3.1 INTRODUCTION

The therapeutic use of Vitamin K revolves around its role as a dietary principle which is essential for the normal plasma levels of the Vitamin K dependent clotting factors. For that reason a therapeutic Vitamin K test can play an important role in the differentiation of obstructive and hepatocellular jaundice, since the values of the clotting factors if reduced can be checked by the administration of Vitamin K. If they are markedly decreased and there is no change in 24 hours a severe hepatocellular insufficiency can be assumed. If, on the contrary, the low values rise to normal following the administration of Vitamin K a severe liver damage can be excluded.

The aim of this chapter is to (a) assess the effect of treatment with Vitamin K on patients with liver disease; (b) comment on the pathways involving the absorption and utilisation of Vitamin K; (c) seek information on the problem of estimating which level of Vitamin K is appropriate to a given clinical situation.

3.2 METHODS AND MATERIALS

3.2.1 Vitamin K

In the study vitamin K was given intravenously as a dose of 10 mgm over a period of 10 minutes.

3.2.2 Samples

Venous blood samples were obtained on two

independent occasions before the vitamin K infusion and at 30 mins, 1, 2, 4, 8 and 24 hours following the infusion. Samples were not collected at any unsociable hours and consideration of the patients' rest periods were taken into account. Samples thus obtained by venepuncture were mixed with 3.8% (w/v) sodium citrate, centrifuged and the plasma removed and stored, care being taken to avoid glass contact. Samples were coded and analysed without recourse to sampling times.

3.2.3 Methods

Assays of factors II, VII, IX, X as well as the one stage prothrombin time were carried out by techniques described in chapter two (Methods).

3.3 PATIENTS

Between December 1974 and September 1975, 14 patients who required surgery or liver biopsy were given vitamin K (10 mgms I.V.). These patients were aged between 35 and 75 years and all had abnormal prothrombin times or ratios (Table 14).

It should be noted that analysis of the patients was carried out at this stage of the investigation without details of clinical diagnosis being available.

3.4 RESULTS

Patients had one stage prothrombin and specific assays of factor II, VII, IX, X carried out on each of the samples collected. Results of these analyses are shown for each patient in Tables 14 and 15(a-n). Each result is the mean of three tests performed on individual samples.

TABLE 14

Prothrombin times (in seconds) at intervals following Vitamin K infusion

No.	Patient	Pre	Pre	Ratio	30 mins	Hours 1	2	4	8	24	Control
1	J.D.	25	25	1.9	26	20	19	17	14.8	13.5	13.0
2	E.T.	16	17	1.4	16	15	15	14	13	12	12.0
3	S.O.	58	62	5.4	63	70	70	73	73	73	11.0
4	W.B.	16.5	16.5	1.5	16.0	14.8	14.8	14.5	14.5	15	11.0
5	F.R.	21	19	1.8	21	21	18	21	22	20	11.0
6	A.S.	16	16	1.33	16	15	14.5	14.5	11.5	12.0	12.0
7	M.C.	28	28	2.5	29	24	20	18	12	11.5	11.0
8	S.W.	45	47	4.0	45	40	32	27	16.5	12.5	11.5
9	M.G.	19	19.5	1.6	20	21	22	20.5	21.5	19	12.0
10	H.H.	18.5	18.5	1.4	19	19	18	19	18.5	18.5	13.0
11	M.T.	29	30.5	2.5	30	26	25	22	16	14	13.0
12	M.M.	28	26	2.25	29	23	18	14	12	12	12.0
13	G.C.	27	27	2.25	27	27	25	24	22	20	12.0
14	S.L.	60	60	5.0	58	28	21	17	14	12	12.0

TABLE 15

Results (in percentages) of Factor II, VII, IX, X assays on individual patients following an infusion of 10 mgm Vitamin K intravenously

Factor	Pre	Pre	½	1	2	4	8	24 Hrs
			(a) Patient J.D.					
II	37	35	38	45	65	72	76	100
VII	29	29	27	41	60	61	100	105
IX	33	30	32	32	41	80	86	120
X	21	20	22	40	55	59	69	110
			(b) Patient E.T.					
II	51	49	48	52	55	64	92	110
VII	45	47	45	54	57	57	92	94
IX	50	50	47	50	48	60	88	120
X	57	56	57	76	85	95	115	130
			(c) Patient J.U.					
II	29	29	30	31	26	25	26	26
VII	6	6	4	4	5	3	5	5
IX	33	30	32	32	30	33	30	33
X	15	14	15	15	18	20	18	18
			(d) Patient W.B.					
II	47	47	46	46	45	47	46	47.5
VII	35	35	35	53	68	68	68	68
IX	22	16	19	27	27	30	31	24
X	37	27	35	37	62	64	57	62
			(e) Patient F.R.					
II	25	29	25	23	31	27	26	27
VII	27	30	29	29	28	28	26	31
IX	43	47	48	46	50	50	48	48
X	29	31	31	24	33	31.5	33	34
			(f) Patient A.S.					
II	80	81	81	90	93	91	100	100
VII	55	54	52	54	53	53	62	76
IX	78	80	84	81	82	86	91	94
X	78	80	80	89	89	110	130	130

TABLE 15 (continued)

Factor	Pre	Pre	½	1	2	4	8	24 Hrs
			(g) Patient M.C.					
II	28	28	30	31	36	41	50	88
VII	12	10	10	14	45	60	96	120
IX	31	33	31	35	40	44	46	100
X	25	24	26	29	50	65	75	130
			(h) Patient S.W.					
II	12	10	12	16	21	21	22	24
VII	2.5	2.5	3	3	4	7	14	100
IX	10	12	11	21	22	34	37	34
X	14	14	17	23	34	42	46	75
			(i) Patient M.G.					
II	21	19	20	20	19.5	20	22	20.5
VII	16	13	14	14	20	20	21	21
IX	30	33	30	30	29.5	35	34	33
X	30	34	30	35	36	36	34	50
			(j) Patient H.H.					
II	80	80	84	81	79	81	83	84
VII	55	50	55	58	58	60	64	60
IX	64	60	60	62	65	64	67	66
X	48	50	48	47	56	78	84	85
			(k) Patient M.T.					
II	30	33	33	38	46	48	50	53
VII	34	32	31	39	42	60	65	82
IX	35	34	34	38	38	46	55	77
X	20	21	23	28	29	33	50	63
			(l) Patient M.M.					
II	23	23	25	26	29	44	49	49
VII	11	13	12	30	48	70	81	81
IX	30	32	32	40	57	65	92	102
X X	14	15	16	34	53	65	59	62

TABLE 15 (continued)

Factor	Pre	Pre	½	1	2	4	8	24	Hrs
			(m) Patient G.S.						
II	15	15	15	19	21	21	24	26	
VII	24	21	24	22	22	25	31	45	
IX	16	16	18	24	24	26	24	23	
X	27	28	27	31	35	37	37	41	
			(n) Patient S.L.						
II	3	1	4	17	19	28	28	56	
VII	4	4	6	13	23	50	76	92	
IX	4	4	3	13	29	31	40	60	
X	3	3	4	17	29	31	40	61	

It is apparent from the results (Tables 14 and 15) that the patients treated with 10 mgm vitamin K intravenously can be divided into two groups -

GROUP ONE : Correction with vitamin K_1

GROUP TWO : No correction with vitamin K_1.

Thus the randomly selected patients can be reclassified as follows :

GROUP ONE	GROUP TWO
J.D.	S.U.
E.T.	W.B.
A.S.	F.R.
M.C.	M.G.
S.W.	H.H.
M.T.	G.S.
M.M.	
S.L.	

3.5 ANALYSIS OF GROUP ONE PATIENTS (Correction with Vitamin K)

3.5.1 One Stage Prothrombin

No correction of the one stage prothrombin was observed during the first hour following the infusion in group one patients (Tables 14 and 15). Although in the majority of patients minimum times for the one stage prothrombin were obtained at 24 hours, in two of the patients (A.S. and M.M.) minimum prothrombin times were obtained at 8 hours post infusion vitamin K_1. Analysis at 24 hours would appear therefore to be the most suitable time to monitor the effect of vitamin K_1 on abnormal prothrombin times or ratios, despite the fact that no analysis was undertaken between 8 and 24 hours.

3.5.2 Analysis of Factor II, VII, IX, X levels

Analysis of factor II, VII, IX, X assay levels at 0, 4, 8, 24 hours post infusion vitamin K_1 (10 mgm I.V.) shows that there is an overall increase in levels (Tables 15 a-n and Table 16). However there

TABLE 16

Factor II, VII, IX, X levels at 0, 4, 8, 24 hours post vitamin K (10 mgm I.V.)

Hours	0		4		8		24	
Factor	Mean %	Std Dev %	Mean %	Std Dev %	Mean %	Std Dev %	Mean %	Std Dev %
II	34.20	26.86	51.10	23.20	58.40	28.40	73.00	30.97
VII	24.00	19.64	52.25	19.20	73.25	27.67	93.70	14.50
IX	34.00	23.52	55.70	20.40	66.88	24.54	90.80	25.10
X	29.00	25.88	61.20	28.40	73.00	32.90	95.25	32.79

does not appear to be any significant difference between the means of the four factors at 0 and 4 hours (P = 0.1). This would indicate that all four factors are increasing at the same rate of synthesis during the first few hours following the administration of the vitamin K_1. Analysis of the differences between the means of the four factors at 0 and 8 hours (P = 0.05) and 0 and 24 hours (P = 0.01) indicates that a post 24 hours analysis will give more relevant clinical information on the ultimate behaviour of the vitamin K dependent clotting factors than an analysis at 8 hours. (Table 16).

3.5.3 t_{LAG} (Time for a significant response to the infusion of vitamin K_1)

Tables 15 (a-n) show the response of the vitamin K dependent factors II, VII, IX, X to the infusion of 10 mgm I.V. vitamin K_1. The responses show that no measurable synthesis of the factors occurs during the first 30 minutes. Between 30 minutes and one hour, however, variable degrees of response involving factors II, VII, X can be observed. Factors VII and X appear to be more sensitive to the vitamin K_1 at this stage than factor II, which only showed slight synthesis in some cases (M.C., E.T. and M.M.). Factor IX did not show significant changes in level during the first 60 minutes after infusion. Between one hour and two hours responses continued with factors II, VII, X building on the variable degrees of response observed during the first hour. Finally, factor IX during this period of analysis began to show an increase in levels in five patients (Table 15 a, b, g, l, n).

These findings suggest a short t_{LAG} for vitamin K before it is utilised in the synthesis pathway involving the vitamin K dependent clotting factors, which contradict those of Poller et al (1976)[94] who have suggested a pronounced improvement takes place at two hours post vitamin K therapy. Further analysis is intended in

order to see if the results are consistent with (a) de novo synthesis of factors II, VII, IX, X immediately following the vitamin K infusion; (b) completion of the vitamin K dependent step in an intracellular protein pool; or (c) completion of the vitamin K dependent step in existing circulating factor II, VII, IX, X.

Practical information derived from t_{LAG} is that it is possible to test for a response to vitamin K within a period of two hours.

3.5.4 Vitamin K infusion : Long term or short term effect

During the study only one infusion of vitamin K (10 mgm I.V.) was given. This was followed by an intensive analysis of effect over a period of 24 hours. It appears from this analysis (section 3.5.2) that the maximum effect of the vitamin K is observed at 24 hours, although the question which remains unanswered is, "Is the dose effect sustained for longer than 24 hours?"

One patient (S.L.) did not have any operative procedure carried out during the seven days post infusion vitamin K. With his permission routine blood samples were collected at 72 hours, six days and seven days post infusion and sent into the laboratory. Results of the analysis are shown in Table 17.

In the previous analysis of mean response at 0, 4, 8, 24 hours (section 3.5.2) the $T_{24\text{-hours}}$ analysis is believed to be reflecting maximum synthesis of the vitamin K dependent factors. From the results obtained on patient S.L., however, it is apparent that the synthesis effect of the vitamin K_1 is retained for at least a further three days for all the vitamin K_1 dependent factors. Individual factor levels between 3 and 6 days indicate that factor II synthesis is

TABLE 17

Effect of 10 mgm Vitamin K on the Vitamin K dependent factors II, VII, IX, X over a period of seven days

	Pre 1	Pre 2	Post ½ Hr	Post 1 Hr	Post 2 Hr	Post 4 Hr	Post 8 Hr	Post 24 Hr	Post 3 Day	Post 6 Day	Post 7 Day
Proth/secs.	60	60	60	28	21	17	14	12	11	13	13
F. II %	3	1	4	17	19	28	28	56	50	67	83
F. VII %	4	4	6	13	23	50	76	92	100	83	70
F. IX %	3	4	3	13	29	31	40	60	60	43	38
F. X %	3	3	4	17	29	31	40	61	60	58	49

sustained and even increased; factors VII and IX show 30% and 22% fall in factor levels respectively and factor X only starts to show a fall off between days 6 and 7. This effect showing a fall off between 3 and 7 days could reflect the individual factor synthesis using up the biologically available vitamin K in which case the maximum effect of one dose of 10 mgm vitamin K_1 (I.V.) appears to be for a period of up to three days. This is then followed by a fall off in levels as the vitamin K_1 effect on synthesis declines. It is not possible to state whether observations made are consistent with dose response characteristics since only one infusion of 10 mgm vitamin K_1 has been used. Information on the length of time the dose is effective can be important in situations where patients may or may not undergo surgical procedures.

3.5.5 $t_{50\% \text{ MAX}}$ (Time for attainment of 50% maximum response)

The estimation of the time taken to attain 50% of maximum response ($t_{50\% \text{ MAX}}$ response) following the infusion of 10 mgm vitamin K_1 (I.V.) was carried out in order to gain further information on the uptake of vitamin K_1 and its subsequent utilisation in the synthesis pathway involving factors II, VII, IX, X. Although this estimation of the time taken for 50% maximum synthesis is empirical it can be extremely useful in comparing the effect of uptake on individual rates of synthesis between the individual factors II, VII, IX, X.

Results of the $t_{50\% \text{ MAX}}$ analysis are shown in Table 18.

The mean $t_{50\% \text{ MAX}}$ values (Table 18) show that overall there appears to be no single factor selected preferentially for the vitamin K_1 to act upon. It

TABLE 18

$t_{50\% \text{ MAX}}$ response to 10 mg Vitamin K_1 I.V.

Patient	Factor	$\frac{\%T24 - \%To}{2}$	$\frac{50\% \text{ MAX}}{(To+(\%T24-To)}{2}$	$t_{50\% \text{ MAX}}$ (Hrs)
J.D.	II	31.5	68.5	3
	VII	29	58	1.75
	IX	43.5	76.5	3.5
	X	44.5	65.5	3
E.T.	II	10	90	6
	VII	15.5	70.5	16
	IX	8	86	5
	X	26	94	7
M.C.	II	30	58	12
	VII	54	66	6
	IX	34.5	65.5	16
	X	52.5	77.5	9
S.W.	II	6	18	15
	VII	46.5	53.5	20
	IX	22	33	4
	X	30.5	44.5	6
M.T.	II	11.5	41.5	1.5
	VII	24	58	4
	IX	21	56	8
	X	11.5	31.5	3
M.M.	II	13	36	4
	VII	35	46	2
	IX	36	66	4.5
	X	24	38	2
S.L.	II	26.5	29.5	8
	VII	44	48	4
	IX	28	32	3
	X	29	32	4

∴ $t_{50\% \text{ MAX}}$ for FII Mean = 5.14 Hrs Std D = 3.84
" " " FVII Mean = 7.34 Hrs Std D = 7.2
" " " FIX Mean = 6.5 Hrs Std D = 4.56
" " " FX Mean = 4.86 Hrs Std D = 2.54

Mean $t_{50\% \text{ MAX}}$ = 5.9 Hrs

would appear that the common core protein takes up the vitamin K_1 and incorporates it into the vitamin K dependent step simultaneously for all the factors, as follows.

	F.II	F.VII	V.IX	F.X	
$t_{50\% \text{ MAX}}$ =	5.14	7.3	6.5	4.9	Hrs

There are of course some patients showing degrees of response varying appreciably from the mean, possibly indicating that even within an apparently homogenous group physiological variations due to utilisation, degradation and incorporation are present. However, overall the $t_{50\% \text{ MAX}}$ is useful in relating uptake, utilisation of the vitamin K_1 and subsequent synthesis of the vitamin K dependent factors.

Additional analysis comparing the $t_{50\% \text{ MAX}}$ times (Table 18) with the maximum responses obtained at T_{24} Hours (Table 16) revealed that over the 24 hour period, the responses to the vitamin K infusion were not linear (Table 19).

The difference between the $t_{50\% \text{ MAX}}$, the estimated $t_{100 \text{ MAX}}$ and $T_{24 \text{ hour}}$ levels suggested the possibility of different rates of synthesis occurring during the uptake and response to vitamin K, i.e. Biphasic response. To investigate this possibility the data of patient J.D. was randomly selected and analysed.

3.5.5(1) Analysis of data from patient J.D.

The rate of appearance of clotting factors was thought to be biphasic; an initial fast phase followed

TABLE 19

$t_{50\% \text{ MAX}}$ compared with the estimated $t_{100\% \text{ MAX}}$

Factor	$t_{50\% \text{ MAX}}$	Estimated $t_{100\% \text{ MAX}}$	Time observed Max.effect
II	5.0 Hrs	10.0 Hrs	24 Hrs
VII	7.3 Hrs	14.6 Hrs	24 Hrs
IX	6.5 Hrs	13.0 Hrs	24 Hrs
X	4.5 Hrs	9.0 Hrs	24 Hrs

by a slower phase. In order to summarise and compare data for different factors an attempt was made to describe the time-course with a two-term exponential equation (equ. 1) :

$$C = C_1(1-e^{-\alpha_1 t}) + C_2(1-e^{-\alpha_2 t}) \quad \ldots\ldots \quad (1)$$

where C is the concentration of clotting factor at time t expressed as a percentage of normal value; C_1 and C_2 represent coefficient terms of the fast and slow components respectively, such that $C_1 + C_2 = 100\%$ of normal value; α and α_2 are the first-order rate constants (hr^{-1}) of the fast and slow components respectively.

To obtain values of C_1, C_2, α and α_2 equation 1 was rearranged in the form of equation 2 :

$$(100 - C) = C_1 e^{-\alpha_1 t} + C_2 e^{-\alpha_2 t} \quad \ldots\ldots \quad (2)$$

Hence, a plot of (100 - C) vs. $t^{(time)}$ was constructed and the two exponential terms isolated by the "method of residuals"[95]. An exponential curve-fitting programme for a Hewlett-Packard HP-65 calculator was used to obtain least squares estimates of each coefficient and exponent term. Half-lives ($T\frac{1}{2}$) for the fast and slow phases were obtained using equation 3 :

$$T\tfrac{1}{2} = \frac{0.693}{\alpha} \quad \ldots\ldots \quad (3)$$

and values for each clotting factor are shown in Table 20.

It should be emphasized that because of the small number of data points the values obtained must be regarded as gross estimates. Further experimentation is required before a biexponential equation can be accepted as an operationally adequate representation of the rates of clotting factor synthesis following Vitamin K infusion.

TABLE 20

Half Lives ($T_{\frac{1}{2}}$) for the fast and slow phases of clotting factor synthesis following vitamin K infusion

	Fast Phase (α_1)	Slow Phase (α_2)
Factor II	2.57 Hrs	23.4 Hrs
Factor VII	2.73 Hrs	58.7 Hrs
Factor IX	2.10 Hrs	28.00 Hrs
Factor X	5.00 Hrs	22.4 Hrs

3.6 ANALYSIS OF THE MEAN CURVE FOR FACTORS II, VII, IX, X

Figure 20 shows the mean overall curve for factors II, VII, IX, X derived from the analysis of all assay results during the period of 24 hours post infusion vitamin K. Using the same assay data, mean curves for each of the individual factors were constructed (Figure 21).

It can be seen that although the shape of the individual factor curves are similar they differ considerably in magnitude. During the early stages of response it would be difficult to separate the individual factors based on percentage difference between them (Table 21). Figure 21 suggests that the synthesis activity (i.e. assay levels) alone at 8 hours would separate the four factors, especially factors II and VII. At 24 hours there are significant differences between factors II and VII, IX, X, but not between factors VII, IX, X themselves (Table 21).

It has been shown in this analysis that the individual factors can be distinguished from each other at various times during the analysis based on (a) response and (b) magnitude of response. This is particularly so between 8 and 24 hours post infusion (Table 21). However can this separation be improved by using further parameters? Does the simple analysis of curves based on the response and magnitude of response represent all the information contained in the patients' assay results? Principal component factor analysis, which is discussed in the following section, enables these questions to be answered.

