THE NATURE OF THE ANTAGONISM BETWEEN VITAMIN K_1 AND INDIRECT ANTICOAGULANTS

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ABSTRACT

The simultaneous administration of an indirect anticoagulant, warfarin, inhibits partially or completely the increase of the plasma level of factor VII by an optimal dose of vitamin K_1 in vitamin K deficient but not in anticoagulant treated rats. When the ratio of vitamin K_1 to warfarin is kept constant, but the doses increased, the inhibition at first increases, but disappears when the doses are increased further. The dose of vitamin K_1 necessary for the disappearance of the inhibition is found to be constant for all dose ratios tested and of the same order of magnitude as that necessary to produce a comparable increase of the plasma level of factor VII in anticoagulant pretreated rats.

The results can be explained by the hypothesis that in addition to its normal site or mechanism of action, larger doses of vitamin K_1 can act by an alternate site or mechanism. While indirect anticoagulants inhibit the action of vitamin K_1 at the normal site by an antagonism which is not of the competitive, but either of the nonequilibrium (competitive irreversible) or noncompetitive type, larger doses of vitamin K_1 reverse the inhibition by acting at the alternate site, which is not inhibited by indirect anticoagulants.

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I. INTRODUCTION AND STATEMENT OF THE PROBLEM

THE MECHANISM OF BLOOD COAGULATION

The classical theory of blood coagulation of Schmidt (97) and Morawitz (81) postulated that four substances, thromboplastin, calcium ions, prothrombin, and fibrinogen interacting in two consecutive reactions are required for the formation of a coagulum or fibrin clot. The first of these reactions (i) involves the activation of prothrombin to thrombin by calcium ions and thromboplastin and is followed by (ii) the transformation of fibrinogen to fibrin by the action of thrombin.

(i) Prothrombin $\xrightarrow{Ca^{++},\ \text{thromboplastin}}$ thrombin

(ii) Fibrinogen $\xrightarrow{\text{thrombin}}$ fibrin

According to this theory blood remains fluid because free thromboplastin is not found in the plasma, but is present in the tissues or in the platelets. Tissue damage or the disintegration of platelets results in the appearance of thromboplastin in the plasma and initiates the clotting process. In 1935 Quick introduced a method for the quantitative measurement of prothrombin *in vitro* based on this theory, by assuming that with optimal concentrations of calcium ion, fibrinogen, and tissue extract as a source of thromboplastin, the time required for fibrin formation would be a function of the prothrombin concentration (90).

More detailed investigation of certain congenital hemorrhagic diseases and an increased interest in blood coagulation brought about by the clinical use of anticoagulants after 1940 showed that many experimental observations could not be explained satisfactorily by the simple classical theory. In particular, these studies revealed that tissue or platelet extracts that had been assumed to possess thromboplastin activity, that is

the ability to transform prothrombin to thrombin, did so only after interacting with several additional plasma factors. It is now generally accepted that the generation of thromboplastin activity can only occur by two different independent mechanisms (14, 15). In the so-called extrinsic mechanism, a tissue factor interacts with several plasma factors, specifically, factors V, VII, and X to form an entity capable of transforming prothrombin to thrombin and which is referred to as thromboplastin or thrombokinase, while in the intrinsic mechanism, the components required for the formation of thromboplastin are a factor present in platelets, platelet factor 3, and several plasma factors, i.e., factors V, VIII, IX, X, XI, and XII. Both mechanisms require factors V and X, while factor VII is required by the extrinsic mechanism and factors VIII, IX, XI, and XII by the intrinsic mechanism only. These interrelations are summarized in the scheme shown in Figure 1.

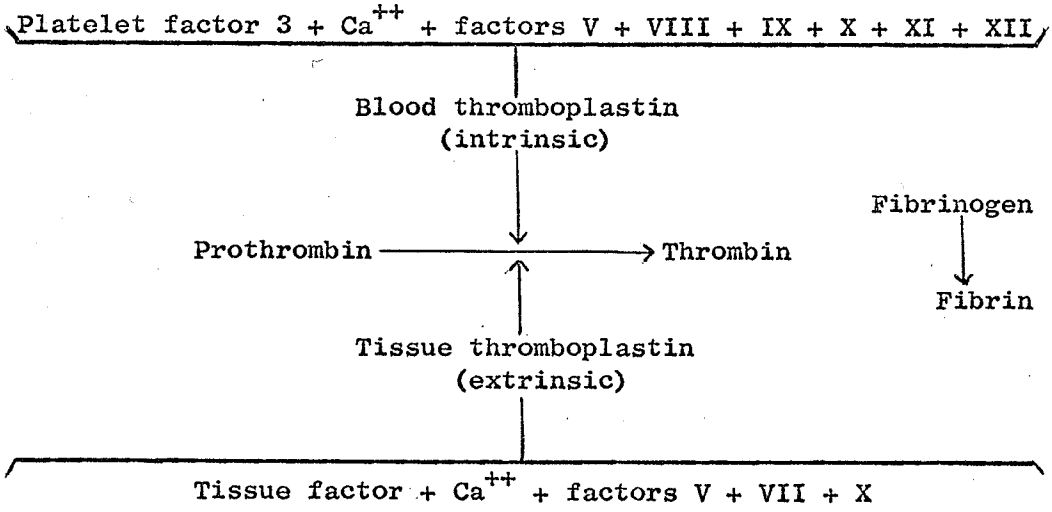


FIGURE I

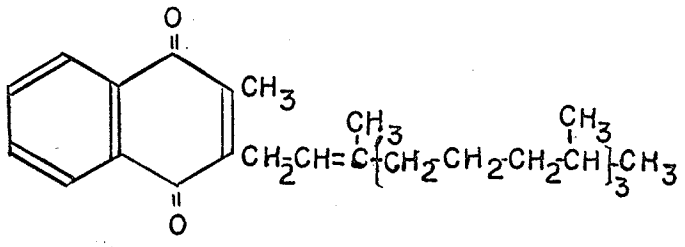
SEQUENCE OF REACTIONS INVOLVED IN EXTRINSIC AND INTRINSIC THROMBOPLASTIN FORMATION

The assumptions on which Quick's method for the estimation of prothrombin are based are therefore not valid, since tissue extract does not possess thromboplastin activity, but is only one of the components necessary for extrinsic thromboplastin formation. Quick's method estimates, therefore, in addition to prothrombin, the combined effect of the concentrations of plasma clotting factors, V, VII, and X on extrinsic thromboplastin formation. The method can be modified to measure the effect of a single factor on extrinsic thromboplastin formation by using reagents which supply an excess of all the factors required with the exception of the one that is estimated. The clotting time will then depend on the concentration of this factor in the sample and under these conditions the assumptions of Quick will be satisfied within certain limits. Such so-called one-stage methods for the determination of prothrombin, factor VII, and factor X have been used in the present investigation.

VITAMIN K

Before 1935 there had been several reports in the literature that chicks kept on a low fat diet developed hemorrhagic symptoms and showed decreased coagulability of the blood (27, 28, 29, 76, 77). In 1936 Dam et al., (30) and Schønheyder (100) showed that the coagulation defect was due to a deficiency of prothrombin. Dam (24, 25) and Almquist (1, 2) in 1935 found that the hemorrhagic symptoms could be prevented by the addition of alfalfa or putrified fish meal to the diet, but none of the known vitamins was found to be effective. Dam assumed the existence of an unknown vitamin required for normal blood coagulation, to which he gave the name vitamin K (Ger. Koagulation) and undertook its isolation. The active substance was found in the nonsaponifiable fraction of ether extracts of alfalfa (24).

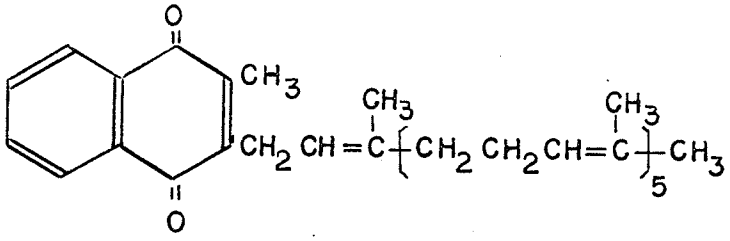
By degradative studies and chemical synthesis the active substance from the alfalfa extracts was shown to be a derivative of 2-methyl-1, 4-naphthoquinone having a phytyl side chain at the 3-position (16, 17, 32, 35, 38, 39, 56, 57).