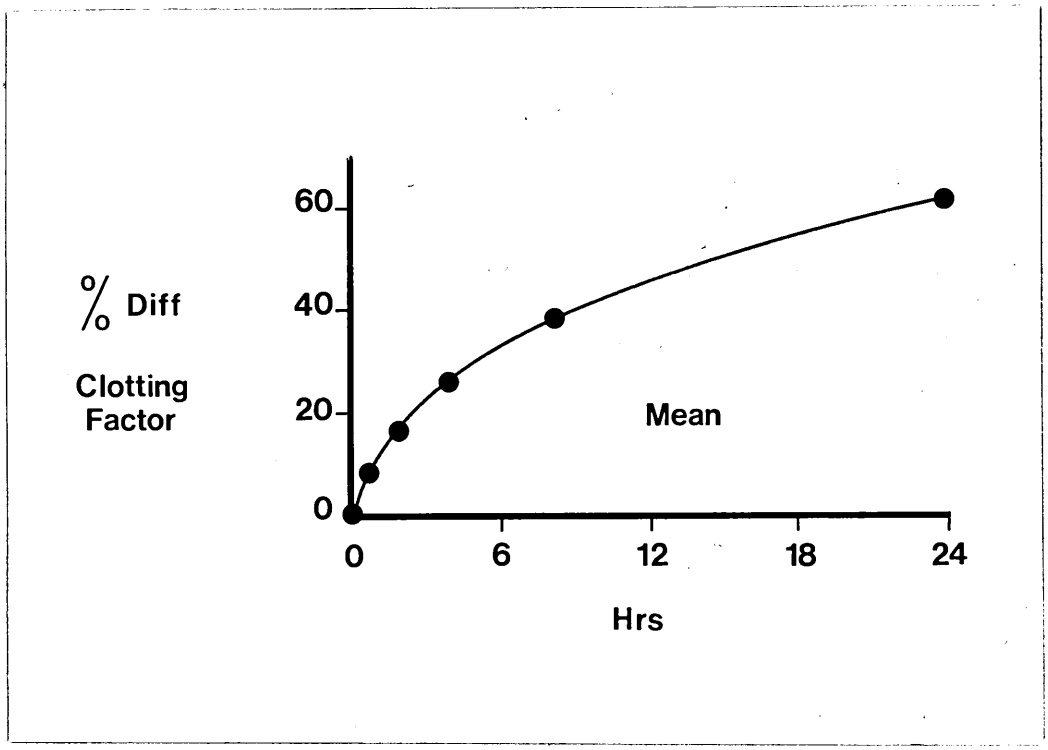


Figure 20

The mean curve for factors II, VII, IX, X derived from the seven patients

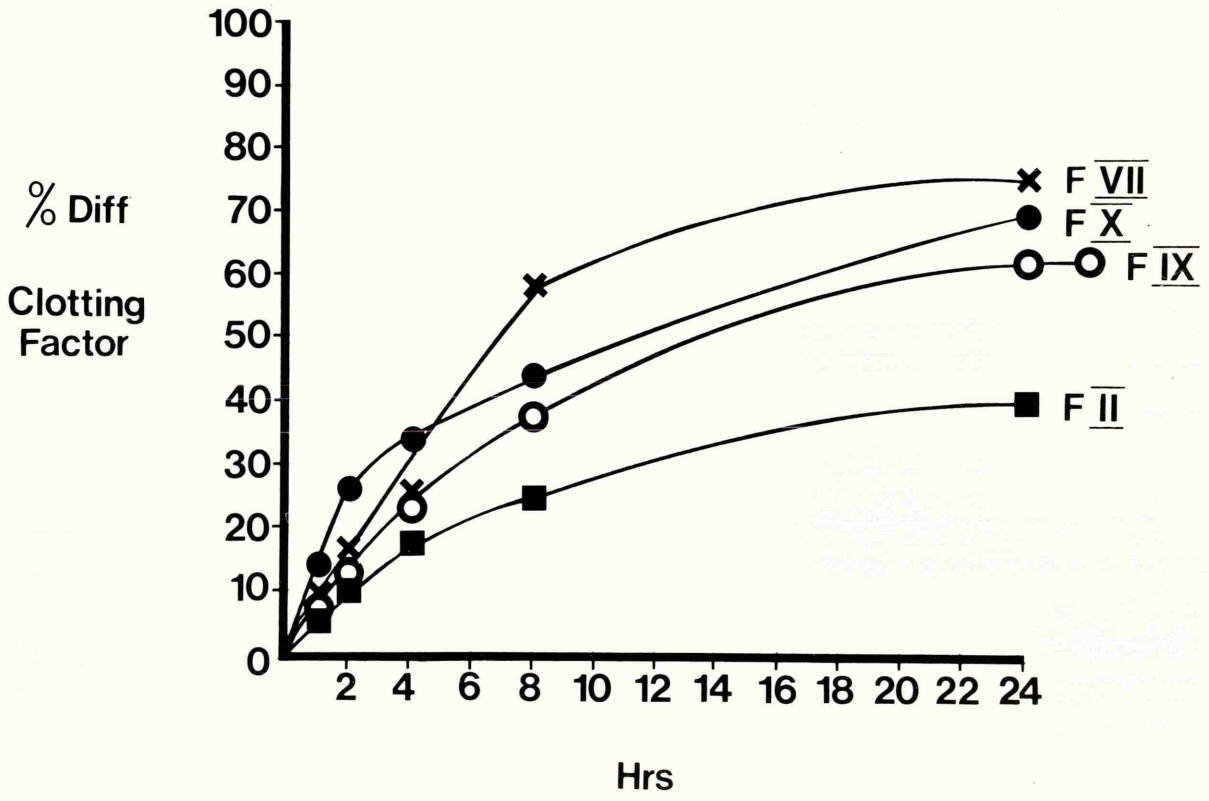


Figure 21

The mean curves for each of the individual clotting factors (% difference)

TABLE 21

Overall mean + individual mean factor levels for factors II, VII, IX, X following Vitamin K_1
(% difference)

T/Hours	Overall Mean for F.II, VII, IX, X	Mean F.II %	Mean F.VII %	Mean F.IX %	Mean F.X %
0	0	0	0	0	0
1	8	5	8	6	13
2	16	10	16	12	26
4	29	18	24	23	34
8	40	25	60	38	45
24	60	40	71	61	70

3.7 PRINCIPAL COMPONENT ANALYSIS

3.7.1 Introduction

The aim of any data analysis is to express the important features of the data by a few parameters. Usually this can be achieved by empirical analysis or by the use of models. Another approach is the method of principal component analysis which has been used in different fields of clinical medicine to analyse similar data. In this type of analysis the data from the four factor levels for all the individual patients between 0 and 24 hours following vitamin K are fed into a computer programmed by the method of Barber[96]. All sets of data to be analysed must have an equal number of data points. The factor II, VII, IX, X assay levels for all the patients obtained at T_0; T_1; T_2; T_4; T_8 and T_{24} hours were used as these data points, ensuring that the frequency at which the data points occurred was more or less proportional to the rate at which the activity (i.e. synthesis) of the clotting factors was changing.

Using this form of analysis it was considered possible to analyse differences between the 0, 4, 8 and 24 hour factor levels critically, in particular the period between 8 and 24 hours when no samples were collected for analysis.

The manner in which the data was gathered together and coded before being fed into the computer is shown in Table 32 (Appendix).

3.7.2 Method of analysis

From the results of all data analysed, the overall mean curve (Figure 22) representing information derived from factor II, VII, IX, X levels, can be drawn. In the principal component analysis this mean curve was subtracted from each of the individual patient records

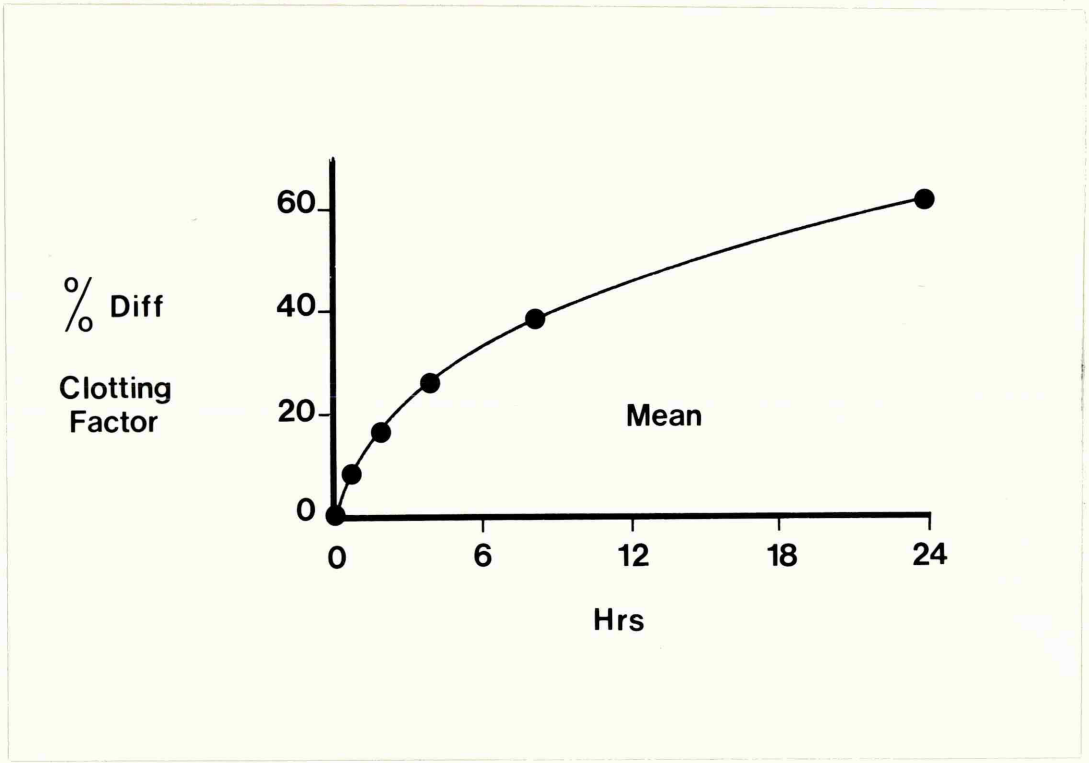


Figure 22

Mean curve of principal curve analysis

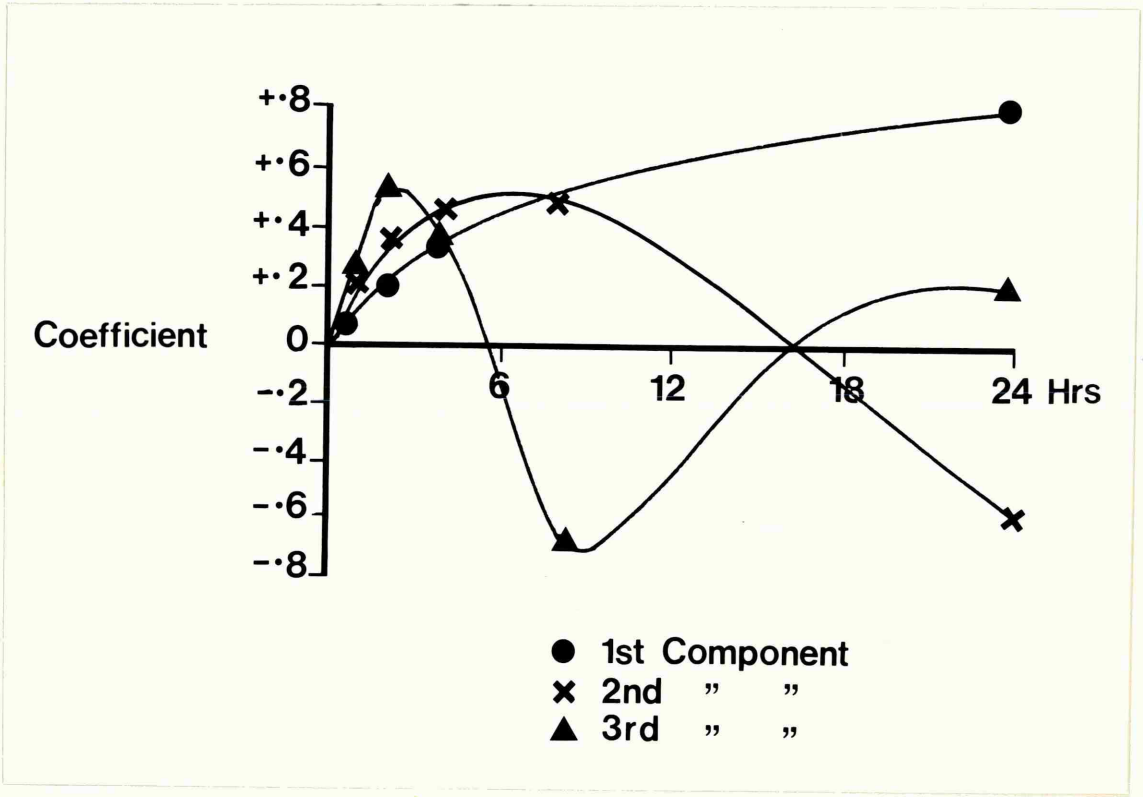


Figure 23

Principal curve components

(i.e. assays) in turn by the computer to give a population of differences from the mean. It is from this population of difference curves that the first three principal components were calculated by the method of Barber[97]. Additional information derived from the analysis is that the method of computation enables the variance associated with each of the three principal components to be calculated. The first component was found to represent 70% of the total variance of the curves, 23% was associated with the second component and 5.5% with the third. (Note that the computation could be stopped when the cumulative sum of the components reached an arbitrary percentage of the sum of the variances, i.e. 98.5%).

3.7.3 Results of principal component analysis

The computer results of the principal component analysis were plotted and are shown in figures 23 and 24. The data relating to the analysis is shown in the appendix.

Each individual patient's factor response curve can now be considered to be made up of the mean curve (figure 22) plus each of the three principal components associated with that individual factor response curve, i.e. :

Principal Components of the Individual Patient

$$\text{Mean Curve Value} + \underset{\text{x Coefficient } (\alpha_1)}{\text{Component 1}} + \underset{\text{x Coefficient } (\alpha_2)}{\text{Component 2}} + \underset{\text{x Coefficient } (\alpha_3)}{\text{Component 3}}$$

The analysis therefore represents data reduction without any loss of information.

To test this hypothesis the data from the factor VII response curve (patient M.M.) was selected at random and analysed. The results of the analyses are shown in tables 22 and 23, and the appendix. Plotting the data from Table 22 revealed the individual

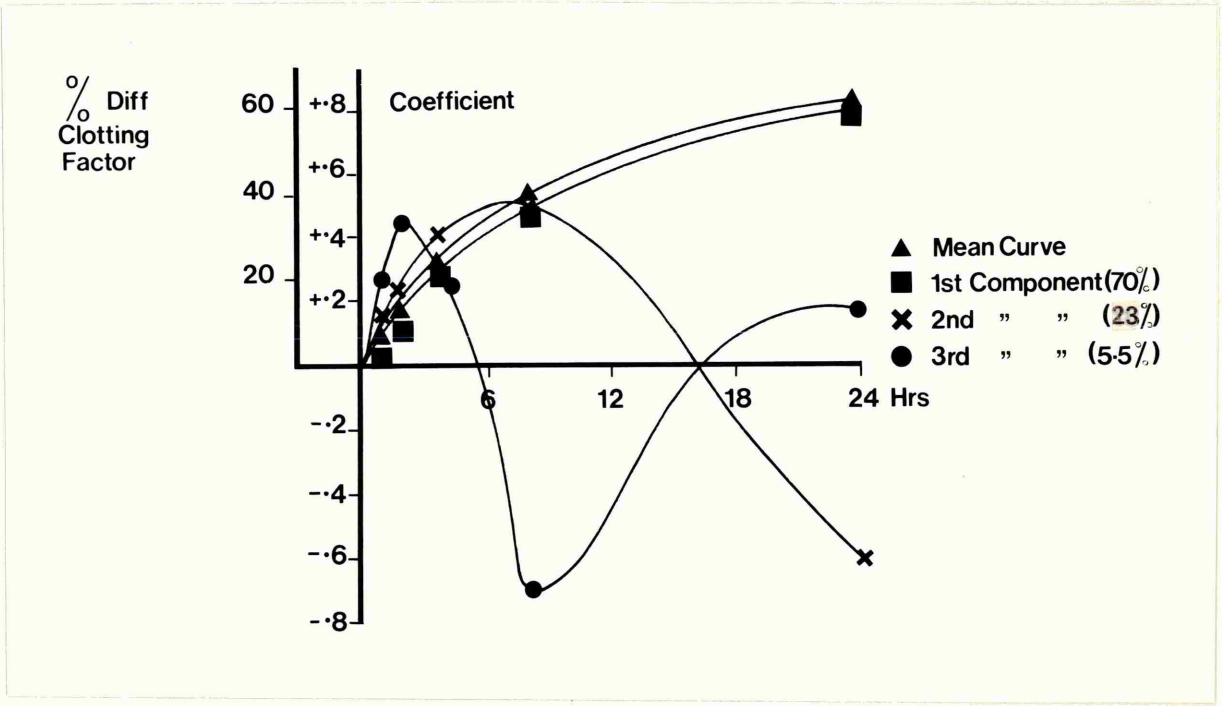


Figure 24.

Mean factor curve and principal components of the curve

TABLE 22

Principal Component Analysis using Data from Patient M.M. (Factor VII)

T/Hours	Mean Curve Values	Principal Components (M.M.)			Sum o Mean Cu + Compone
		Component 1 x α_1	Component 2 x α_2	Component 3 x α_3	
0	0.0	0.0	0.0	0.0	0
1	7.84	1.81	8.24	1.42	19.3
2	16.50	6.36	13.72	2.91	39.0
4	26.57	11.77	16.58	1.81	57.7
8	39.36	17.16	17.78	- 3.67	70.6
24	63.59	27.6	- 21.81	0.75	70.3

TABLE 23

Comparison of Sum of Mean Curve + Principal Components with Actual Data Curve of F.VII for Patient M.M.

T/Hours	Sum of Mean Curve + Principal Components	% Rise in F.VII Observed (M.M.)
0	0.0	0.0
1	19.3	20.0
2	39.0	37.0
4	57.7	60.0
8	70.6	71.0
24	70.1	70.0

characteristics of the three principal components (Figure 25). In addition, comparison of the actual data (factor VII response curve) with the mean response curve was made (Figure 26a). Completing the analysis is the plot of factor VII levels (actual data patient M.M.) and the principal component fit (sum of the mean curve + principal components of patient M.M.). The closeness of fit between the actual data and the principal component analysis (Figure 26b) supports this hypothesis.

From the values obtained for the variance associated with each of the three principal components (70%, 23%, 5.5%) it is obvious that the first component will reflect most of the data. As can be seen from the analysis of the principal components (Figure 23) at approximately 16 hours, the second and third components are equal to zero, leaving the first component to represent the response data. An analysis at this time would show information represented by the first principal component only. Furthermore, in support of this finding derived from all the patients' data, the principal component data of factor VII (patient M.M., Figure 25) also shows that between 17 and 18 hours the values of the second and third components approach zero, leaving the first component to represent all the information regarding factor VII levels at that time. Both overall and an individual factor VII analysis for one patient show similar findings regarding the first principal component. These findings suggest that results of factor assays between 16 and 18 hours would be represented by this component; therefore it could be argued that an analysis at 16 - 18 hours may give an accurate indication of the ultimate behaviour of factors II, VII, IX, X following the administration of vitamin K_1 (10 mgm I.V.).

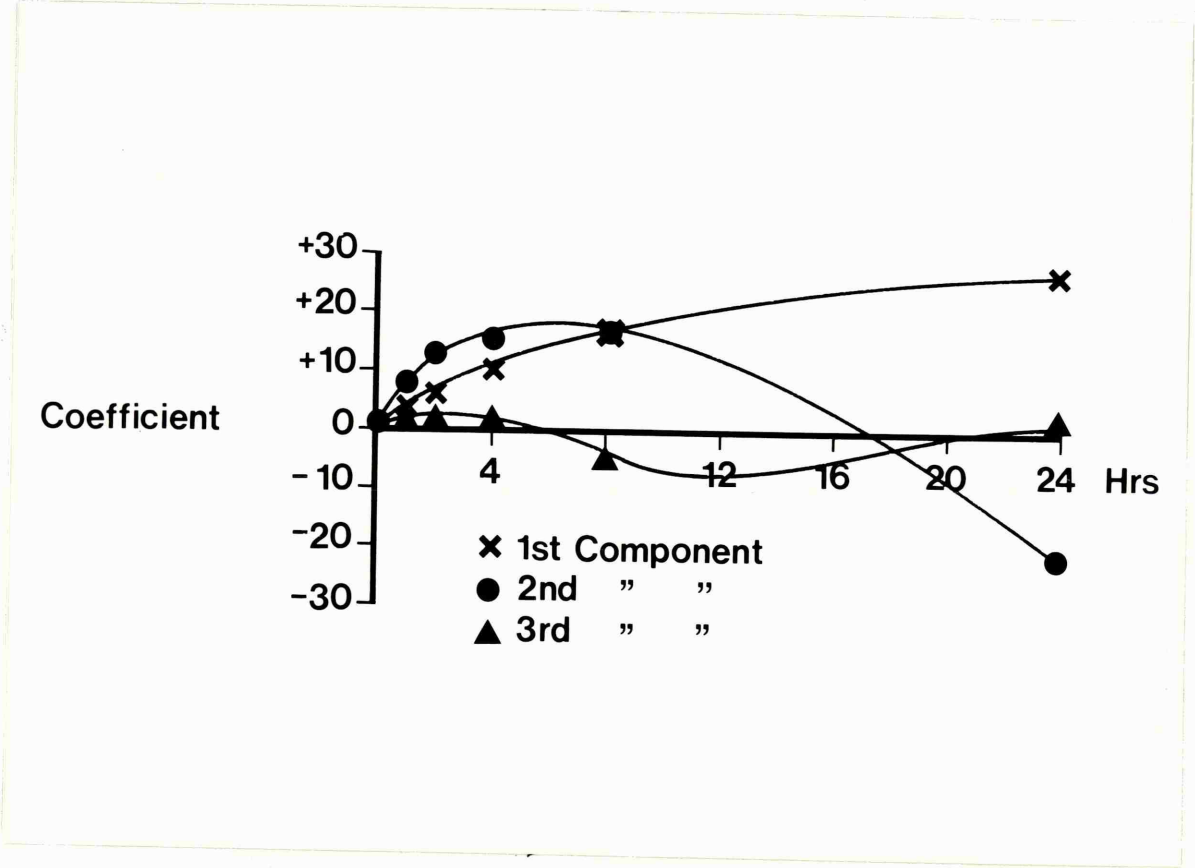
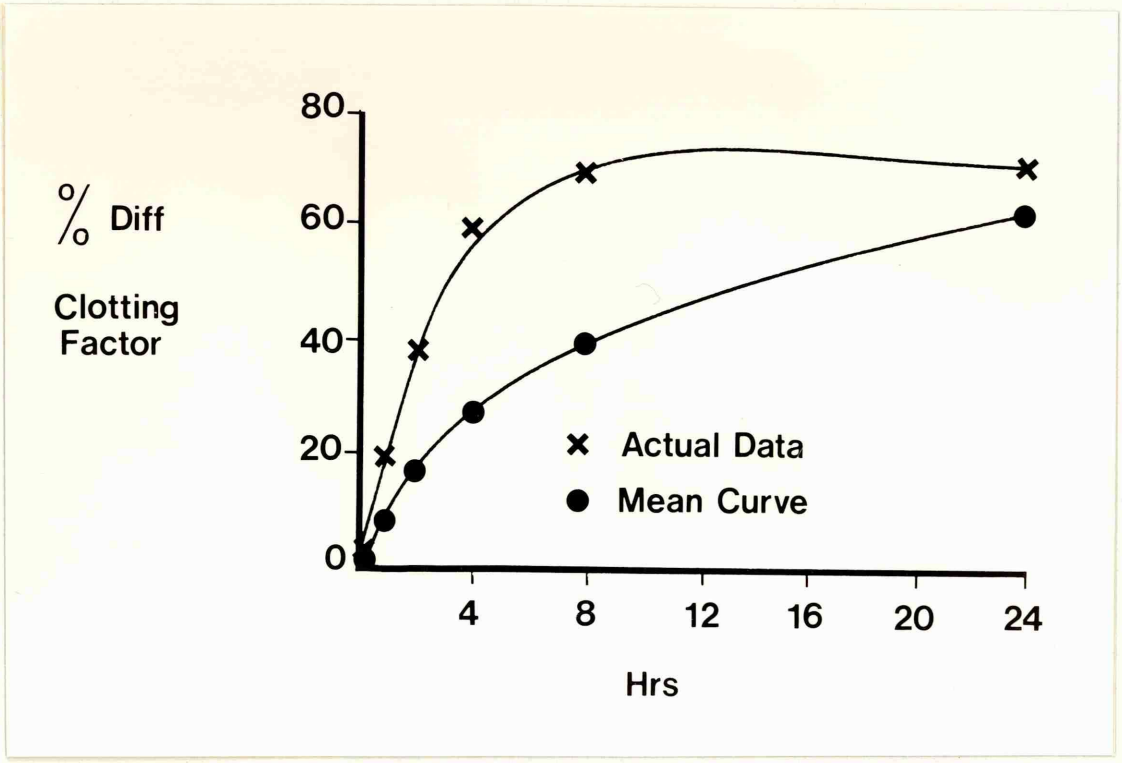
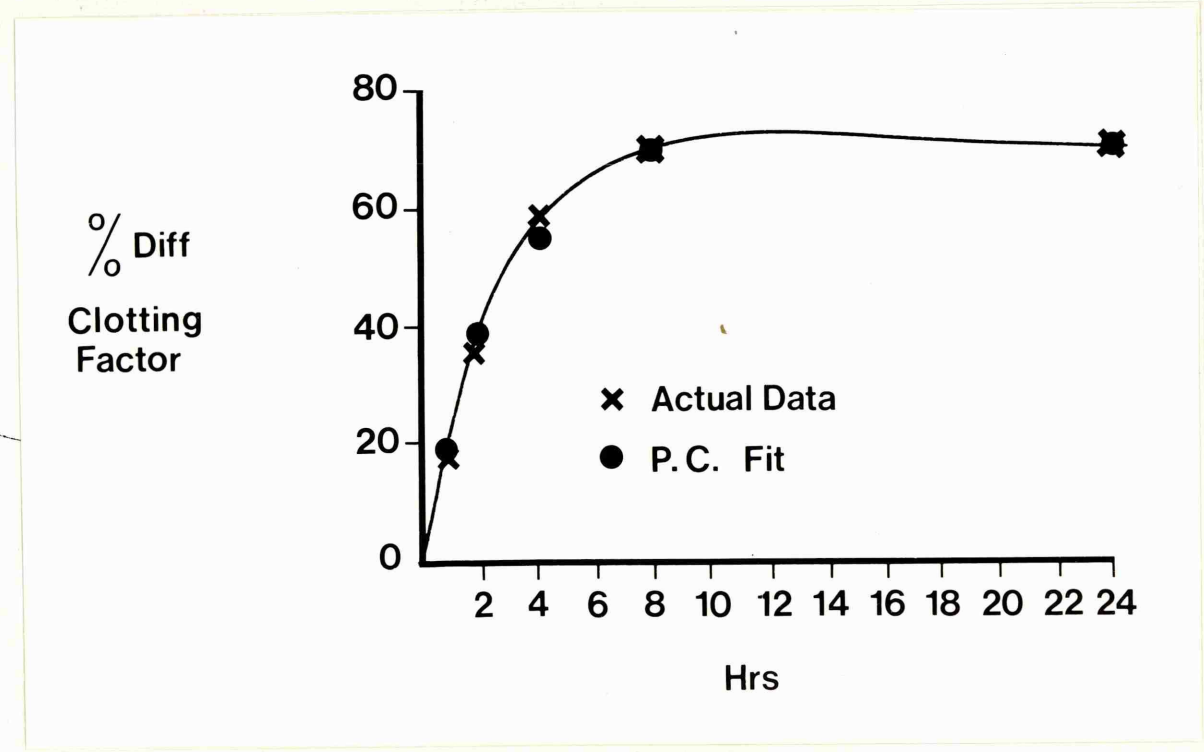


Figure 25

1st, 2nd and 3rd Principal Components
(Patient M.M.)



a. Comparison of actual data and mean curve



b. Closeness of fit between the actual data and the principal component analysis

Figure 26

Analysis of data, patient M.M.

3.8 ANALYSIS OF GROUP TWO PATIENTS (Non-correction with vitamin K)

3.8.1 One stage prothrombin

No appreciable shortening of the prothrombin times or ratios occurred in this group of patients apart from patients W.B. and G.S., in whom partial corrections were observed (Tables 14 and 15).

3.8.2 Specific assays (Table 24)

Following the administration of vitamin K no significant differences between the factor levels at T_o and T_{24} were observed in the majority of the group (t-test $p = < 0.1 > 0.05$; Correlation coefficient $r = 0.94$). From the observations made in group one patients (sections 3.4 and 3.5.2) it could be argued that there is no need to follow the response to vitamin K in group two patients beyond the four hour analysis. Significant differences between the two groups of patients are highlighted by observing the differences between levels of factor II at four hours and 24 hours. A significance value of $p = 0.1$ (not biologically significant) was obtained with group two patients whilst a similar analysis with group one patients revealed a value of $p = 0.001$ (a highly significant difference between the levels).

One significant finding amongst the group two patients involving specific assays of factors VII and X, is that certain patients (W.B., M.G., H.H., G.S.) appeared to respond to the vitamin K_1. Although this slight response did not affect the overall statistical analysis in any way, the mean levels of factors VII and X increased by 10% and 17% respectively. The change in activity involving factors VII and X might be due to different diseases being combined with cell

TABLE 24

Specific factor assays at 0 hours (T_0) and 24 hours (T_{24})

(post infusion vitamin K 10 mgm I.V.)

Factor	Specific Factor Levels (T_0)		Specific Factor Levels (T_{24})	
	Mean %	S.D. %	Mean %	S.D. %
II	36	24	38	24
VII	27	16.3	38	24
IX	33	19	37	16.5
X	31	11	48	23

damage and secondary deficiency of vitamin K. Levels of factor II and IX analysed at the same time showed no significant increase.

3.9 DISCUSSION

Two important groups of liver disease are associated with low circulating levels of the vitamin K dependent clotting factors II, VII, IX, X[98]. Although these groups were discussed in the Introduction (Chapter 1) they will be briefly classified here as they provide the background for the rational use of therapeutic vitamin K infusions.

Group one : Hepatic disease associated with biliary obstruction, e.g. malabsorption due to the absence of bile.

Group two : Hepatic disease associated with functional insufficiency, e.g. hepatitis, cirrhosis.

It was stated in the Introduction to this chapter that dependent on the type of response to vitamin K, a differential diagnosis between obstructive (Group one) and hepatocellular jaundice (Group two) can be achieved. This presumption, however, did not emphasise the possible interrelationships which may exist in the liver disease process, e.g. that hepatic insufficiency may also play a secondary role to biliary insufficiency thus complicating the clinical picture.

From the results obtained following the infusion of 10 mgms vitamin K_1 (I.V.) it was possible to classify a group of fourteen liver disease patients randomly selected into two groups, viz :

Vitamin K

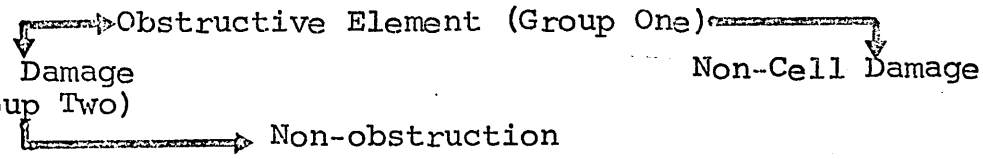
Group One	Group Two
1. Response to vitamin K	1. Non-response to vitamin K

This simple classification would support the previous assessment of the value of the test[99]. Furthermore, one could say that in the group of liver disease patients the vitamin K infusion study allows a further tentative classification of the patients to emerge, viz :

Vitamin K

Group One	Group Two
1. Vitamin K response	1. Non-response to vitamin K
2. Possible obstructive element	2. Possible cellular insufficiency

Looking at the levels of clotting factors and type of response obtained in group one patients (S.L., M.T., M.M.) and comparing them with the partial increase in clotting factors, i.e. factor VII, X amongst group two patients (W.B., H.H., G.S.), it is possible that there may be a further classification to be made, as follows :



Recourse was made at this stage to the clinical diagnosis made at the time of operation or biopsy. Comparison was made between the initial clinical diagnosis, laboratory diagnosis and the findings at surgery or biopsy (Table 25). The good correlation between laboratory and biopsy diagnosis appears to confirm the value of the vitamin K_1 infusion test in assisting clinical diagnosis.

Amongst the group two (non-responsive to vitamin K) patient G.S. (Hepatitis) appears to be one patient showing possible interrelationships between cell damage and obstructive pathway according to the response to vitamin K_1. The interrelationship between cell damage and obstructive element in hepatitis has been described by Sherlock[100] as one of a temporary inflammatory condition, affecting uptake and utilisation of vitamin K.

TABLE 25

Patient	Biopsy	Correction by Vit.K Infusion
Group One		
J.D.	Obstructive Jaundice	Corrected
E.T.	Obstructive Jaundice	Corrected
A.S.	Aggressive Hepatitis	Corrected
M.C.	Obstructive Jaundice	Corrected
S.W.	Obstructive Jaundice	Partially Corrected
M.T.	Obstructive Jaundice	Corrected
M.M.	Obstructive Jaundice	Corrected
S.L.	Obstructive Jaundice	Corrected
Group Two		
J.U.	Liver failure	NOT Corrected
H.H.	Cirrhosis	NOT Corrected
F.R.	Biliary Stasis	NOT Corrected
M.G.	Cirrhosis	NOT Corrected
W.B.	Cirrhosis	Partial Correction
G.S.	Hepatitis	Partial Correction

Other findings of interest in group two patients W.B., H.H. and M.G. involve a selective response by factors VII and X to vitamin K. Retrospective clinical analysis indicated a diagnosis of cirrhosis (chronic liver disease). The difference between these and other patients in the group may be due to different mechanisms for coagulation disorders in liver diseases involving for example, synthesis pathways, inhibitory mechanisms, or indeed selective interference with certain synthesis pathways (II, VII, IX, X) at different stages of the disease.

One of the patients in group one (A.S.) who had a response to vitamin K was clinically diagnosed as aggressive hepatitis. Initially the patient had low normal levels of factors II, VII, IX, X which still responded to vitamin K, indicating that despite synthesis continuing and vitamin K being available, extra vitamin K created a boost to synthesis. Once more it could be argued that an interrelationship between cell damage and an obstructive element had been established. The method by which it manifests itself is thought to be temporary local inflammatory conditions affecting extra hepatic absorption of the vitamin K.

Analysis of the t_{LAG} provided information on the uptake and subsequent utilisation of the vitamin K. A short t_{LAG} between 30 minutes and 2 hours was established and is supported by the observation that following the absorption of an oral dose of 1 mgm tritium-labelled vitamin K_1, measurable amounts were detected in the plasma within 30 minutes[101]. It would appear therefore that vitamin K becomes biologically available by both intravenous infusion and oral ingestion pathways in an extremely short period of time.

Once the vitamin K is biologically available the $t_{50\%}$ study indicates that factors II, VII, IX, X have similar rates of synthesis, during the first 4-6 hours post infusion (Table 18). The fact that all factors

utilise the available vitamin K to achieve a fast response phase followed by a slower synthesis phase (Table 20) appears to suggest feed back mechanisms interplaying with the ultracellular synthesis of the clotting factors. As the low levels normalise, the slower α_2 phase (Table 20) obviously is necessary to control a possible hypercoagulation state manifesting itself. It is also believed that as the intracellular level of biological vitamin K builds up it also provides a feed-back mechanism affecting further uptake and utilisation[102].

Although the response to vitamin K appears to be smooth with a non-selective preference to any single protein, the response against time (Table 15a-n) appears to show occasional resting periods of synthesis. If this is so, these findings could reinforce the hypothesis that blood coagulation belongs to a set of systems that are complicated enough to show non-linear characteristics[103]. This means that in such a system there exist varying relationships between the concentration of some factors (e.g. factor VII) as a substate and its effect on reaction velocities, i.e. enough is produced to initiate a reaction pathway, but an excess will result in a feed-back mechanism affecting further substrate production. This will therefore control potentially explosive reactions as is required by a HOMEOSTATIC mechanism. In simple systems an increase in substrate concentration will always result in an increase in reaction velocity; non-linear systems do not exhibit this by having multiple steady states.

Initially the investigation was to analyse the effect of the vitamin K infusion. However, following the analysis of data, in particular the difference between factor levels at 0, 4, 8 and 24 hours, it was realised that the maximum effect of vitamin K appeared to be at or near 24 hours. Further analysis involving

the use of computer aided analysis, i.e. principal component analysis, showed that it was possible to represent seven patients' data by a single mean curve without loss of information (section 3.7). In addition an evaluation following the infusion of vitamin K_1 using principal component analysis further suggested that it might not be necessary to wait until 24 hours post infusion before final analysis of the response. Considering the previous vague type of analysis of T_0 then T_{24} hours, the saving of up to eight hours without significant loss of information would appear to enhance vitamin K infusion studies clinically.

The response of a single infusion of 10 mgms vitamin K_1 in one patient (S.L.) showed that the one injection sustained the synthesis pathway for up to seven days, with a maximum effect appearing at three days. Since the amount of vitamin K that is required to maintain normal levels of the vitamin K dependent clotting factors is believed to be 0.1 - 0.5 μ/Kg/day[104] the infusion of 10 mgms should satisfy requirements for a period of longer than the observed response. Therefore it could be considered that (a) a limited amount of vitamin K is used intracellularly; (b) once the cell is saturated with vitamin K the rest is excreted quickly via the urine or faeces; (c) as the vitamin K is degraded in the synthesis of clotting factors it is not replaced in liver disease states where the storage potential to vitamin K may be reduced. More studies will be needed to prove that dose dependence to vitamin K can be observed in infusion studies and this can only be solved by altering the common dose of 10 mgms I.V. and analysing response and uptake. This type of investigation could be valuable in predicting the therapeutic dose regime required to correct abnormal prothrombin times, particularly in anticoagulant overdose.

Although the use of vitamin K infusion in patients with liver disease is common, no standard procedure appears to have been established in clinical regimes. Problems arise from :

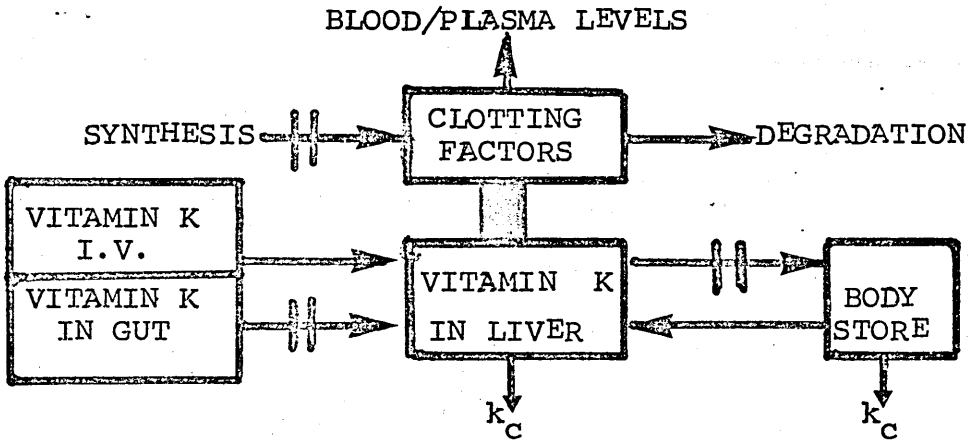
(a) Type of vitamin K preparation used;

(b) The dose required, which appears to be left entirely to individual choice;

(c) Dose effect analysis, which is usually based on the estimation at T_0 and a subsequent analysis at $T_{24\ hours}$.

It would be more scientific if preparations of vitamin K_1 to be used as a test of assessing hepatic function, could also be selected with a view to elucidating pathways involving completeness of absorption (oral) or utilisation (intravenous). It must be remembered for example that intestinal absorption of vitamin K is limited; indeed, a dose of 1 - 5 mgms intravenously is probably equivalent to 50 mgms given orally[105]. This must be taken into account when using vitamin K_1 to assess hepatic function.

Drawing conclusions from this type of study is difficult. Most of the information has been derived empirically and commented upon. However, experimental models from information produced in this study showing the absorption of the vitamin K_1 and its subsequent utilisation could be as follows :

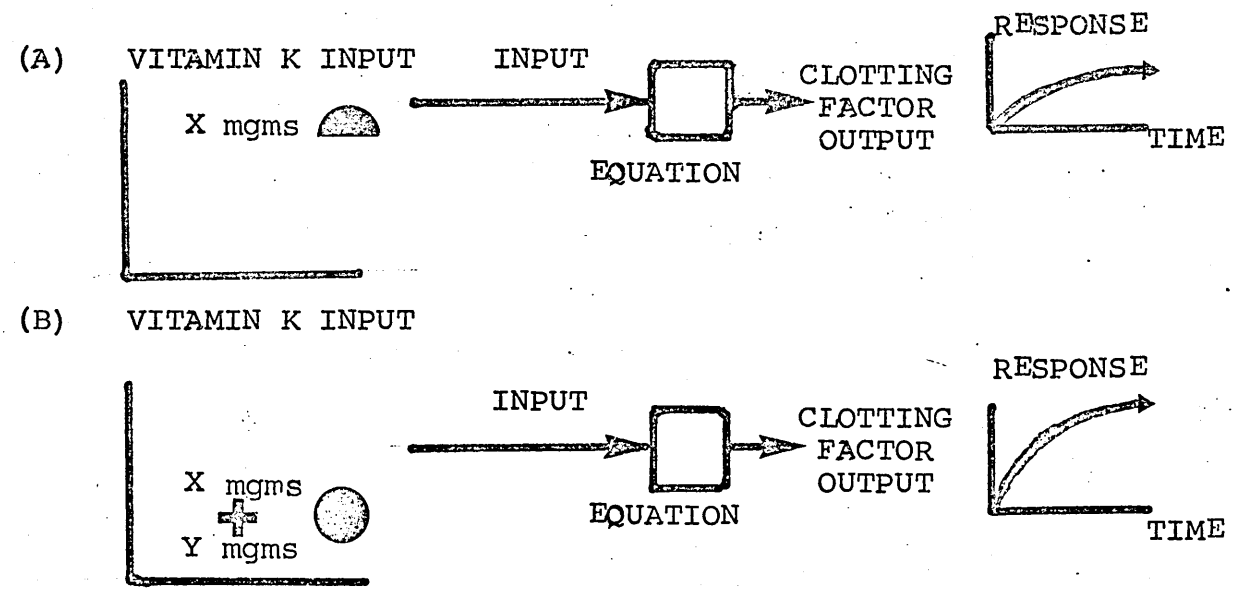


ABSORPTION AND UTILISATION PATHWAYS

POSSIBLE INHIBITORY MECHANISMS DEVELOPED DURING DISEASE PROCESSES

This type of model illustrates the complexity in assessing absorption, uptake and utilisation of vitamin K_1, especially in disease states.

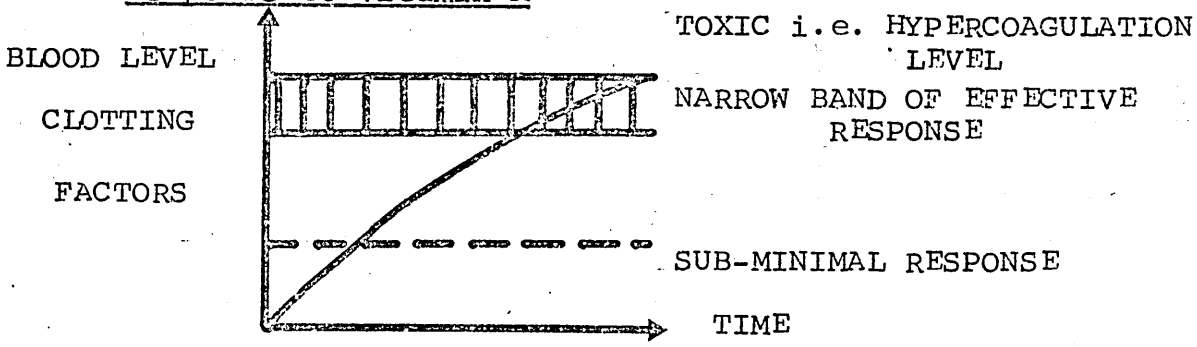
Similar models could also be used in assessing different vitamin K_1 inputs, i.e. dose, as follows :



This type of vitamin K_1 dose input assessment would be useful in deciding whether the response to vitamin K_1 is related to dose, i.e. concentration dependent and how this is affected in liver disease states.