Vitamin K_1 (2-methyl-3-phytyl-1, 4-naphthoquinone)

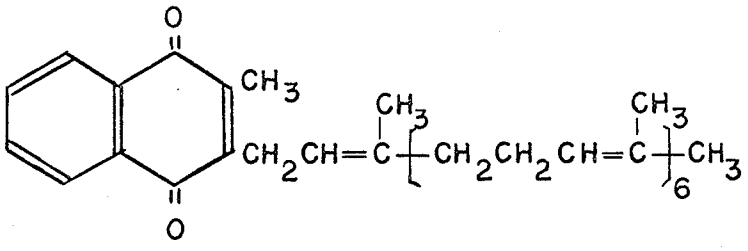
To this compound the name vitamin K_1 was given.

A similar compound differing only in the nature of the side chain at the 3-position was isolated from bacteria, identified as 2-methyl-3-difarnesyl-1, 4-naphthoquinone, and given the name vitamin K_2 (78).



Vitamin K_2 (2-methyl-3-difarnesyl-1, 4-naphthoquinone)

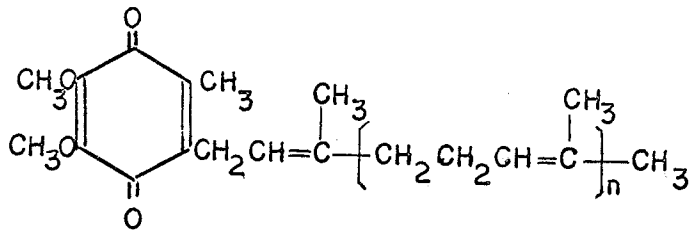
Recent improved techniques for the isolation and identification of biological substances from complex biological mixtures have shown that vitamin K_2 was not a single substance but a mixture of two naphthoquinones, one having a farnesyl-digeranyl side chain and the other the previously identified difarnesyl compound (50, 51).



Vitamin K_2 (2-methyl-3-farnesyl, digeranyl-1, 4-naphthoquinone)

Another analogue of vitamin K_2 with a side chain of 50 carbon atoms has been isolated (52). It therefore appears that in nature bacteria synthesize a series of vitamin K_2 analogues that differ only in the length of the side chain at the 3-position. On the other hand no homologues of vitamin K_1 have been isolated.

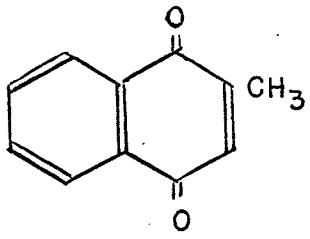
These were the first quinones shown to possess biological activity in higher organisms. It was only after more than fifteen years that Crane (23) and Morton (82) isolated a substituted benzoquinone, ubiquinone or coenzyme Q, which functions in terminal electron transport.



ubiquinone (coenzyme Q)

Both vitamin K_1 and vitamin K_2 were found to be active in restoring the prothrombin level to normal in vitamin K deficient chicks.

In 1939 Almquist (3-6) observed that the aromatic moeity of vitamin K_1, 2-methyl-1, 4-naphthoquinone, (Menadione) could prevent the hemorrhagic symptoms in the vitamin K deficient chick. This compound is known as vitamin K_3, although the designation as a vitamin is open to some criticism since it does not occur in nature.



Vitamin K_3 (2-methyl-1,4-naphthoquinone)

THE NATURE OF VITAMIN K DEFICIENCY

Although vitamin K deficiency could be produced readily in chicks by feeding a vitamin K deficient diet, Dam and co-workers (30) failed to produce a deficiency when they fed similar diets for long periods to young rats, guinea pigs, rabbits, dogs, and swine. Greaves (41), however, reported a small incidence (12/77) of vitamin K deficiency in rats kept on vitamin K deficient diet for forty-five weeks. These failures arose from the fact the vitamin can be obtained from intestinal bacterial synthesis. For this reason also, in humans, simple dietary deficiency of vitamin K rarely occurs. However, it had been noted clinically from very early times that in patients with chronic biliary obstruction hemorrhage was frequently a serious complication (94). In 1935 Quick (90) showed that the hemorrhagic symptoms associated with biliary obstruction were related to a low concentration of plasma prothrombin. Following the isolation of vitamin K, it was soon shown that in the absence of bile the vitamin is not absorbed from the gastro-intestinal tract.