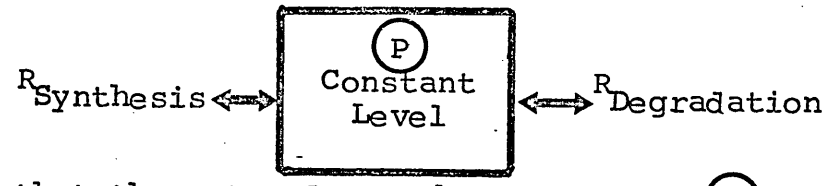
Of extreme value could be the assessment of the mechanisms by which the response to vitamin K_1 is controlled. When the initial high rates of synthesis in the fast phase (α_1) are followed by a slower phase (α_2) it could be controlled by either of the following pathways.

(A) Critical level of plasma clotting factors in response to vitamin K



(B) Circulating plasma level control mechanism

Perhaps the circulating plasma level (P) reflects the balace between $Rate_{Synthesis}$ and $Rate_{Degradation}$ of the clotting factors as follows :



and that the rate of net change R_{Net} of (P) at any time may be described by

$$R_{Net} = R_{Synthesis} - R_{Degradation}$$

This means that a constant level of clotting factor is observed when $R_{Synthesis} \equiv R_{Degradation}$. If, however, $R_{Synthesis}$ decreases (i.e. liver disease,

obstructive jaundice) then $R_{Synthesis} < R_{Degradation}$ and the plasma level (P) falls. On compensation with the liver disease state minimum levels of (P) are found when $R_{Synthesis}$ again equals $R_{Degradation}$. If, however, it is possible to utilise vitamin K_1, e.g. obstructive jaundice, then $R_{Synthesis}$ is stimulated until $R_{Synthesis} > R_{Degradation}$ and (P) increases until it reaches normal levels where $R_{Synthesis}$ is the normal synthesis rate and equals $R_{Degradation}$. This could explain one of the mechanisms by which the utilisation of vitamin K_1 followed by subsequent synthesis is regulated once the levels approach normality, avoiding hypercoagulation status.

The overall assessment of the study shows that apart from aiding diagnosis and prognosis it has provided valuable information on the complex relationships involved in liver disease with vitamin K absorption and utilisation.

CHAPTER IV

STUDY OF PROTEINS INDUCED BY VITAMIN K ABSENCE IN LIVER DISEASE (PIVKA)

4.1 INTRODUCTION

The existence of proteins induced by vitamin K absence (PIVKA) was suggested from experiments on the kinetics of the blood coagulation process in 1964. These proteins were considered to be analogous to the vitamin K dependent factors II, VII, IX, X and will be referred to as PIVKA II, PIVKA VII, PIVKA IX, PIVKA X respectively.

More recently in 1968 the presence of the PIVKA analogues was detected in the plasma of patients on oral anticoagulants[106]. These experiments indicated that whilst the presence of oral anticoagulants *in vivo* interfered primarily with the synthesis of Factors II, VII, IX, X, resulting in decreased levels in circulation, it could also result in detectable amounts of PIVKA protein being released into the plasma.

In this study the demonstration and quantitation of PIVKA in liver disease will be attempted using the following techniques:

(a) Modified thrombotest according to Hemker[107];

(b) One-dimensional quantitative immuno-electrophoresis of factor II;

(c) Two-dimensional quantitative immuno-electrophoresis of factor II.

It is hoped that these studies will show whether abnormal protein synthesis in patients with liver disease exists. Furthermore it is hoped to discuss the results

in the context of (a) effects of such abnormal synthesis on the haemostatic mechanism; (b) whether any functional abnormalities in patients with liver disease, as assessed by clinical criteria, are paralleled by the production of functional abnormalities in clotting proteins.

The study will be divided into two parts :

(1) The production and characterisation of a human antiserum against the vitamin K dependent clotting factor II.

(2) The use of the prepared antiserum and the modified thrombotest to demonstrate the presence of proteins induced by vitamin K absence in liver disease.

PART ONE

Production and Characterisation of Antisera against the Vitamin K Dependent Clotting Factor II

4.2 INTRODUCTION

In order to quantitate immunologically the plasma level of factor II in the absence of vitamin K (PIVKA II) it was necessary to produce and characterise antisera to human factor II, which will be described here.

The problems associated with the production of monospecific antibodies to clotting factors can be particularly difficult to resolve. In recent years several investigators[108] have succeeded in producing antisera to human prothrombin, bovine prothrombin, human factor VII, human factor IX and human factor X. These involve preparing either (a) concentrates of individual clotting factor, or (b) using prepared concentrates with more than one clotting factor and absorbing out contaminants at a later stage in the antibody preparation. These concentrates are then used as antigens for the

preparation of antisera in rabbits, sheep, goats, cows[109].

4.3 MATERIALS

4.3.1 Plasma samples

Plasma samples were prepared by collecting blood from a pool of 25 normal laboratory staff. The blood collected was mixed in the ratio of 9 mls blood to 1 ml of 3.8% (W/V) sodium citrate. Following anticoagulation and mixing, the plasma was separated by centrifugation at 3000 r.p.m. for 15 mins. at 4°C, pooled and then stored at -20°C in 1 ml aliquots in plastic containers.

4.3.2 Chemicals

Except where mentioned chemicals were of pure grade and obtained from B.D.H., SIGMA or MERCK.

4.3.3 Antiserum and normal rabbit serum

Collection and preparation of antiserum and normal rabbit serum was performed by shaving the marginal ear vein of the rabbit and then transversely cutting the vein. After allowing blood to flow freely into sterile universal containers the bleeding was stopped. Up to 45 mls of blood were drawn from the rabbits using this technique at any one collection. One hour following the collection, the blood was centrifuged at 3000 r.p.m. for 15 minutes and the serum left on the clot overnight at room temperature. The serum was harvested and stored at -20°C in 1 ml amounts, after the addition of 0.01% final concentration of sodium methiolate, in plastic containers.

4.3.4 Special apparatus and reagents

Pharmacia chromatography columns K15/30 (1.5 cm/30 cm) K25/40 (2.5 x 40 cms), K25/100 (2.5 x 100 cm) were used in fractionation techniques, with the fractions collected in the CAMLAB refrigerated fraction collector. The LKB "UVICORD" was used for monitoring absorbency of column fractions at 280 Nm, this being recorded simultaneously on an L.K.B. recorder. Apparatus for polyacrylamide electrophoresis both analytical and preparative was obtained from Shandon Instruments.

Spectrophotometric analysis was carried out on a Pye-Unicam SP.1800 spectrophotometer. Concentration of protein samples was carried out in Amicon ultrafiltration apparatus, using verious U.M. membrane filters. Electrophoretic equipment used for immunoelectrophoresis, i.e. electrophoresis chamber and power pack, was supplied by Behring Diagnostics, Hoechst Pharmaceuticals.

4.4 METHODS

4.4.1 Factor II, VII, IX, X assays were carried out using the techniques described in Chapter 2 (Methods and Material).

4.4.2 Immunodiffusion was performed by a modified Ouchterlony[110] technique in which glass slides 3x1x0.1 inches were covered with 3 mls 1% agarose in 0.05 m barbitone acetate buffer pH 8.6 (oxoid). Wells 3 mms in diameter were cut in the agarose and 10 μl of undiluted antiserum or antigen were placed in the wells. The diffusion was allowed to proceed for 48 hours at R. temperature. Slides were dried and stained by Coomassie blue.

4.4.3 One dimensional quantitative immuno-electrophoresis in 1% agarose was performed according to Laurell[111] on glass plates 8 x 8 x 0.2 cms.

4.4.4 Two dimensional crossed immunoelectrophoresis was carried out by the method of Lowell[112] and Clarke and Freeman[113]. Electrophoresis was carried out in the first dimension in 1% agarose in 0.05 m barbitone acetate buffer pH 8.6 on glass slides 8 x 8 x 0.2 cms at 240 V for three hours. The gel containing the separated proteins was transferred to a clean glass slide and subjected to immunoelectrophoresis at right angles into 1% agarose containing specific antiserum in the same buffer. Calcium lactate 2.5 mM was present during the first dimension when it was necessary to separate proteins in the presence of calcium. The second dimension immunoelectrophoresis was carried out at 110 V for 16 - 18 hours with cooling and the slides were dried and stained with Coomassie blue.

4.4.5 SDS polyacrylamide gel electrophoresis was carried out according to the method of Nils Olav Solum[114], using 5% gels.

4.5 PRODUCTION AND CHARACTERISATION OF ANTIBODY TO FACTOR II

4.5.1 Introduction

The four factors, II, VII, IX, X, comprising the prothrombin complex have many points in common; for example, in adsorption techniques using inorganic absorbants such as barium sulphate, aluminium hydroxide, they are adsorbed together on to the inorganic powders.

According to Devillee et al[115] in the presence of Cd^{2+}, aluminium hydroxide adsorbs less of the factor II than factors VII, IX, X. Using this procedure factor II was separated and purified employing column chromatography and then used to raise specific antibodies to factor II as follows.

4.5.2 Purification procedure (Devilee et al[115])

(a) All procedures were carried out in a cold room.

(b) To 250 mls of normal plasma is added successively 1/100 volume of 0.1 M cadmium sulphate and 1/5 volume of aluminium hydroxide in the form of 20% (W/V) moist gel.

(c) After stirring for 10 mins, the $AlOH_3$ was removed by centrifugation for 15 mins at 6000 x g.

(d) The cadmium from the supernatant (Supernatant A) was removed by adding 3 gms solid sodium oxalate per 100 mls and then centrifuging to remove the cadmium oxalate precipitate (15 mins, 6000 x g) to give Supernatant B .

(e) From the supernatant B, prothrombin (Factor II) was adsorbed on to 20% (W/V) moist gel $AlOH_3$, which was then sedimented as above.

(f) The $AlOH_3$ sediment was washed first with 1/5 volume of 0.1 M EDTA (pH 8.0) and then with 1/5 volume of 0.15 M NaCl.

(g) The proteins adsorbed were eluted with 1/20 volume 0.25 M sodium-potassium phosphate buffer (pH 8.0) and the $AlOH_3$ was removed.

(h) The eluate was dialysed overnight against 0.1 M NaCl in 0.01 M sodium-potassium phosphate buffer pH 6.8 and applied to a 9 x 1 cms column containing DEAE-SEPHADEX A50, which was previously equilibrated with 0.1 M NaCl in 0.01 M sodium phosphate buffer pH 6.8. Initially 50 mls of this buffer was followed by a linear gradient in the same buffer of 0.0 - 0.6 M in $NaCl_2$.

(i) Fractions were collected in 4.0 ml volume and monitored for absorbance at 280 NM using LKB uvicord equipment.

(j) Prothrombin eluted as a single peak (Fig. 27) in fractions 38 - 50, total volume = 40 mls, which were collected, pooled and concentrated to 8 - 10 mls using Amicon equipment.

4.5.3 Properties of the purified material

The plasma, supernatant A, supernatant B $Al(OH_3)$ eluant and sephadex A-50 were examined using a specific assay of factor II in order to show purification of the factor II during adsorption and chromatography procedures.

From the results of table 26 it can be seen that approximately 35% of the starting prothrombin was recovered in the sephadex A-50 eluate. At the same time only minimal, if any, contamination with factor VII, IX, X (< 1%) was detected in the final preparation of factor II before concentration.

4.5.4 Injection schedule for raising antibodies to human factor II

1 ml of factor II and 1 ml of Freund adjuvant were mixed for 5 minutes and then 1 ml of this mixture was injected into each haunch of a New Zealand white male rabbit, 3 - 5 kgs in weight. The rabbits were then :

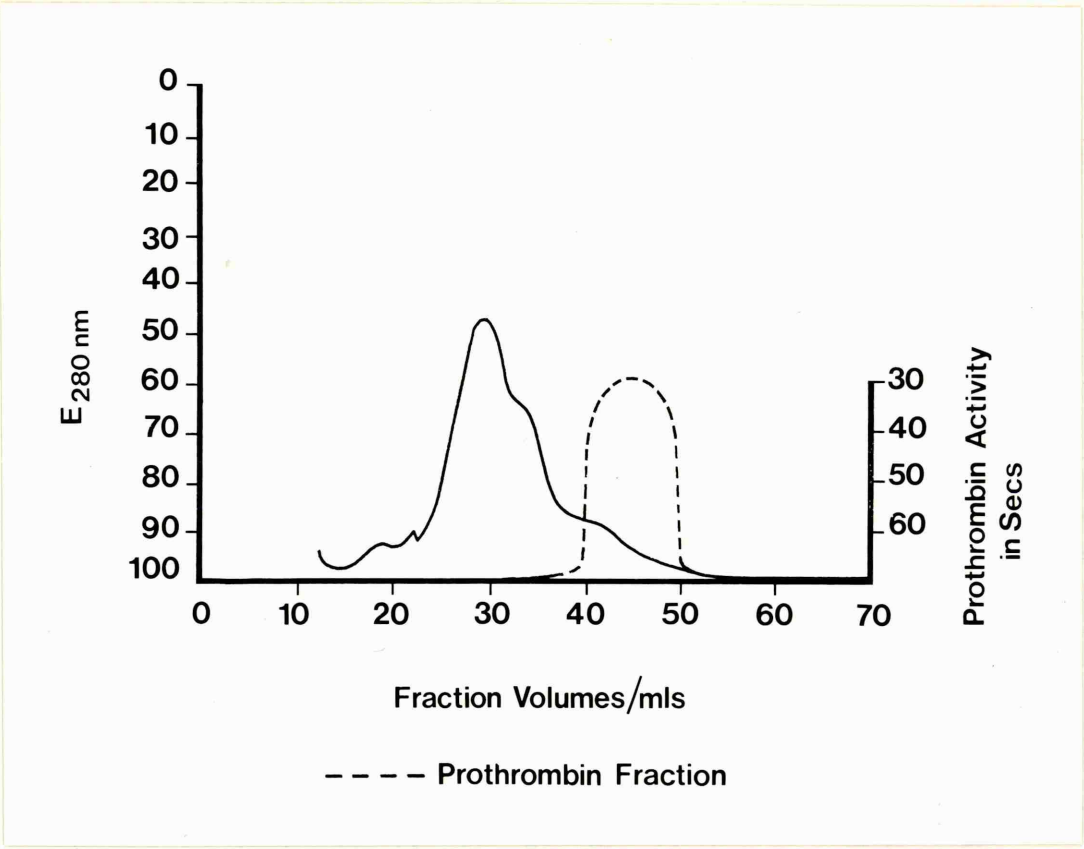
(a) Left for 14 days;

(b) Bled in order to assess antibody production prior to a repeat injection of factor II and Freund adjuvant;

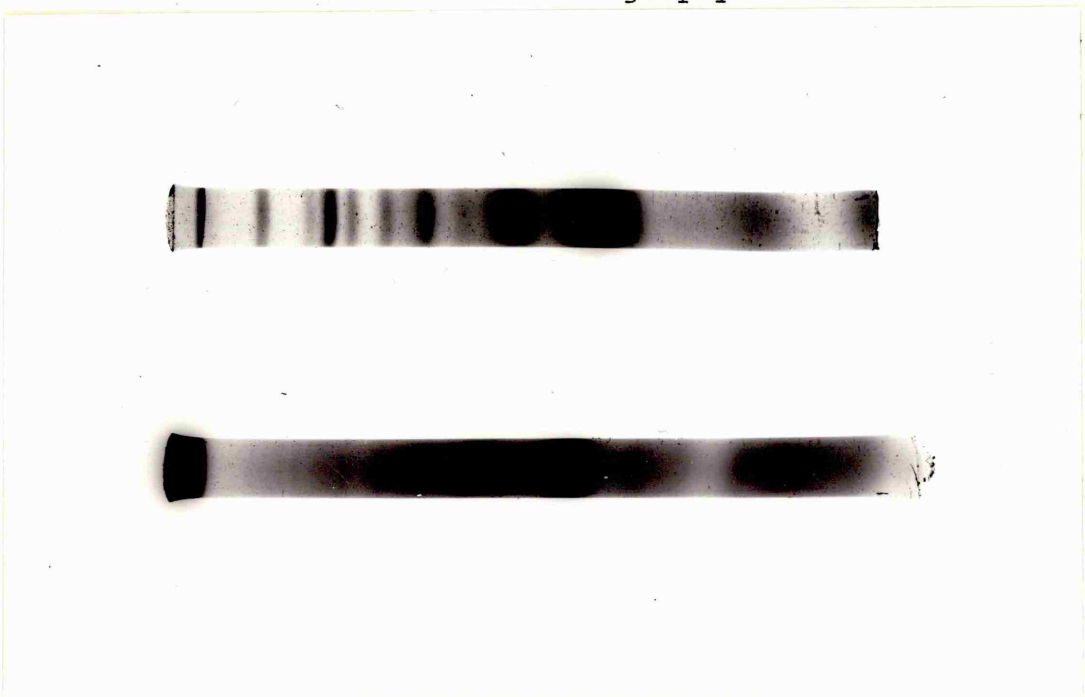
(c) Left for a further 14 days;

(d) Bled once more to assess antibody production, followed by

(e) An injection of 1 ml of factor II intravenously with no Freund adjuvant;



a. DEAE-SEPHADEX A-50 Chromatography of Prothrombin



b. Polyacrylamide gel electrophoresis of human Factor II

(i) Sample before chromatography
(ii) Sample after chromatography with one peak

Figure 27

diffusion technique of Oucherlony. Results of the double immunodiffusion experiment with the antibody to factor II are shown in figure 28.

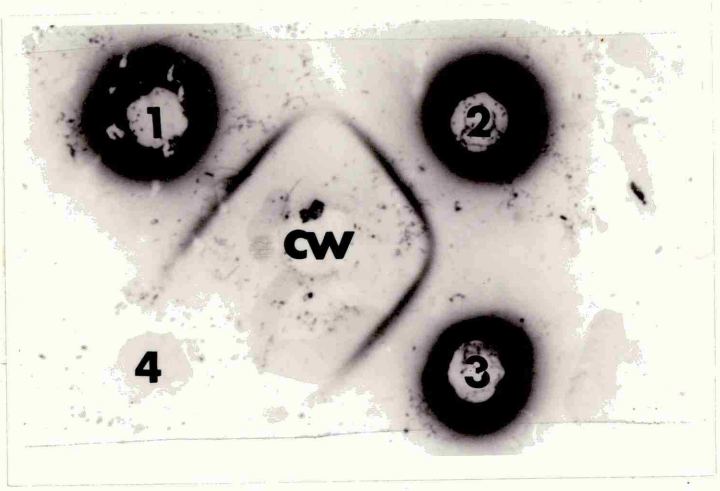


Figure 28

Double immunodiffusion (Oucherlony) in 1% agarose gels using wells of 3 mms in diameter

CENTRE WELL	contained test antibody to factor II
WELL ONE	Normal plasma 1/5 dilution
WELL TWO	Normal plasma 1/5 dilution
WELL THREE	Normal plasma 1/5 dilution
WELL FOUR	Normal aged serum

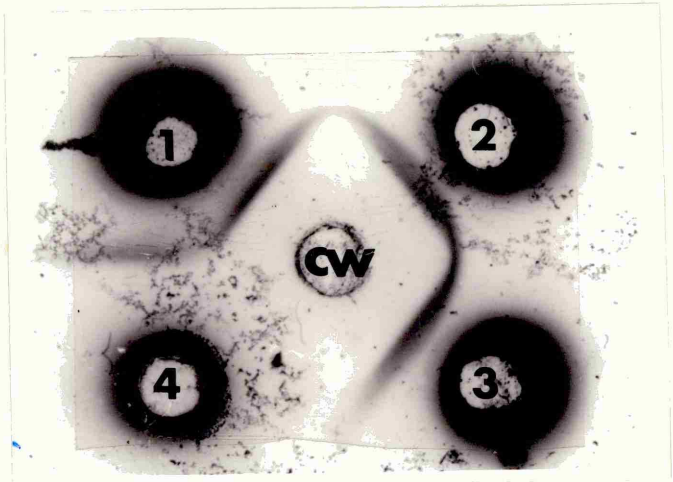


Figure 29

CENTRE WELL	Test antibody to factor II
WELL ONE	Normal plasma 1/5 dilution
WELL TWO	Normal plasma 1/5 dilution
WELL THREE	Normal plasma 1/5 dilution
WELL FOUR	Reference antibody to factor II

The antibody raised against human factor II showed a single precipitation line with normal plasma and no line with aged normal serum (Figure 28). A reaction of identity was shown by fusion of the precipitates between wells containing the antibody, reference antibody and normal plasma (Figure 29).

4.6.3 Assessment of specificity of absorption using two dimensional immunoelectrophoresis

Prior to the testing for specificity the antibody was absorbed with absorbed normal serum as described in section 4.5. Two-dimensional immuno-electrophoresis was used to demonstrate the removal of contaminants for the antisera following the absorption. The antisera was tested pre- and post-absorption using the two-dimensional immunoelectrophoresis technique.

4.6.4 Results

The reaction of the antibody to factor II (which had not been absorbed with normal serum) with normal plasma is shown in figure 30.

Figure 31 shows the reaction of the antibody to human factor II (post-absorption with normal serum) and normal plasma.

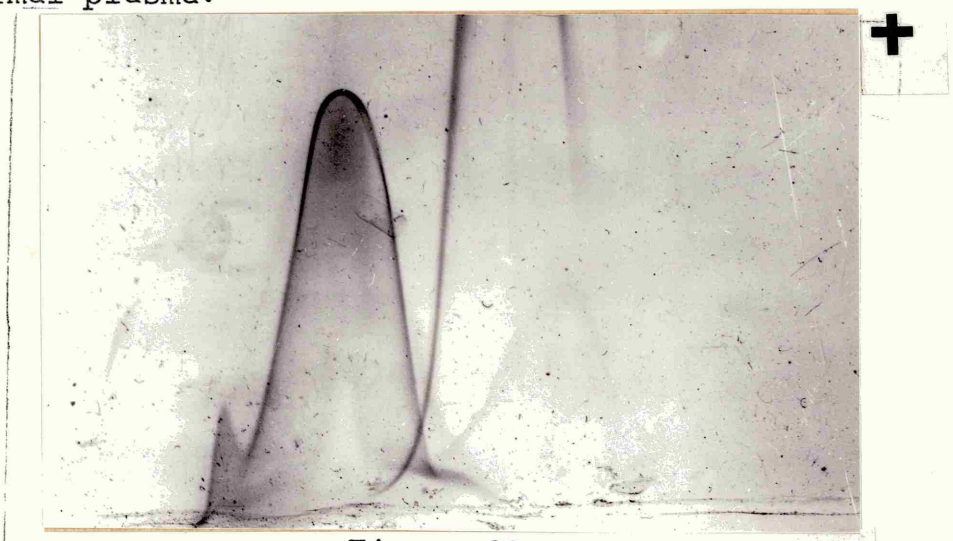


Figure 30

Rabbit antisera to factor II prior to absorption
Two-dimensional immunoelectrophoresis against
normal plasma

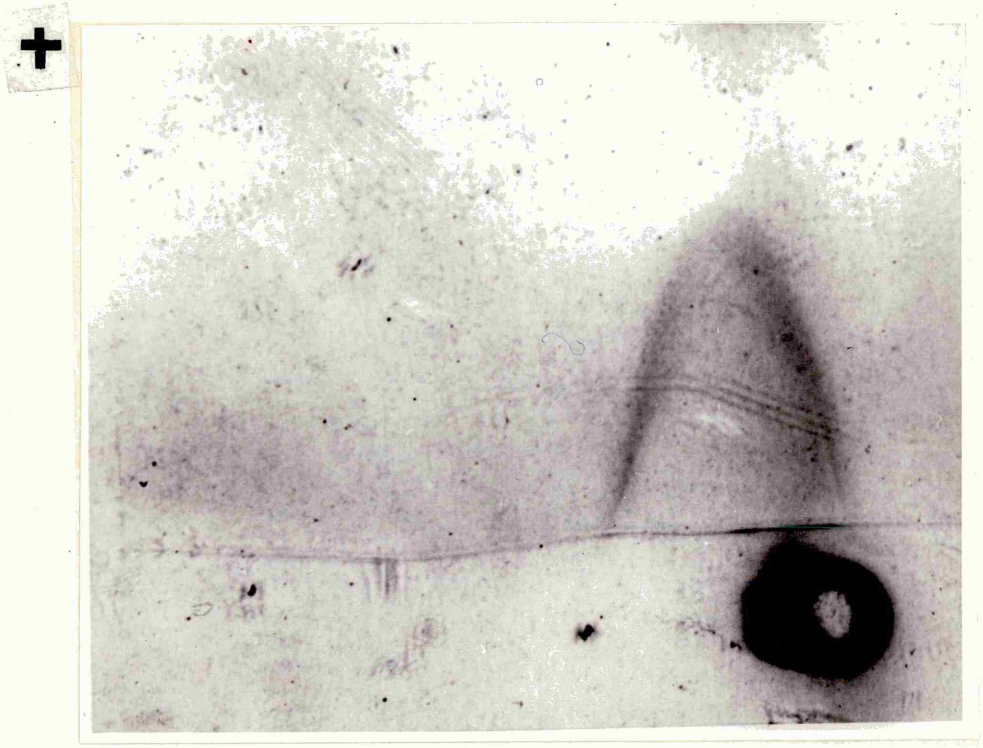


Figure 31

Rabbit Antisera to Factor II post adsorption with adsorbed normal serum
Two-dimensional immunoelectrophoresis against normal plasma

The results show that following specific adsorption with adsorbed normal serum only one precipitation line was observed against normal plasma (Figure 31).

4.6.5 Summary

The antibody to human factor II gave a specific reaction of identity with a reference antibody to factor II. In addition, following specific adsorption of contaminants with normal serum, only one reaction was observed against normal plasma in the two-dimensional immunoelectrophoresis technique.

4.6.6 Neutralisation of coagulation factor activity by antisera

Antisera were treated as described under Materials and Methods. When 0.1 ml antiserum was incubated for 1 hour at 37°C with 0.9 mls normal pooled human plasma (NHP), diluted 1:5 with veronal acetate buffer pH 7.35, factor II, VII, IX and X activities were assayed according to assay methods (Chapter two). In the control experiment 0.1 ml normal rabbit serum (NRS) or 0.1 ml buffer were used instead of antiserum.

Clotting times (t_c) for each clotting factor assay have been given.

INCUBATION	t_c-II	t_c-VII	t_c-IX	t_c-X
NHP + SALINE (NOT INCUBATED)	20"	29"	110"	34.1"
NHP + anti II	72"	27"	114"	34.6"
NHP + NRS	20"	32"	110"	34.0"
NHP + Buffer	21"	30"	118"	34.0"
Buffer	90"	90"	180"	96"

4.6.7 Summary

Under the test conditions chosen (0.1 ml antiserum + 0.9 mls NHP diluted 1:5 with veronal acetate buffer pH 7.35) a residual factor II of 2% was calculated from a reference plot at the clotting time determined. Thus anti-factor II neutralised about 98% of factor II activity whereas no neutralisation of factors VII, IX, X was observed.

Conclusion These findings, 4.6.2, 4.6.3 and 4.6.6, suggest that an immunospecific antiserum to factor II had been produced in the rabbit.

PART TWO

Study of Proteins induced by Vitamin K Absence (PIVKA) in Liver Disease

4.7 INTRODUCTION

In this section the demonstration of PIVKA by the modified thrombotest is described. Using immunological assays the demonstration and quantitation of PIVKA II have been attempted. Characteristics of PIVKA in relation to liver disease and the haemostatic mechanism are also discussed.

4.8 METHODS

4.8.1 PIVKA screening test

Using modified thrombotest, the PIVKA screening test was carried out as follows :

Dilutions of plasma in glyoxaline buffer tested against Thrombotest reagent, produced clotting times which when plotted as t(time) against D (dilutions) produced a straight line. These clotting times (i.e. the modified thrombotest) can be used as an overall assessment of the levels of factors II, VII, IX, X in liver disease, since the slope of the line is proportional to the substrate (i.e. complex of factors II, VII, IX, X) concentration in the plasmas tested. Extrapolation to infinite substrate concentrate (intercept of the line obtained with the ordinate) will give the minimum clotting time (t_{min}) (Figure 32).

Plasma with different substrate concentrations all display the same value for t_{min} (Fig. 32). This holds for plasma from patients with hepatic damage of varying severity. In vitamin K deficiency, however, Hemker[116] suggested that the modified thrombotest was found to be elevated, suggesting the presence of an

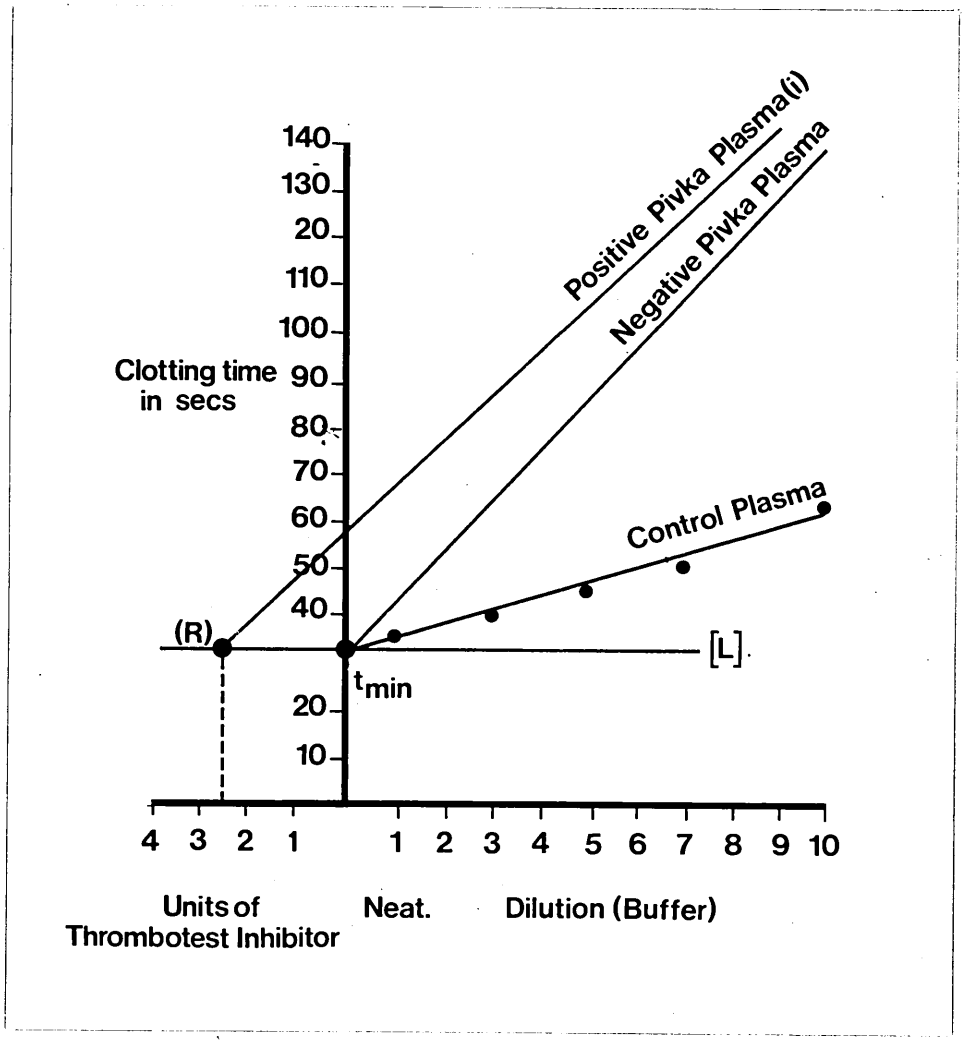


Figure 32

PIVKA screening test

INHIBITOR, tentatively called PREPROTHROMBIN, but subsequently named PIVKA. The quantitation of the inhibitor is carried out as follows :

> When a line (L) is drawn through the t_{MIN} of normal plasma parallel to the Abscissa, the lines obtained from the values of a plasma containing the inhibitor (i) will intercept (L) at a distance (R) left of the ordinate[117]. The length of (R) is proportional to the amount of inhibitor present, independent of the amount of substrate.

Reagents

Thrombotest supplied by B.D.H.
Glyoxaline buffer pH 7.35
Control plasma (non-contacted)

Technique

Prepare NEAT, 1/3, 1/5, 1/7, 1/10 dilutions of control and patients plasma in glyoxaline buffer. Prewarm the thrombotest 0.25 mls in plastic tubes ready for use. Add 0.05 mls of a plasma to the thrombotest and start the stopwatch. Mix quickly and leave for 30 seconds; then time clot formation. Repeat with dilutions.

4.8.2 One-stage factor assays were carried out by techniques described in Chapter 2.

4.8.3 One-dimensional and two-dimensional immuno-electrophoresis was carried out according to the techniques described in section 4.4.

4.9 USE OF MODIFIED THROMBOTEST AS PIVKA SCREEN

4.9.1 Results

Table 27 shows inhibitor unit values from a time-dilution (t-D) plot estimation using thrombotest, obtained in a group of liver disease patients. Controls included normal plasmas and plasma from patients under prolonged anticoagulant therapy (abnormal control group).

4.9.2 Analysis

Preliminary studies for PIVKA inhibitors using the modified thrombotest show that in patients undergoing anticoagulant therapy, detectable amounts of PIVKA were present ($N = 19$ $\bar{x} = 3.15$ units Table 27) as would be expected in the presence of vitamin K antagonist therapy. The normal group results ($N = 19$ $\bar{x} = 0.1$ $S.D = 0.11$ Table 27) indicated that our normal population gave a normal range of 0 - 0.32 units of PIVKA as detected with modified thrombotest. Liver disease patients showed abnormal levels in only 23 out of the 38 patients tested (Table 27) indicating that the PIVKA was NOT present in all types of liver disease ($N = 38$ $\bar{x} = 1.02$).

Since Hemker suggested that the thrombotest detected an increase in the amount of circulating precursor protein, e.g. PIVKA, which under normal conditions is converted by the vitamin K dependent step, these results in the liver disease patient indicate the absence of the vitamin K dependent step in certain liver disease conditions.

4.9.3 PIVKA screen in non-obstructive and obstructive liver disease

In the previous analysis the random group of liver disease patients gave results which indicated that some of the group had significant inhibitor protein present. A highly selected group of liver disease

TABLE 27

PIVKA Screen Inhibitor Values obtained using Modified Thrombotest Results

Anticoagulent Patients	Liver Disease Patients		Normal Control Plasma
Units	Units	Units	Units
3.1	1.8	0.0	0.0
2.8	1.4	0.0	0.0
2.3	0.7	0.0	0.4
5.0	2.0	0.0	0.1
2.4	1.9	1.0	0.0
3.1	0.4	2.4	0.0
2.0	0.1	0.0	0.1
6.0	0.2	0.1	0.0
3.8	0.0	0.0	0.0
3.0	0.1	1.3	0.0
5.0	0.1	1.5	0.2
1.2	1.3	0.2	0.3
1.3	2.0	0.4	0.1
2.4	1.2	1.8	0.0
2.6	2.9	1.0	0.0
3.2	1.5	0.5	0.1
7.0	1.0	0.4	0.1
2.2	4.0	0.0	0.0
1.5	5.5	0.0	0.0
N = 19	N = 38		N = 19
x = 3.15	x = 1.02		x = 0.07
SD = 1.58	SD = 1.22		Sd = 0.11

patients were next tested using the same techniques. The group was made up as follows :

Patients	No. Tested
Obstructive jaundice patients	6
Non-obstructive jaundice patients	6

4.9.4 Results

The results obtained using the modified thrombotest are shown in table 28.

TABLE 28

PIVKA Screen Inhibitor Values (units/ml) obtained in Control, Obstructive and Nonobstructive Liver Disease Patients

	Mean $\pm$	2.S.D.
Controls	0.10	0.12
Obstructive Jaundice	3.17	2.40
Nonobstructive Jaundice	0.05	0.05

4.9.5 Analysis

The modified thrombotest indicated that more PIVKA material was detected in obstructive liver disease ($\bar{x} = 3.17 \pm$ S.D. = 2.40) than in either non-obstructive liver disease states or the normal control group.

4.9.6 PIVKA Screen in a Patient receiving Vitamin K Therapy

(Estimation of inhibitor in a patient receiving vitamin K)

Table 29 shows the results of the modified thrombotest on plasma obtained from a patient receiving

10 mgms vitamin K_1 (I.V.). Samples of plasma were obtained at 0, 1, 2, 4, 8, 24 hours as described in Chapter 3 (Materials and Methods). These results were plotted as shown in Figure 33.

TABLE 29

Results of the Modified Thrombotest in a Patient receiving Vitamin K_1 10 mgm (I.V.)

Plasma Specimen	Clotting Times Obtained (DILUTION/GLYOXALINE)			PIVKA Value/Units
	N	1/2	1/3	
Pre Vit.K Infusion	61"	74"	90"	1.3
Post 1 Hr "	56"	65"	78"	1.0
Post 2 Hr "	50"	58"	70"	0.7
Post 4 Hr "	43"	51"	58"	0.5
Post 8 Hr "	40"	47"	53"	0.2
Post 24 Hrs "	34"	38"	43"	0.1
Control Plasma	34"	41"	45"	0.0

4.9.7 Analysis

The results of the modified thrombotest carried out on the plasmas of a patient receiving 10 mg vitamin K_1 (I.V.) (Table 29 and Figure 33) revealed gradual changes in the inhibitor level during therapy (Pre level = 1.3 units; Post 24 hours = 0.1 units). As well as changes in the inhibitor lines the plot of the clotting times alters during the therapy. The reciprocal slope of the lines, (expressed as the co-tangent, i.e. 1/TAN of the angle between this line and the abscissa) can be obtained for both

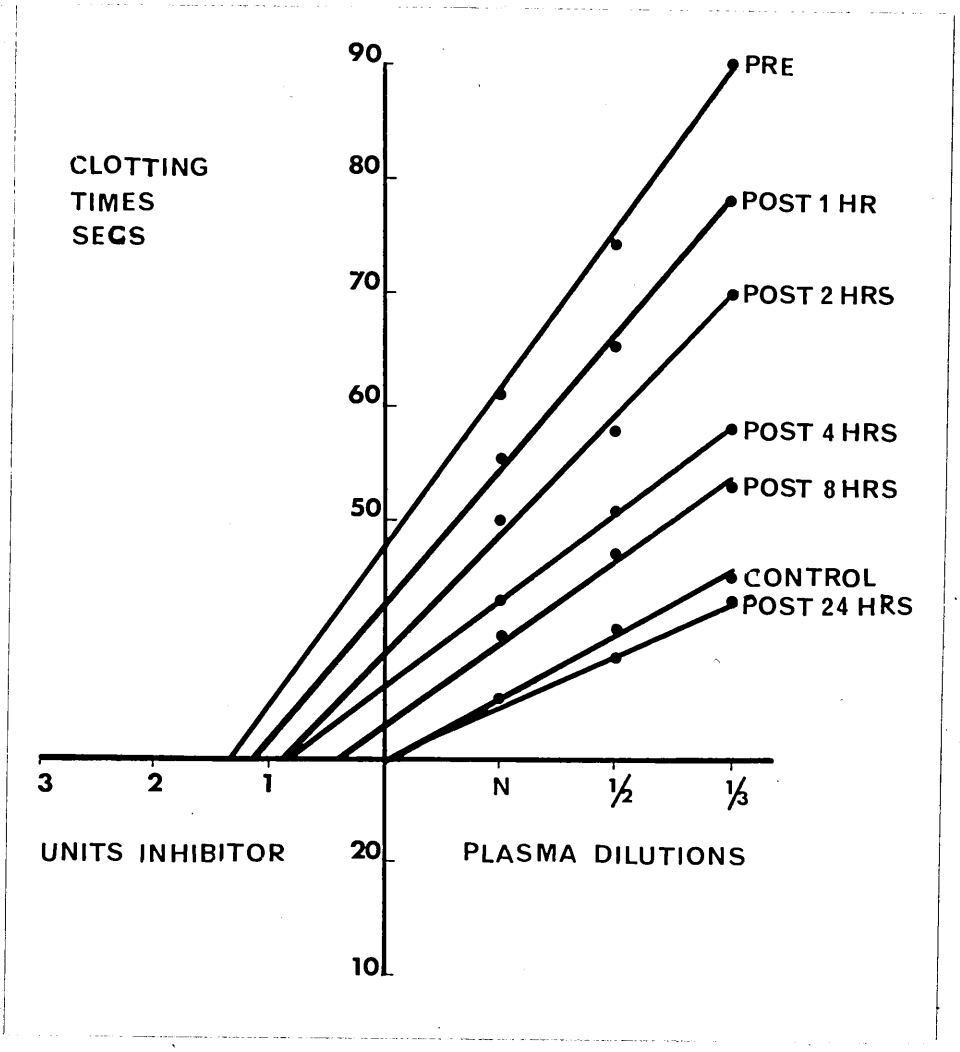


Figure 33

Results of the Modified Thrombotest in a Patient receiving Vitamin K_1 10 mgm (I.V.)

the patient α_X and the control α_N. The ratio between α_X and α_N is thought to indicate the amount of factor X present in the unknown plasma[118], e.g.

$$\text{Pre sample} = \frac{\alpha_X \text{ (Pre)}}{\alpha_N \text{ (Control)}} = \text{Ratio} \equiv \text{Factor X level}$$

The ratio of $\frac{\alpha x}{\alpha N}$ for all the samples was analysed and the equivalent amount of factor X was calculated. This was then compared with the actual assay of factor X obtained in the clotting factor assay. The results are shown in Table 30.

TABLE 30

The relationship between the ratio of $\frac{\alpha_X}{\alpha_N}$ and factor X assay using Thrombotest

Sample	Ratio $\frac{\alpha_X}{\alpha_N}$	Equivalent Factor X	Clotting F.X Assay
		%	%
Pre	0.33	33	31
Post 1 Hr	0.44	44	40
Post 2 Hr	0.50	50	55
Post 4 Hr	0.72	72	57
Post 8 Hr	0.74	74	69
Post 24 Hr	1.14	114	110

The ratios expressed as equivalent amounts of factor X correlate with the actual assay of factor X clotting activity. This confirms the findings of Hemker et al[116], although their investigations were not conducted on a patient receiving vitamin K. It also confirms that the modified thrombotest is extremely sensitive to the circulating plasma level of factor X. In particular, the form of the circulating X

is important since factor X has been shown to be rate-limiting for the velocity of the reaction[119] PIVKA X in circulation would cause inhibition to take place at the coagulation sequence at which factor X is rate limiting and work as a competitive inhibitor. This will be discussed later in the thesis.

4.10 ONE-DIMENSIONAL IMMUNOELECTROPHORETIC ANALYSIS OF FACTOR II USING ANTI-HUMAN FACTOR II ANTIBODIES

4.10.1 Obstructive Jaundice Patients

Using a homogenous distribution of factor II antisera in 1% agarose, the amount of immuno-reactive factor II was determined by one-dimensional electrophoresis. The peak height (in mms) shows a linear relationship to the concentration of antigen applied, therefore the determination allows quantitation of the factor II antigen present.