Various workers attempted to produce vitamin K deficiency experimentally by utilizing the requirement of bile for the absorption of vitamin K. Greaves (40) showed that bile-fistula rats maintained on a vitamin K deficient diet became vitamin K deficient in less than a week. In dogs, cholecystnephrostomy (55) i.e., the diversion of bile to the hilus of the kidney produced a similar effect (93). However, these procedures were difficult, and there was also the possibility of liver dysfunction due to bile stasis and of multiple deficiencies due to the absence of bile.

Several years ago an attempt to modify this operation in rats by draining the bile into the vas deferens was made by us (67). Although a few rats developed a mild vitamin K deficiency, in a high percentage of these animals the drainage tube became blocked, the animals showed signs of severe jaundice and were generally in poor condition for experimental studies.

Other workers fed large doses of mineral oil to rats in an attempt to produce vitamin K deficiency (26, 36, 83). In our hands this method was not satisfactory because the absorption not only of vitamin K but also of other essential lipids was interfered with, and the animals again were generally in poor condition (67).

Barnes and co-workers (13) produced vitamin K deficiency in the rat by placing a plastic cup over the tail and anus of the rat to prevent coprophagy and feeding a vitamin K deficient diet. Their results demonstrated that the rat obtains a major part of its vitamin K requirement by coprophagy. Evidence that the bacterial flora of the intestine was an important source of vitamin K has been obtained also from studies with germ-free rats. Such animals became vitamin K deficient when a vitamin K deficient diet was fed and coprophagy allowed (43, 44).

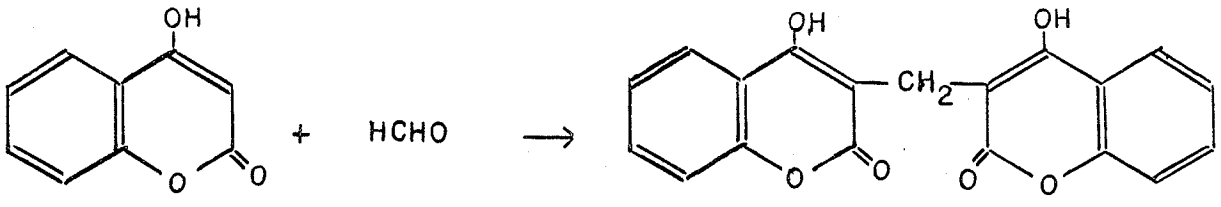
Metta and Johnson (79) improved on the rather laborious technique of Barnes by designing a cage which prevents or reduces coprophagy while at the same time the animals are not unduely restrained. Under these conditions feeding a vitamin K deficient diet results in a high incidence of vitamin K deficiency, and at present this is undoubtedly the simplest

and most reliable method for producing vitamin K deficiency in rats.

COUMARIN ANTICOAGULANTS

Another aspect of blood coagulation has come from studies of a hemorrhagic syndrome encountered in cattle. Schofield in 1922 (98, 99) traced the syndrome to the feeding of spoiled sweet clover hay, Melilotus sp., and in 1931 Roderick (96) demonstrated by quantitative precipitation of prothrombin that the delayed coagulability of the blood was related to a reduced concentration of prothrombin in the plasma. In 1939 Campbell et al., (18, 19) and Link (62) isolated from spoiled sweet clover the substance responsible for the hemorrhagic syndrome.