4.10.2 Results

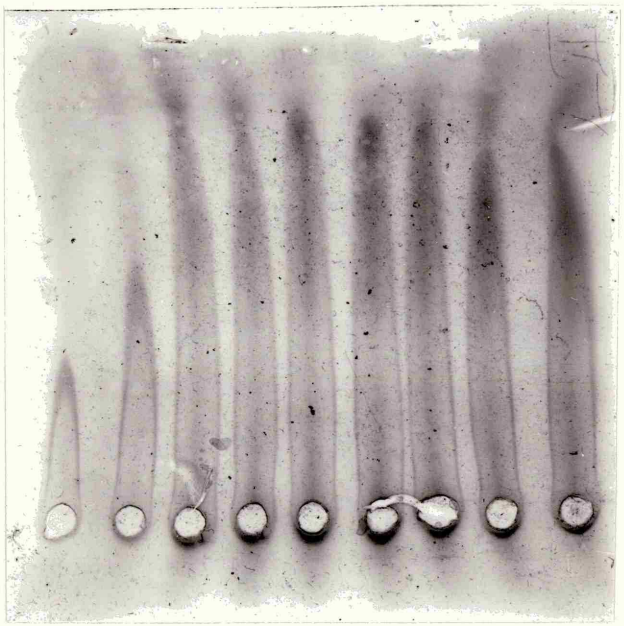
Samples were immunoelectrophoresed and the results analysed (Figure 34). Prior to electrophoresis the samples were diluted 1:5 in barbitone acetate buffer pH 8.6 prior to application.

4.10.3 Comment

In all the obstructive jaundice plasmas tested, excess antigenic factor II was detected in relation to the amount detected by specific factor II assays. It would appear therefore that the plasma contains a functionally abnormal vitamin K dependent factor II.

4.10.4 Non-obstructive liver disease

Figure 35 shows the results of I.D. electrophoresis with various liver disease states (non-obstructive jaundice).



1 2 3 4 5 6 7 8 9

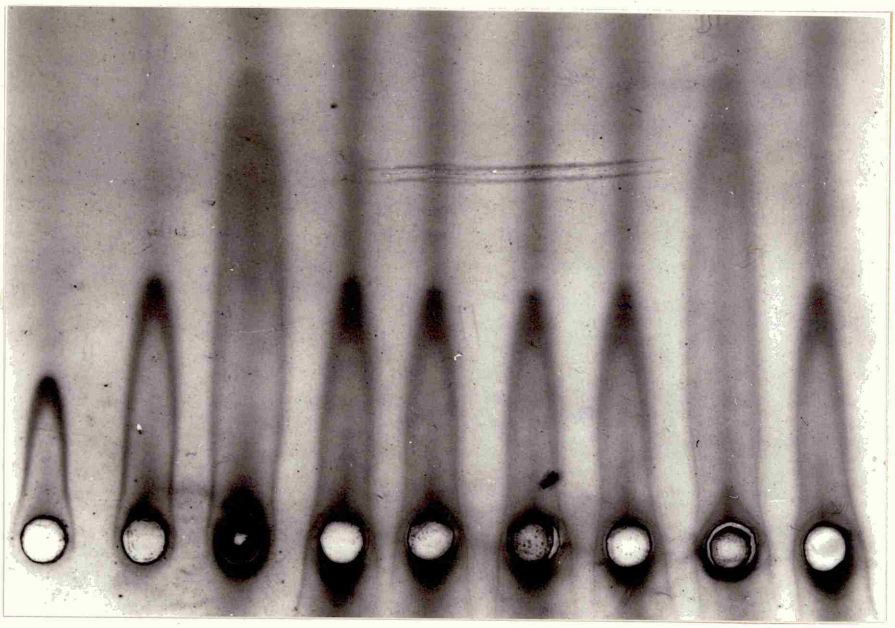
Figure 34

Obstructive Jaundice Patients

Quantitative one-dimensional immunoelectrophoresis according to Laurell in the presence of anti-human factor II

Samples 10 μl placed in the numbered wells

			Antigenic II		Factor II Assay
Well	1	Control plasma	1/50	(10%)	110%
"	2	" "	1/10	(50%)	
"	3	" "	1/5	(100%)	
"	4	Patient No. 1	1/5	(90%)	37%
"	5	" " 2	1/5	(105%)	12%
"	6	" " 3	1/5	(96%)	28%
"	7	" " 4	1/5	(93%)	30%
"	8	" " 5	1/5	(83%)	18%
"	9	" " 6	1/5	(90%)	28%



1 2 3 4 5 6 7 8 9

Figure 35

Non-obstructive Liver Disease Patients

Quantitative one-dimensional immunoelectrophoresis according to Laurell in the presence of anti-factor II

Samples 10 μl placed in the wells

			Antigenic II	Factor II Assay
Well	1	Control plasma	1/50 (10%)	
"	2	" "	1/10 (50%)	
"	3	" "	1/5 (100%)	110%
"	4	Chronic Act. Hepatitis	(50%)	59%
"	5	Primary Biliary Cirrhosis	(52%)	50%
"	6	" " "	(47%)	40%
"	7	" " "	(49%)	58%
"	8	Hepatitis	(100%)	70%
"	9	Alcoholic Cirrhosis	(54%)	41%

4.10.5 Comments

In the non-obstructive jaundice plasma tested there appeared to be a direct relationship between the concentration of antigen in factor II and clotting activity, indicating that the patients were synthesizing decreased amounts of a structurally normal factor II. One exception to this was patient No. 8 in whom it was believed that some form of vitamin K deficiency was also present.

4.10.6 Anticoagulant patients

Figure 36 shows the result of one-dimensional electrophoresis with anti-human factor II in patients on anticoagulants. (The arcs within the rockets in my view are a photographic artefact.)

4.10.7 Comment

In plasma from patients on oral anticoagulant an excess of antigenic factor II to clotting factor II was detected. These findings support the hypothesis that anticoagulants compete with vitamin K and precursor proteins (PIVKA) are produced as a by-product instead of normal factor II. The levels detected did not appear to correlate with the degree of anticoagulant the patient was being maintained on (personal observation).

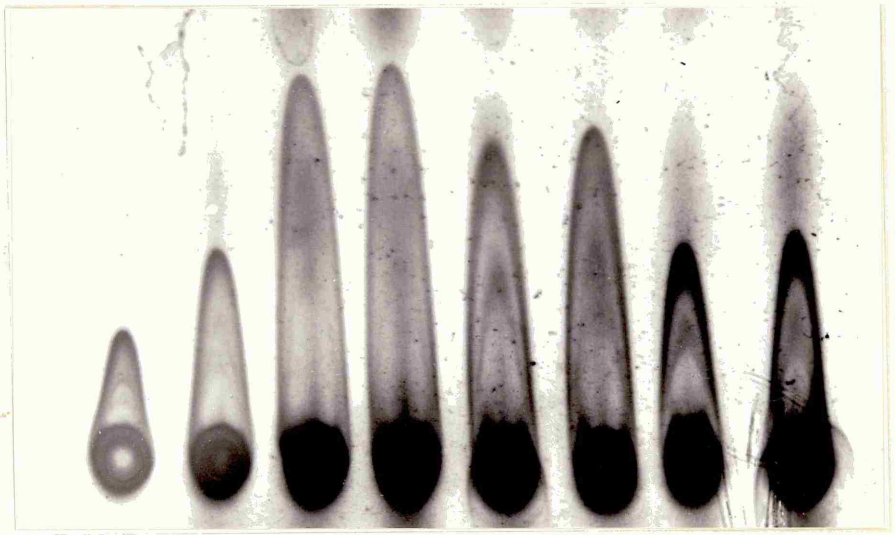
4.10.8 One-dimensional immunoelectrophoresis in a plasma from a patient receiving vitamin K therapy

(Patient J.D. receiving 10 mgm vitamin K_1 (I.V.))

Figure 37 shows the results of one-dimensional electrophoresis with anti-human factor II in a patient (J.D.) who had received 10 mgms of vitamin K_1 (I.V.). Samples were taken at pre, post 1, 2, 4, 8, 24 hours infusion (Chapter 3, Methods and Materials).

4.10.9 Comment

Analysis of factor II antigen levels in a patient receiving vitamin K_1 (10 mgm I.V.) reveals that excess



1 2 3 4 5 6 7 8

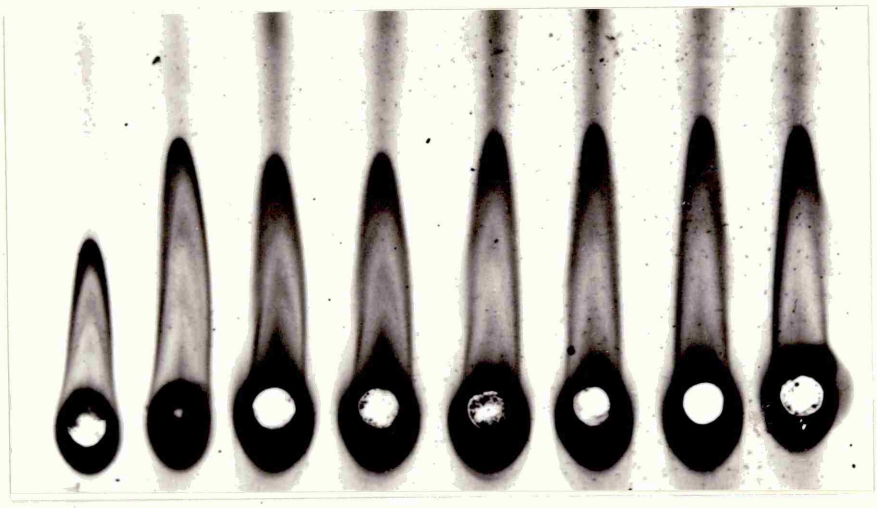
Figure 36

Oral Anticoagulant Patients

Quantitative one-dimensional immunoelectrophoresis according to Laurell in the presence of anti-human factor II

Samples 10 μl placed in the numbered wells

		Antigenic II		Factor II
Well 1	Control plasma	1/20	(50%)	
" 2	" "	1/10	(100%)	110%
" 3	Anticoagulant plasma	1/10	(210%)	30%
" 4	" "		(220%)	21%
" 5	" "		(150%)	37%
" 6	" "		(170%)	42%
" 7	" "		(92%)	34%
" 8	" "		(88%)	30%



1 2 3 4 5 6 7 8

Figure 37

One-dimensional immunoelectrophoresis according to Laurell in the presence of anti-human Factor II in a patient receiving vitamin K_1

Samples 10 μl placed in the numbered wells

			Antigenic F.II	Factor II Assay
Well	1	Control	1/20 (50%)	
"	2	"	1/10 (100%)	110%
"	3	J.D. Pre Vit. K	88%	36%
"	4	" Post	1 Hr 88%	45%
"	5	" "	2 Hrs 91%	65%
"	6	" "	4 Hrs 96%	72%
"	7	" "	8 Hrs 91%	76%
"	8	" "	24 Hrs 91%	100%

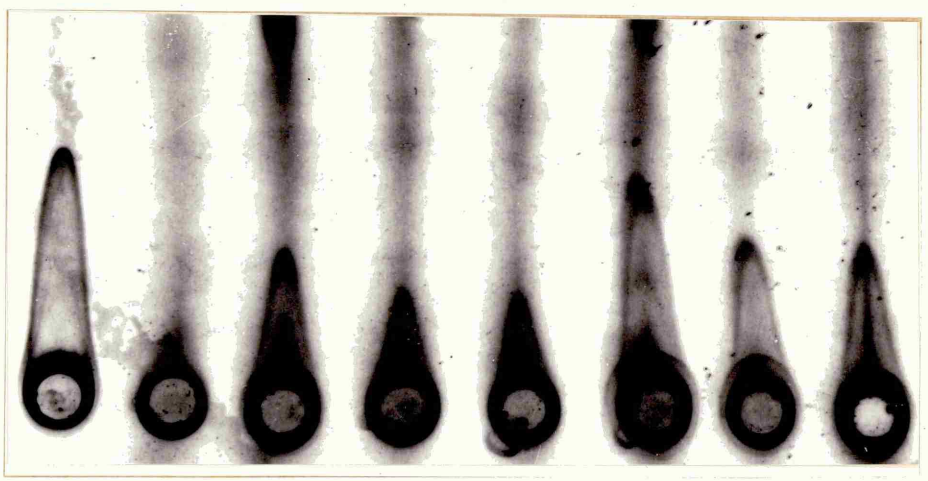
antigenic factor II was present and that the concentration did not alter during the infusion study. However, the clotting factor II did change during the infusion over the same period of time. The ratio of clotting factor II activity/antigenic factor II changed during the infusion from 0.42 (Pre) to 1.09 (Post) indicating that the main effect of the vitamin K therapy is on the functional part of the molecule rather than the antigenic structure.

4.10.10 Adsorption characteristic of antigenic factor II in plasma from obstructive jaundice patient

Figure 38 shows the results of one-dimensional electrophoresis with anti-human factor II antibody against plasma which had been adsorbed with $ALOH_3$. The adsorption of plasma using $ALOH_3$ to remove normal factor II was as follows : To 0.95 ml of plasma add 0.05 ml of 20% moist gel alum hydroxide; stir for 5 minutes then leave for 30 minutes at $4^{o}C$. Centrifuge and remove supernatant adsorbed plasma.

4.10.11 Comments

Figure 38 shows the reaction of plasma which had been adsorbed with $ALOH_3$ with anti-human factor II antibody. Aluminium hydroxide should adsorb out normal factor II and this is observed in the control pre and post adsorption (factor II $\bar{=}$ 100% pre; $<$ 5% post adsorption) indicating normal antigen present. In the plasma from patients with obstructive jaundice it was observed that detectable amounts of antigen reacted with the antibody post adsorption indicating abnormal factor II (PIVKA II) present in these plasma. It also indicated that obstructive jaundice plasma contained an atypical immuno-reactive form of factor II which did not have the adsorption characteristics of normal factor II and was physico-chemically different from the normal molecule.



1 2 3 4 5 6 7 8

Figure 38

One-dimensional electrophoresis with antihuman factor II antibody against obstructive jaundice plasmas which had been adsorbed with $ALOH_3$

Samples 10 μl placed in the numbered wells

Well	1	Control plasma before adsorption with $ALOH_3$
"	2	" " after " " "
"	3	Obstructive jaundice plasma after adsorption with $ALOH_3$
"	4	" " " " " " "
"	5	" " " " " " "
"	6	" " " " " " "
"	7	" " " " " " "
"	8	" " " " " " "

4.11 TWO-DIMENSIONAL IMMUNOELECTROPHORESIS OF FACTOR II IN THE PRESENCE OF CALCIUM LACTATE

Samples obtained from patient S.L. who had received 10 mgm vitamin K_1 (I.V.) were subjected to two-dimensional immunoelectrophoresis (Materials and Methods, 4.4.4. One significant difference to the technique described was the addition of calcium lactate 2 mM/l to the barbitone acetate buffer during electrophoresis in the first dimension. Following this, the gels were excised and placed against the first dimension electrophoresis was agarose containing antisera to factor II. Electrophoresis was then continued in the second dimension. Glass plates were used, 210 x 10 x 1.5 cms, in order that all the samples collected from the infusion could be analysed in one run (samples analysed included Pre, Post 1, 2, 8, 24, 72 hours; 6 days). Following electrophoresis the plates were dried and stained with Coomassie blue.

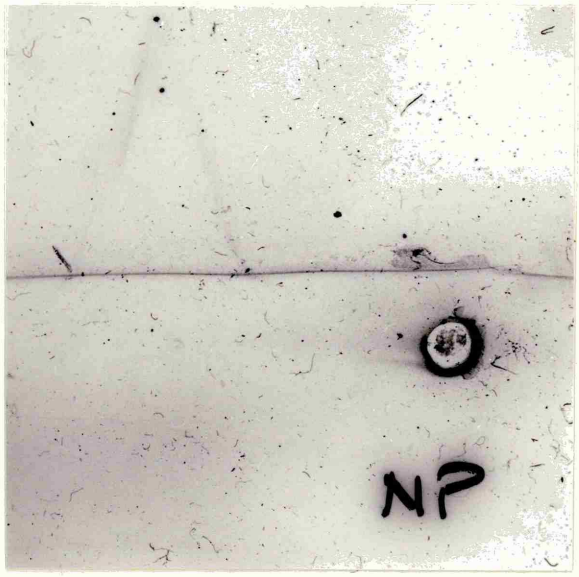
4.11.1 Results

Figure 39a-h shows the result of two-dimensional immunoelectrophoresis analysis of plasmas obtained during the injection of 10 mgms vitamin K_1 (I.V.) on patient S.L.

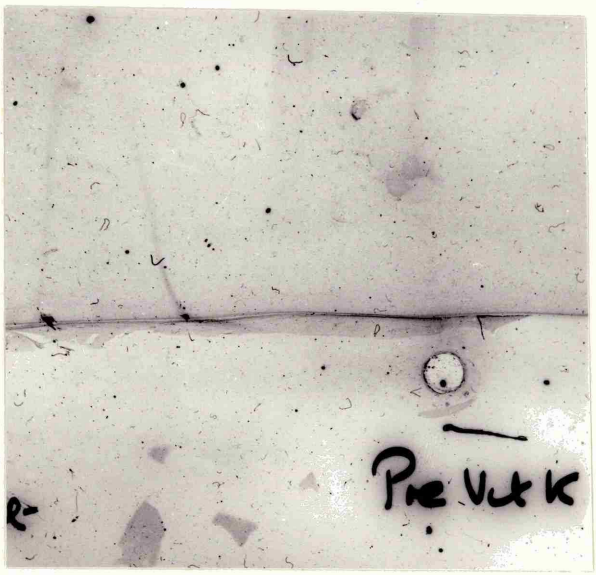
4.11.2 Analysis of the two-dimensional immunoelectrophoresis of factor II

It was observed that normal control plasma gave a single peak with a mobility from the origin of 3.0 cms and that the pre sample (S.L.) showed a peak with higher mobility 4.5 cms. Thus the patient's pre sample contained a single type of factor II antigen, with faster mobility in the presence of calcium lactate, e.g. PIVKA II.

Analysis of the post 1 hour sample revealed that two precipitation peaks were present. The main peak



(a) Normal pooled plasma (100%)
antigen mobility 3.0 cms

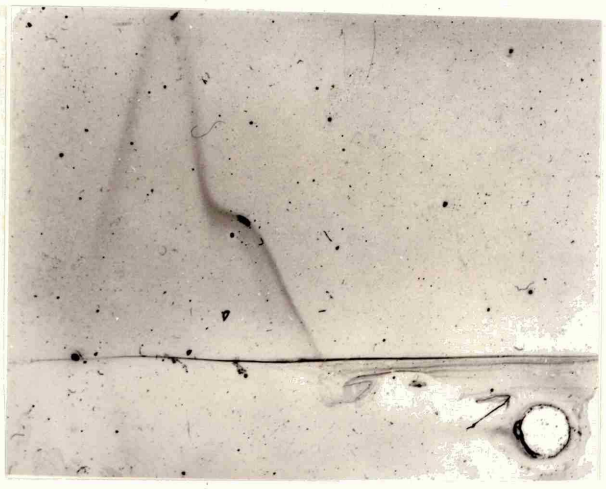


(b) Patient pre vitamin K
antigen mobility 4.5 cms

Figure 39.

Two-dimensional immunoelectrophoresis in the presence of 2.5 mm calcium lactate (first dimension) and anti-human factor II antibody (second dimension)

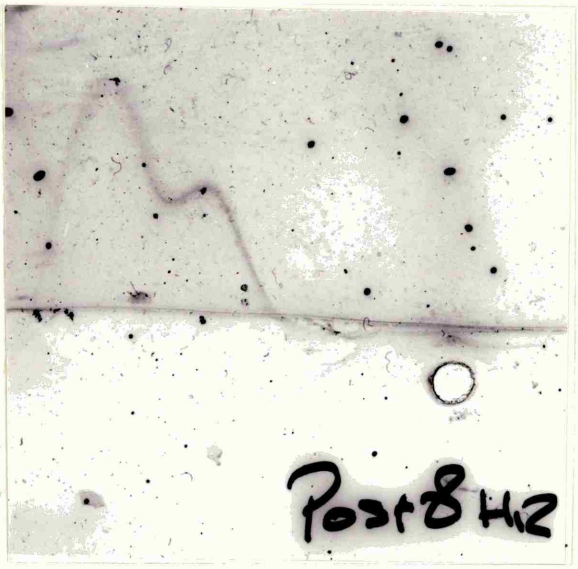
(c) Post 1 hour vitamin K



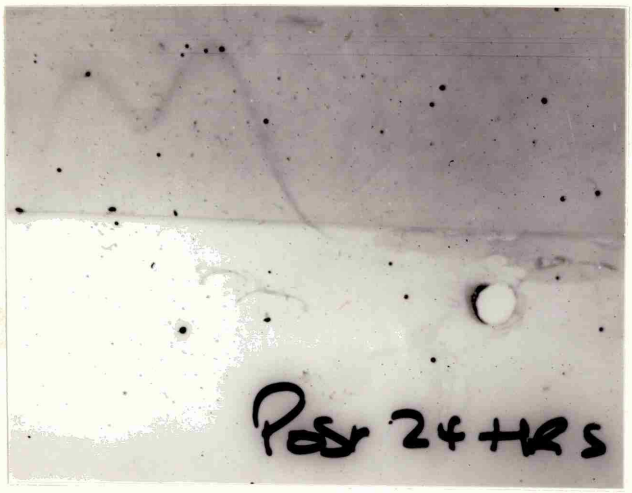
(d) Post 2 hours vitamin K

Figure 39 (continued)

Two-dimensional immunoelectrophoresis in the presence of 2.5 mm calcium lactate (first dimension) and anti-human factor II antibody (second dimension)



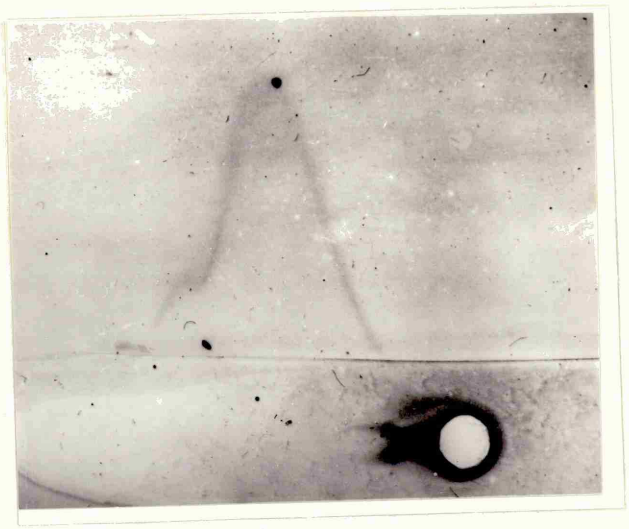
(e) Post 8 hours vitamin K



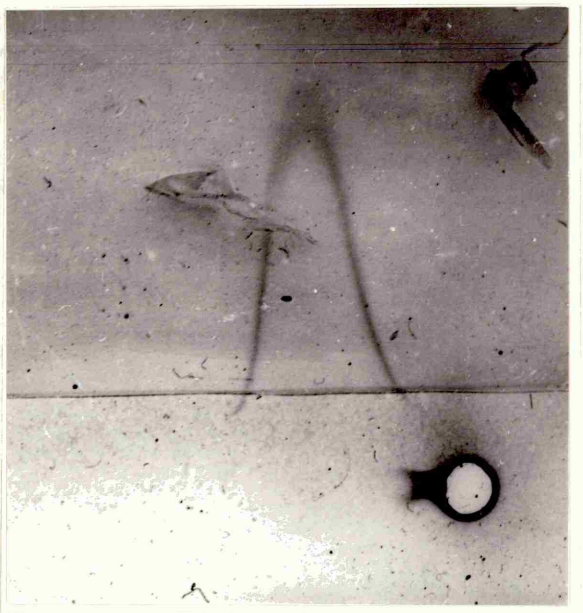
(f) Post 24 hours vitamin K

Figure 39 (continued)

Two-dimensional immunoelectrophoresis in the presence of 2.5 mm calcium lactate (first dimension) and anti-human factor II antibody (second dimension)



(g) Post 72 hours vitamin K



(h) Post 6 days vitamin K

Figure 39 continued

Two-dimensional immunoelectrophoresis in the presence of 2.5 mm calcium lactate (first dimension) and anti-human factor II antibody (second dimension)

still had a mobility of 4.5 cms indicating PIVKA II but a small peak with the mobility of 3.0 cms was observed. This occupied the same position as the normal control plasma, indicating that a small amount of normal factor II had been synthesised within one hour of the vitamin K_1 infusion. Further analysis of post 2 and 8 hours indicated that the normal peak was increasing whilst the PIVKA II was decreasing. Finally, at 24 hours post infusion the normal peak had increased until it was the main constituent of the double peak (> 50% of the total factor II present). Even at this stage, however, PIVKA II was still present. At 72 hours the main clotting protein present was normal factor II with only a small peak observed in the PIVKA II mobility position. It was the final analysis at 6 days which revealed a single peak of normal factor II. These results gave evidence that normal factor II was formed progressively during vitamin K infusion in a patient who was previously vitamin K deficient.

Close examination of the two-dimensional immuno-electrophoresis reveals that as the area of normal factor II protein increases during the vitamin K therapy the abnormal PIVKA II decreases. Since the one-dimensional immunoelectrophoresis analysis (section 4.10.9) revealed that values of the antigenic protein remained constant during the vitamin K_1 therapy the findings in the two-dimensional immuno-electrophoresis suggest that the change from PIVKA II to normal II takes place whilst a constant amount of protein is being synthesised. In order to interpret this observation an evaluation of the interrelationship between PIVKA II and normal factor II during vitamin K_1 therapy using two-dimensional immuno-electrophoresis was carried out.

4.11.3 Relationship between PIVKA II and normal factor II during an infusion of 10 mgms vitamin K_1 (10 μg I.V.)

Each individual analysis using the two-dimensional Laurell technique gave values (Figure 39 a-h) which had been derived by multiplying Height x Base of the precipitation areas. In the analysis the total area of precipitation was expressed as 100% (PIVKA II + Normal II) and then PIVKA II and normal II were expressed as a percentage of the total precipitation. Thus each separate analysis was normalised and variations between them were not considered. The normalised values obtained from each separate analysis, e.g. Pre, Post 1 hr, 2 hr, 8 hr, 24 hr, 72 hr, 144 hr are shown in table 31.

TABLE 31

PIVKA II and normal Factor II values obtained using two-dimensional immunoelectrophoresis during vitamin K therapy

Time/Infusion	% Area/PIVKA II	% Area/Normal F.II
Hours	%	%
0	100	0
1	89.6	10.3
2	72.2	25.8
8	66.0	34.0
24	40.6	59.3
72	12.3	87.3
144	0	100

The values of PIVKA II and normal factor II were plotted against each other as shown in figure 40.

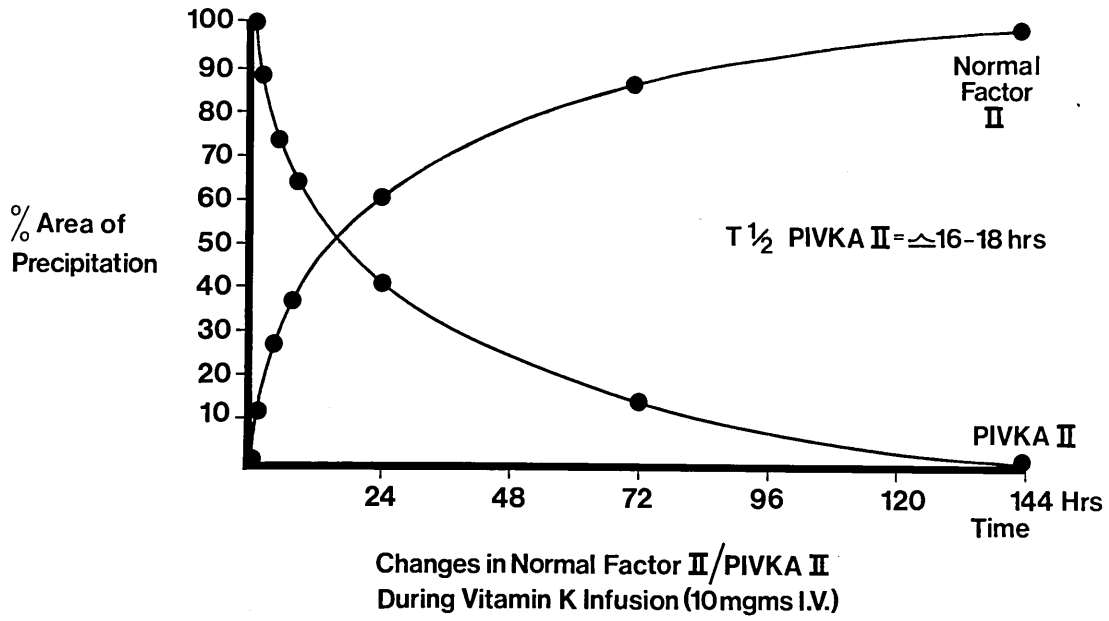

100
90
80
70
60
50
40
30
20
10
% Area of Precipitation
Normal Factor II
T ½ PIVKA II = ≏16-18 hrs
PIVKA II
24
48
72
96
120
144 Hrs
Time
Changes in Normal Factor II/PIVKA II
During Vitamin K Infusion (10 mgms I.V.)

Figure 40

Measurement of the area of precipitation defined by the two-dimensional immunoelectrophoresis showed that between sample variations associated with the technique and their effect on the accuracy of measurement were difficult to evaluate. The normalisation for total area in each individual analysis compared only the relationship between PIVKA II/Normal II and was considered valid.

Plotting the values obtained for PIVKA II : Normal Factor II from table 31 gave apparent mirror images between the decline of PIVKA II and the production of normal factor II (Figure 40). Further analysis indicated that the $T_{\frac{1}{2}}$ for PIVKA II was about 16-18 hours, a much shorter time than the $T_{\frac{1}{2}}$ of normal factor II of 80 hours.

4.12 DISCUSSION

The special association that the liver has with the synthesis of clotting factors has been well established[120]. In addition the relationship between disease processes involving the liver and haemostatic defects have been the subject of comprehensive reviews[121,122]. Of even more significance have been recent studies involving patients on anticoagulant therapy indicating that the reduction of the vitamin K dependent clotting factor levels is accompanied by the production of a common precursor protein possessing antigenic determinants for each of the vitamin K dependent clotting factors, i.e. PIVKA protein[123,124].

The purpose of this study was to determine, by using the various techniques described, whether liver disease results in the production of normal or abnormal forms of the vitamin K dependent clotting

factors and to discuss the interpretation of the laboratory findings in relation to

(a) the haemostatic mechanism
(b) effect of vitamin K on the abnormal protein.

4.12.1 General findings in liver disease

From the results of one- and two-dimensional immunoelectrophoresis and modified thrombotest (PIVKA screen) obstructive jaundice is associated with low procoagulant activity of the vitamin K dependent clotting factor II, together with normal concentration of an immunologically cross reacting protein associated with the appropriate clotting factor, i.e. PIVKA, which is influenced by vitamin K_1.

Using the same laboratory techniques it was shown that patients without an obstructive element in non-obstructive liver disease appeared to synthesise decreased amounts of a structurally normal protein which is NOT influenced by vitamin K_1.

4.12.2 Modified thrombotest in liver disease

Although the modified thrombotest appeared to be sensitive to inhibitor activity in PIVKA in liver disease (section 4.8) no clear pattern emerged between the arbitrary level of inhibitor detected and the extent of liver dysfunction. In addition, recent reports suggest that this test can also be sensitive to other non-competitive inhibitor, e.g. fibrinogen degradation products[125]. This hypothesis has not been tested in this study. When using the modified thrombotest in the abnormal control group (anticoagulant patients) larger amounts of inhibitor were detected than appeared to be present in liver disease (Table 27). An explanation for this could be that in anticoagulant activity the drug is given regularly in a controlled regime, thus systematically inhibiting the normal

protein synthesis of factors II, VII, IX, X, so effectively that large amounts of PIVKA protein build up and eventually are released. On the other hand, the variation in the amounts of inhibitor detected in liver disease could merely reflect the variable effect of the various types of disease processes, i.e. in simple vitamin K_1 deficiency there would be large amounts of inhibitor building up in the cell and then released. This is supported by the similar mean levels of inhibitor found in obstructive jaundice and anti-coagulant therapy (Tables 27 and 28). On the other hand, in combined forms of vitamin K deficiency and some intracellular damage such large amounts of inhibitor would not be formed. No estimation of inhibitor levels over a given period of time were attempted due to the problems of sampling, although such an estimation would be useful in mixed forms of liver disease.

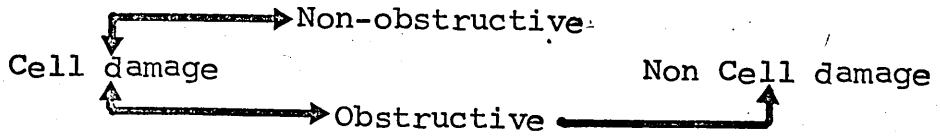
In routine use the modified thrombotest is simple to carry out providing certain technial safe-guards are taken, e.g. NON-CONTACT and fresh samples for estimation. The test appears to be very sensitive to abnormal factor X (Table 30) as shown by the relationship between the ratio $\frac{\alpha X}{\alpha N}$ and factor X and could be used to correlate changes in factor X in vitamin K therapy. During vitamin K_1 therapy the modified thrombotest was used to monitor the effect of the therapy on circulating levels of the inhibitor and this it appeared to do (Table 29).

4.12.3 One-dimensional immunoelectrophoresis of factor II according to Laurell

Quantitation of the amount of antigen related to factor II using an antibody raised against human factor II has been made from one-dimensional immuno-electrophoresis according to Laurell.

One-dimensional immunoelectrophoresis using the anti-factor II, gave normal Laurell rocket heights for the obstructive jaundice plasmas tested. Since the height of the peak can be expressed as a function of concentration[126] this meant normal antigenic factor II concentrations were present (Figure 34). The concentrations recorded were equal to 75% - 105% antigenic factor II. Comparison with the coagulation factor II assays for the same samples (from 12 - 37% factor II) revealed that more excess antigenic material was present that could be detected by specific assay. This production of an abnormal factor II molecule in large quantities in obstructive jaundice means that a molecule is released into circulation, with a lack of procoagulant factor II activity. This molecule may not have the same capability to take part in <u>in vivo</u> coagulation as normal factor II and may even inhibit the central part of the coagulation mechanism, i.e. $X^A \longrightarrow II \longrightarrow II^A$.

In contrast analysis of the non-obstructive group of patients (Figure 35) showed that good correlation existed between antigenic factor II detected and clotting factor activity. One exception to this was patient No. 8, in whom excess antigenic factor II over clotting activity was detected. These findings indicated, as stated in the analysis, that in non-obstructive liver disease the liver synthesises small amounts of a <u>structurally</u> normal protein. This protein, despite its low level in circulation is capable of a normal role in the coagulation pathway. With the finding that one of this group had an <u>abnormal</u> molecule in circulation (patient No. 8 ratio = 0.7) we have demonstrated once more that complex relationships can exist in liver disease, e.g.



in which the main clinical diagnosis of non-obstructive

liver disease is co-existent with a form of non-absorption or non-utilisation of bio-available vitamin K_1.

One-dimensional immunoelectrophoresis of plasma taken from a patient receiving vitamin K_1 (10 mgms I.V.) revealed the presence of a constant amount (91%) of antigenic factor II during the therapy (Figure 37). These findings indicate that despite the rapid increase in clotting factor II activity during the therapy there was no extra increase in the area of antibody precipitation. A ratio between $\frac{\text{clotting FII}}{\text{antigenic FII}}$ was estimated and showed a value for (Pre) ratio of 0.42. At the completion of the therapy the ratio had altered to 1.09 but the antigenic factor concentration for pre and post 24 estimations were 88% and 91% respectively. It may be that rapid conformational changes occur to the same molecule at or around the vitamin K dependent step once vitamin K_1 is made available in the liver cell. This concept will be discussed more fully later in conjunction with PIVKA (Liver disease) and the haemostatic mechanisms.

The adsorption characteristics of the PIVKA II protein detected in obstructive jaundice were tested with aluminium hydroxide adsorption. Under normal circumstances aluminium hydroxide will remove normal factor II from plasma, leaving a level of $< 5\%$. As can be seen from figure 38, the obstructive jaundice plasma revealed that following $AlOH_3$ adsorption appreciable ($> 5\%$) amounts of antigenic factor II were still present. One could conclude therefore that the PIVKA II is physico-chemically dissimilar to normal factor II and enhances the possibility of its having a non-physiological capability within the coagulation process resulting in e.g. incomplete binding at the $X^A + II + V + Ca^{++}$ + phospholipid complex and the consequent reduction of thrombin generation.

The abnormal control group, i.e. anticoagulant therapy revealed excess antigenic factor II in relation to clotting activity. Since this is being used as a control group in liver disease analysis, perhaps one should comment on the fact that more antigen was detected in some of the lightly anticoagulated patients than was present in the heavily anticoagulated group (Figure 36). In other words, the effect of the anticoagulant therapy did not appear to relate to the level of production of PIVKA protein, the levels detected being a function of individual response to anticoagulant therapy.

4.1.2.4 Two-dimensional immunoelectrophoresis of factor II

Two-dimensional immunoelectrophoresis according to Laurell revealed that in the presence of calcium lactate the presence of PIVKA II was demonstrated in obstructive jaundice (Figure 39b). Calcium ions retard the mobility of normal factor II, leaving the mobility of PIVKA II unaffected. The reason why calcium ions do not influence the mobility of PIVKA II as they do with normal factor II is not exactly known, but it may indicate a low binding capacity of PIVKA II for calcium ions. Using this technique it is possible to split isomers, i.e. PIVKA II from normal II which is not possible using the one-dimensional method. By splitting the components away from each other PIVKA II concentrate in relation to normal factor II within the same molecule can be estimated.

Using plasma samples from a patient receiving vitamin K_1 (10 mgms I.V.), two-dimensional analysis was carried out (Figure 39a-h). They revealed that initially (pre vitamin K_1) all the antigenic factor II present was made up of PIVKA II (mobility 4.5 cms), indicating the presence of a molecule with low procoagulant activity and abnormal mobility. Using an accepted

method[126] for calculating areas of immunoprecipitation, e.g. Height x width of the peaks, it was possible to calculate the amount of PIVKA II to normal II present during the therapy. The analysis showed that 12% of normal II was detected within one hour of therapy. Furthermore the analysis revealed that as PIVKA II concentration declined normal factor II increased (Figure 39a-h and Table 31). Considering the fact already established (Figure 37) using one-dimensional immunoelectrophoresis, that during vitamin K_1 therapy the total amount of antigenic protein did not significantly alter, this finding in the two-dimensional analysis would appear to indicate that the ratio between normal II and PIVKA II changes within a constant amount of antigen. In other words immediate conformational changes rather than new protein synthesis (although this does not preclude on-going protein synthesis) takes place. From the two-dimensional analysis a $T_{\frac{1}{2}}$ for PIVKA II was estimated at 17 hours. This compares with one reported $T_{\frac{1}{2}}$ PIVKA II of 18 hours by Lavergne and Josso[127]. Comparison with the quoted physiological $T_{\frac{1}{2}}$ for factor II in the literature of 50 - 80 hours (Introduction) reveals that the half life of the PIVKA's relative to those of the normal factors are of considerable interest[128]. Since it is possible that plasma level of a plasma clotting factor depends on the formula

$$\underset{\text{(available level)}}{P} = \text{Rate Synthesis} - \underset{\text{(degradation)}}{\text{Rate}}$$

(chapter 3) perhaps extra degradation in PIVKA's takes place and one could conclude that the missing vitamin K dependent step not only reduces procoagulant activity but may also have an effect on the physiological role of factor II. This may result in increased degradation due to the ineffective use of the molecule in the normal coagulation pathway or perhaps the production of an unstable configurational change in the molecule, rendering it more susceptible to degradation. In the last stage

of the analysis, by normalising the data from the total precipitation area and then plotting it (Figure 40) it was evident that at the rate PIVKA II disappeared normal factor II appeared to replace it at the same rate; therefore it is possible that during vitamin K therapy the half lives of PIVKA II and normal factor II are very similar. It appears therefore that the vitamin K dependent clotting factor may seem to function as allosteric enzymes in the clotting mechanism and that their specific function depends on one active site, i.e. serine in order to allow specific activation and an alternative site, i.e. γ glutamic acid residues to allow specific binding. This concept will be discussed later in the chapter in relation to the haemostatic mechanism.

4.12.5 PIVKA proteins and the haemostatic mechanism

Interpretation of the laboratory data on PIVKA protein in relation to the haemostatic mechanism is perhaps best explained by first of all considering the fundamental enzymatic activity of clotting proteins, in particular the vitamin K dependent proteins II, VII, IX, X. In the previous section observations on the changing relationship between PIVKA II and normal II during vitamin K therapy allowed the suggestion to be made that the vitamin K dependent clotting factors function as allosteric enzymes in the coagulation mechanism (section 4.12.4). This inference can now be explained in terms of enzyme structure and function, as follows :

The stereochemical structure of the side chains of the enzyme protein at one circumscribed position on the molecule allows the substrate to bind and thus undergo changes. This site is called the active site. The structure of the active site is determined not only by the presence of side chains but also by the relative juxtaposition of these side chain residues to each other.

In turn the whole tertiary structure of the enzyme will determine this particular juxtaposition which will alter with conformational changes on the part of the enzyme[128]. Whilst the active sites of factors II, VII, IX, X are not considered to be altered in the absence of vitamin K_1, what is altered particularly in factor II is believed to be the postribosomal vitamin K dependent step in which the glutamic acid residues are altered into γ carboxyglutamic acid residues. In practical terms the omission of this step means incomplete binding of the molecule to the phospholipid micelles (phospholipid-water interphase). This incomplete binding via the γ carboxyglutamic acid residues to the anionic sites at the phospholipid interphases means that the molecule of prothrombin (PIVKA II) will not fit into the complex of X and V spatially. This fit is highly desirable in order that X^A can activate the active serine site and convert II ——> II^A quickly. Outside the complex, X^A in free solution can still activate factor II but only at a rate of < 1% of that developed in the complex of

$$X^A + II + V + Ca^{++} + \text{Phospholipid}$$ (Chapter one).

Diagramatically this complex arrangement sequence can be represented by figure 41.

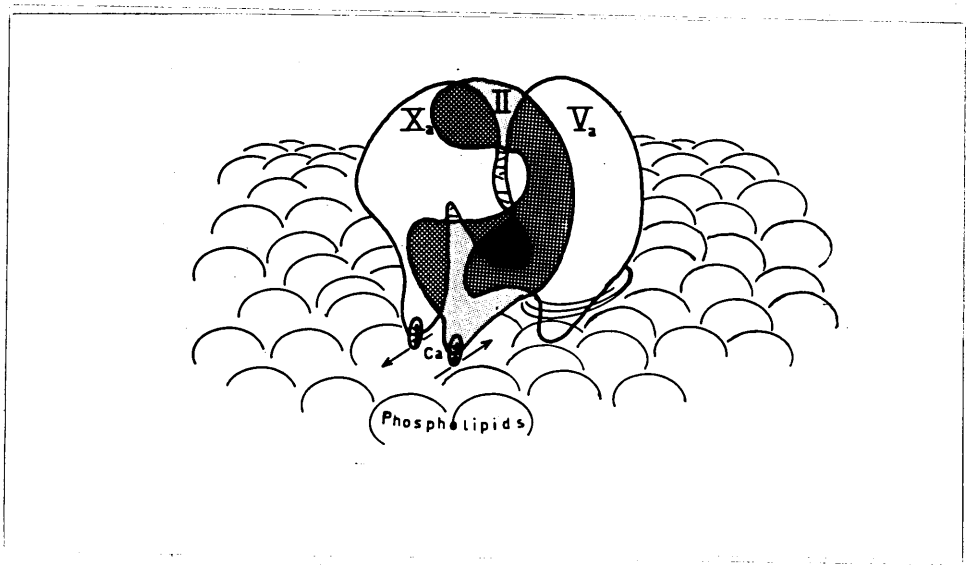


Figure 41

From this explanation it is obvious that the importance of the vitamin K dependent site is to enhance the conversion from pro-enzyme state to enzyme state of the vitamin K dependent clotting factors (i.e. II ——> II^A, X ——> X^A, IX ——> IX^A, VII ——> VII^A) by bringing about a change in the tertiary structure at the vitamin K dependent site. This in turn allows the correct juxtaposition of the amino acid residues at the active site.

If this is so, the vitamin K dependent clotting factors can be classified as heterophilic enzymes; that is, they possess not only binding site for the substrate (active site) but other sites physically separate at which a modular molecule binds. The effect of the modular molecule, e.g. phospholipid, is to accelerate the catalytic activity. Additionally vitamin K dependent clotting factors may act as allosteric enzymes (the term allosteric meaning other active space or other site) and in vitamin K deficiency states this important second site for the modular molecule to bind is absent. Thus the reduced potential to form, II ——> II^A, X ——> X^A, IX ——> IX^A, VII ——> VII^A, could be interpreted as reflecting the effect of the missing site.

The rate at which the synthesis of normal factor II re-establishes itself during vitamin K therapy is worth considering as it may indicate what really happens when the biologically active vitamin K is made available.

Chemically active vitamin K (phylloquinone) is absorbed and utilised as a dietary principle in the vitamin K dependent clotting factor synthesis (Chapter 3) and as a by-product of the vitamin K step in protein synthesis phylloquinone oxide is produced[129] (Figure 42). This oxide needs to be reduced back to phylloquinone in order to revert to its biologically active form and this continuous oxidation and reduction of the vitamin K is utilised in the vitamin K dependent step. In the transformation of coagulant factor precursor to vitamin K

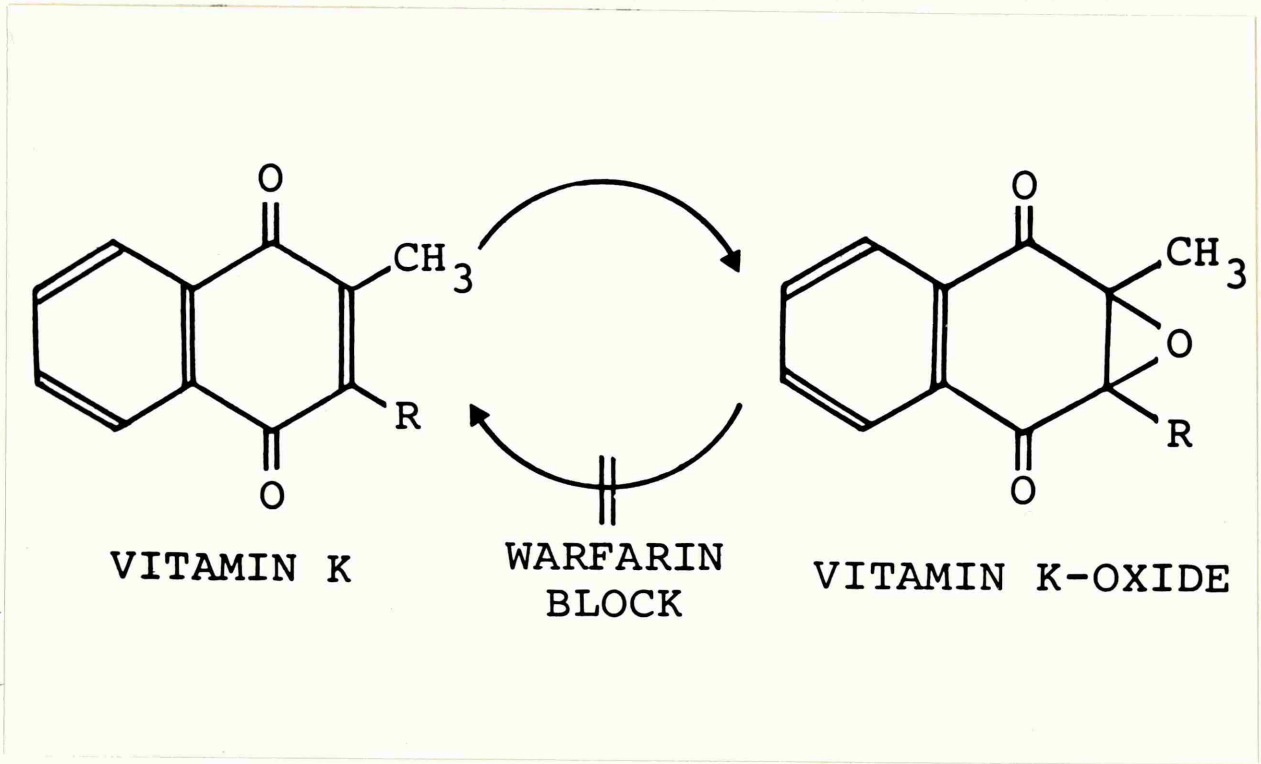


Figure 42

Conversion of vitamin K to vitamin K oxide

dependent clotting factors II, VII, IX, X, vitamin K reductases energised by NADPH are required. The main reductases are DT-Diaphorase and it is a possibility that these enzymes are also sensitive to liver disturbances such as hepatitis which could account for the suggestion of co-existent vitamin K deficiency in non-obstructive jaundice. The model shown in Figure 43 presents the findings of Bell, Matchinger, Ernster et al[129] relating to the transformation of the vitamin K dependent clotting factors.

In vitamin K deficiency the PIVKA protein is believed to be continuously produced and stored before being released without its vitamin K dependent step being completed as circulating PIVKA. The incomplete protein could therefore be stored in the smooth endoplasmic reticulum or plasma membrane itself. More important, it is only a partially completed normal protein, at the storage stage, having taken about 175 - 225 minutes to reach that stage of protein biosynthesis[130]. This would leave a period from about 175 - 225 minutes to 240 minutes during protein biosynthesis in which to complete the molecular finalisation step, a total of 15 - 65 minutes[130]. As it has already been shown (Results, Chapters 3 and 4) normal factor II, VII, IX, X was detected within one hour of vitamin K. Therefore the availability of vitamin K produces conformational changes in the existing intracellular PIVKA molecule probably by the immediate restoration of the carboxylation of glutamic acid residues rather than initiate new synthesis, a pathway which would require at least 240 minutes to complete.

Effectively, then, the vitamin K oxidation-reoxidation reaction, presumably at the microsomal level, produces immediate conformational changes which have been implied by the one-dimensional and two-dimensional immunoelectrophoresis findings (sections 4.10 and 4.11) rather than institute the new protein synthesis pathway. This immediate alteration in the stereochemical structure thus allows the active molecule to be completed and released into the plasma circulation when required.

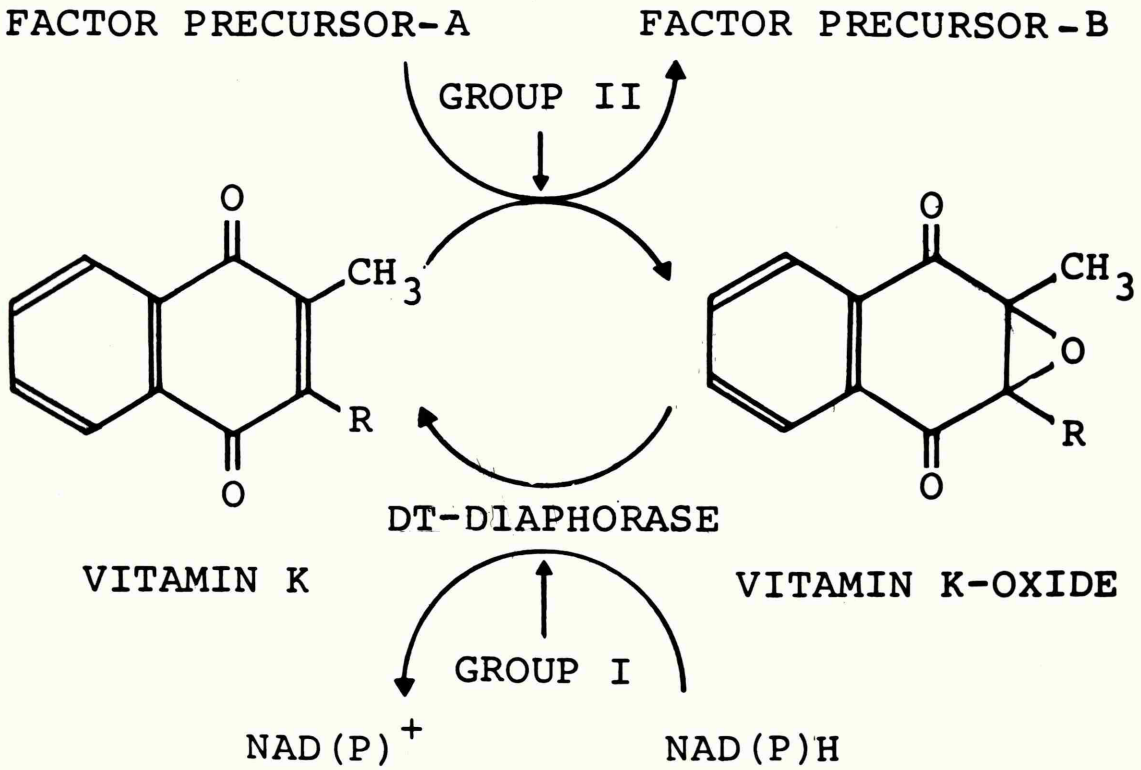


Figure 43

Production of the vitamin K dependent clotting factors incorporating the vitamin K dependent step

CONCLUSIONS

The important factor which has emerged from this study of PIVKA proteins and liver disease is that PIVKA protein is formed and released in certain liver disease states. On the basis of levels of PIVKA as detected by the modified thrombotest it was possible to show two groups of liver disease patients.

A. GROUP ONE - PIVKA POSITIVE (mainly obstructive jaundice + some non-obstructive jaundice)

This group was characterised by :

1. Abnormal PIVKA levels (similar to those found in anticoagulant patients);
2. Normal synthesis of abnormal vitamin K dependent clotting factor II;
3. Response to vitamin K (chapters 3 and 4).

In group one patients, vitamin K administration is followed by :

a) appearance of normal coagulation protein within one hour;

b) disappearance of the abnormal coagulation protein.

The rates of appearance and disappearance of the respective proteins are identical.

B. GROUP TWO - PIVKA NEGATIVE (non-obstructive jaundice)

Group two patients were characterised by :

1. Normal PIVKA levels;
2. Decreased synthesis of a structurally normal protein which is not influenced by vitamin K;
3. A possibility of inter-relationship between non-obstructive jaundice and vitamin K deficiency or non-utilisation.

The present findings are consistent with the view that vitamin K apparently produces, by virtue of the incorporation of the critical vitamin K dependent step into the synthesis of the vitamin K dependent clotting factors, a conformational change in the clotting protein. This is brought about by the carboxylation of the glutamic acid residues (Figure 10). The existence of these γ-carboxyglutamic acid residues means that prothrombin's calcium binding is enhanced and this in turn allows calcium ion-dependent phospholipid binding by prothrombin to take place (Figure 41). Functionally this means that one part of the allosteric enzyme is aligned critically causing correct juxtaposition of the alternative site (active serine) in the complex being formed within the coagulation pathway (Figure 41). This in turn enhances substrate affinity.

It is concluded that without this spatial organisation caused by the γ-carboxyglutamic acid residues a reduced potential to form activated coagulation factors results[131]. Hence the biological importance of the vitamin K dependent step.

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APPENDIX 1

In the study on the effect of liver disease on the synthesis of Vitamin K dependent clotting factors II, VII, IX, X (Chapter II) the diagnosis was made by a member of the clinical unit, who then referred the patient for coagulation studies. Definitions of the liver disorders studied (Table 5, 2.4.3) are as follows :-

Cirrhosis : Diffuse hepatic fibrosis due to the presence of a great deal of fibrous tissue. This may be the result of a number of pathological processes.

Obstructive Biliary Cirrhosis : Patients with chronic extra-hepatic biliary obstruction, carcinoma of the head of the pancreas or surgical injury to the common duct may develop diffuse hepatic fibrosis.

Primary Biliary Cirrhosis : A rare disease in which the primary disorder appears to be diffuse intrahepatic biliary obstruction of unknown cause.

Alcoholic Cirrhosis : Alcohol as a liver poison produces liver damage in patients who consume it in large quantities. The number who proceed to cirrhosis, however, is small (about 10%). The cause for this progression is unknown and probably genetic in origin.

Hepato-Cellular Jaundice : Jaundice is an increase in the amount of bilirubin in the blood, resulting in yellow discolouration of the eyes, skin and body fluids. If too little bilirubin is removed from the blood in its passage through the liver, jaundice may result. This disorder can occur in two ways : the function of the liver cells may be deranged, so that they cannot take up bilirubin from the blood (hepato-cellular jaundice) or an obstruction in the common bile duct may dam the flow of bile, so that the bilirubin-glucuronides excreted by the liver diffuse back into the blood (obstructive jaundice).

Obstructive Jaundice : When the common bile duct is obstructed the rising pressure in the biliary system causes bile to flow back into the peri-lobular lymphatics and by this route into the blood stream. Common causes of the occlusion of the common bile duct are gallstones and carcinoma of the head of the pancreas. A rare cause is chronic pancreatitis.

Liver Failure : This term refers to failure of one or more of the many physiological functions of the liver.

Schistosomiasis : A disease due to infestation with trematodes (flukes) of the genus Schistosoma. During the life cycle they may pass via the bloodstream into the liver where they develop within two months into worms. This infestation may be followed by enlargement of the liver and spleen. Cirrhosis of the liver with ascites is a late complication of the disease.

Liver disease : Disseminated intravascular coagulation

Laboratory and clinical evidence suggest that intravascular coagulation is found in liver disease. Possible pathogenic mechanisms include vascular and haemodynamic changes which could contribute by exposing blood to expanded collaterals and distorted splanchnic circulation in the presence of abnormal hepatic clearing mechanisms (cirrhosis). In acute liver failure exposure of blood to necrotic hepatocytes could trigger local intravascular coagulation[122].

Carcinoma : Intrahepatic carcinoma can result in hepatic or obstructive jaundice by causing deranged liver cell function or occlusion of the common bile duct. The carcinoma may be intra- or extra-hepatic in nature.

Pancreatic failure involving the liver : Jaundice occurs in 85% of patients with cancer of the head of the pancreas and in 20 - 40% of patients with cancer of its body and tail. In the former group the jaundice is mainly the result of partial or complete obstruction of the common bile duct, whereas in the latter the jaundice results from hepatic metastases.

Chronic hepatitis : The term 'chronic hepatitis' describes a wide spectrum of pathological changes in the liver of varied aetiology and with varied clinical features.

Chronic persistent hepatitis : A chronic inflammatory condition affecting the portal tracts with preservation of the normal lobular architecture of the liver and little or no fibrosis.

Chronic active hepatitis : A variety of chronic aggressive hepatitis in which the pathological appearances are particularly florid and there is a conspicuous infiltration with lymphocytes and plasma cells.

Viral hepatitis : Two conditions of infective hepatitis and serum hepatitis may cause acute parenchymal liver disease, resulting in hepatic cell degeneration or necrosis. The severity of the illness following hepatic injury varies with the nature and virulence of the infecting organism.

APPENDIX TWO

TABLE 32

Patient identification and data used in the principal components analysis

Patient	Factor	Record No.	Code
J.D.	II	2	0
	VII	3	1
	IX	4	2
	X	5	3
E.T.	II	6	0
	VII	7	1
	IX	8	2
	X	9	3
M.D.	II	10	0
	VII	11	1
	IX	12	2
	X	13	3
S.W.	II	14	0
	VII	15	1
	IX	16	2
	X	17	3
M.T.	II	18	0
	VII	19	1
	IX	20	2
	X	21	3
E.M.	II	22	0
	VII	23	1
	IX	24	2
	X	25	3
S.L.	II	26	0
	VII	27	1
	IX	28	2
	X	29	3

TABLE 33

Computer printout used in calculating the sum of the variances and the three principal components associated with the variances

```
PATIENT NUMBER 0
STOP
R
APPENSED FACTORS FACT1 FACT2 FACT3 FACT4
R
PJHEIGT
NAME OF DATAFILE  FACTORSINT
R
RENAME LAMDA LAMDACA
R
PJHEIGT
NAME OF DATAFILE  FACTORS
NUMBER OF PATIENTS 33--28
NUMBER OF DATAPOINTS 6
NUMBER OF EIGENVALUES 3
EIGENVALUES OPTION 1
UPDATE OPTION 0
TEST OPTION 1
SUM OF VARIANCES                0.1360975529100529E  4
        5
        1           0.9454978951112657E  3
        4
        2           0.3046262553619128E  3
        5
        3           0.7459390075408318E  2
NUMBER OF EIGS FOR RECONSTRUCTION0
    0.000000E  0    0.000000E  0
    0.100000E  1
STOP
R
PRCOEFFS
FIRST RECORD 1
LAST RECORD 28
NUMBER OF EIGS 3
```

TABLE 34

Computer printout of the 1st, 2nd, 3rd coefficients associated with each individual clotting factor

Record No.	Code	1st Coeffic.	2nd Coeffic.	3rd Coeffic.	
2	0	0.1418	0.4920	1.1256	
6	0	-0.2624	-0.4578	-1.6870	
10	0	-0.6064	-1.0740	-0.1289	
14	0	-1.9874	0.3674	0.2773	Factor II
18	0	-1.6145	0.4687	0.3464	
22	0	-1.2997	0.4808	-0.5835	
26	0	-0.4253	0.1412	0.7097	
3	1	1.0035	1.0442	-0.9565	
7	1	0.0277	-0.2747	-1.2545	
11	1	1.3932	-1.3044	-0.3889	
15	1	0.1110	-2.8449	0.9120	Factor VII
19	1	-0.5003	0.1300	0.0974	
23	1	1.1443	2.0831	0.6102	
27	1	1.3712	0.6617	-1.2323	
4	2	1.1649	0.0769	-0.2614	
8	2	0.3713	-0.5558	-2.8499	
12	2	-0.4711	-1.4970	0.8051	
16	2	-0.8614	0.1063	0.2738	Factor IX
24	2	0.6826	0.8992	-0.6161	
28	2	-0.6473	0.0403	0.2855	
20	2	-1.2967	-0.3271	-0.5156	
5	3	0.9988	0.0449	1.3443	
9	3	0.7475	0.8880	0.1878	
13	3	2.0551	-1.4884	1.2238	
17	3	-0.1672	0.0564	0.8828	Factor X
21	3	-0.9343	-0.1513	-0.7066	
25	3	-0.0544	1.5096	1.0699	
29	3	-0.0844	0.4858	1.0253	

TABLE 35

Computer printout of the data used for the mean curve and the three principal components of the curve

(Figures 22, 23 and 24)

1	
0.00000000E 0	
0.79392855E 1	
0.16200900E 2	
0.26571437E 2	Mean Curve
0.39357137E 2	
0.63588928E 2	
2	
0.00000000E 0	
0.51232301E -1	
0.18020047E 0	
0.33441599E 0	1st Component
0.49757756E 0	
0.78422700E 0	
3	
0.00000000E 0	
0.22666955E 0	
0.37730328E 0	
0.45601300E 0	2nd Component
0.48827666E 0	
-0.59991722E 0	
4	
0.00000000E 0	
0.26865120E 0	
0.55207328E 0	
0.34273318E 0	3rd Component
-0.59667035E 0	
0.14221575E 0	

Presentation of the data for the calculations of components 1 x α_1; 2 x α_2; 3 x α_3

(Table 22, Section 3.7.3)

Calculations of α_1, α_2, α_3, use the following information :-

A. Component 1, 2 or 3

(Table 35 - Records of the three components 1st, 2nd, 3rd).

B. α_1, α_2, α_3 which are derived from

$$\text{Coefficient}^{*} \times \{\text{Variance}^{**} \text{ associated with each of the components}\}^{\frac{1}{2}}$$

* Coefficients (Record 23, Table 34)

1st Coefficient = 1.1443

2nd Coefficient = 2.0831

3rd Coefficient = 0.6102

** Variances (Table 33)

1st Component = 0.945497895 E 3

2nd Component = 0.304626255 E 3

3rd Component = 0.74593900 E 2

Examples

Calculation of α_1 = 1.1443 x 0.945497895 E 3

= 1.1443 x $945.497895^{\frac{1}{2}}$

= 35.19 (Table 22)

Calculation of

Component 1 x α_1 = 0.5132301 E -1 x 35.19 (Table 35 - 1st Component)

= 0.05132301 x 35.19

= 1.81 (Component 1 x α_1 Table 22)

The student attended the following post-graduate courses at Sheffield University and Loughborough University of Technology :-

"Post-graduate lectures in Haematology" during term time

"Statistics" 7 hour evening lecture series

"Gel Filtration and Electrophoresis"
7 day course

Please arrange completion and return to Mrs B. T. Chatwin Asst. Registrar.

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