The observation that a low coumarin content sweet clover species did not become hemorrhagic when subjected to artificial spoilage suggested that the hemorrhagic substance might arise from coumarin (102). From the molecular formula and some of the chemical properties of the substance isolated it appeared likely that it was a derivative of 4-hydroxycoumarin. Link and co-workers synthesized various coumarin derivatives (48). The compound formed by the condensation of 4-hydroxycoumarin with formaldehyde to give methylene-bis-3,3'-(4-hydroxycoumarin),



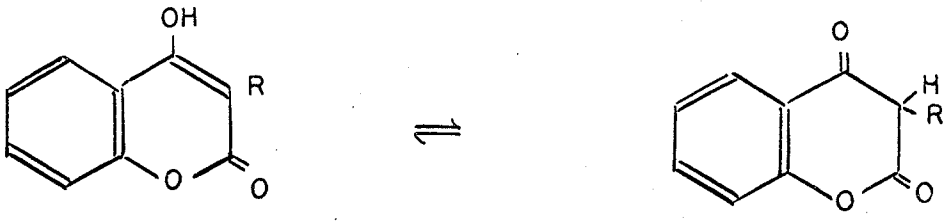
4-hydroxycoumarin formaldehyde (methylene-bis-4-hydroxycoumarin) dicoumarol

dicoumarol, was found to have identical chemical and biological properties with the substance isolated from spoiled sweet clover (103). It is of interest that this compound had been synthesized already by Anschütz in 1903 (7).

At the time these investigations were in progress, heparin had just been introduced for the clinical treatment of conditions requiring reduced coagulability of the blood (54). Because of the short duration of action of heparin and the necessity for parenteral administration, dicoumarol was tried as a substitute. However, the mode of action of heparin and dicoumarol is different. Heparin has an immediate direct effect on the clotting process, acting as an antithrombin and possibly also as an antithromboplastin. In contrast, dicoumarol has no direct effect on the clotting process, but interferes with the synthesis or secretion of certain clotting factors at the cellular level (34, 47, 49). Therefore, the anticoagulant effect appears gradually with the decrease in the plasma level of these factors, and depends on the difference between the rate of secretion into and the rate of disappearance from the plasma. Dicoumarol and related drugs therefore became known as indirect anticoagulants.

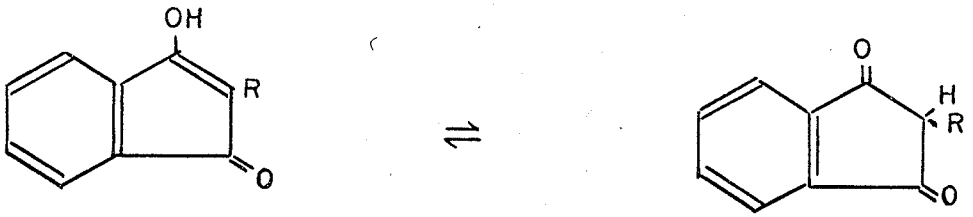
Numerous analogues of dicoumarol were synthesized to establish the structural requirements for activity and to find more active compounds. In addition to derivatives of 4-hydroxycoumarin, the derivatives of 1,3-indanedione were also found to possess anticoagulant activity. Both of these groups of compounds possess a β-dicarbonyl configuration capable of enolization. Activity is markedly increased by an aliphatic or aromatic substituent in the α-position, but the structural requirements of this substituent do not appear to be very specific and no satisfactory

explanation for the relation between chemical structure and anticoagulant activity has been given (49).



enol ⇌ keto

4-hydroxycoumarin



enol ⇌ keto

indanedione

THE RELATION BETWEEN VITAMIN K AND INDIRECT ANTICOAGULANTS

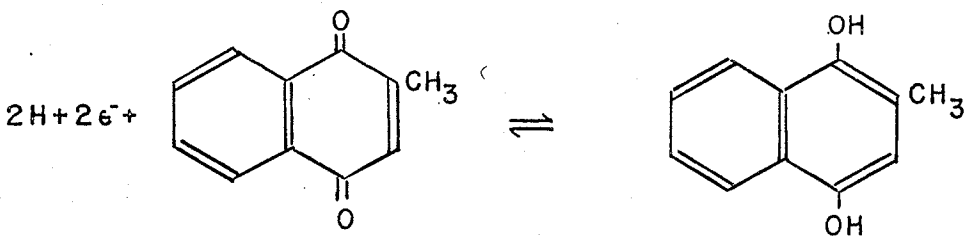
While vitamin K and dicoumarol were discovered concurrently by different groups of investigators, a relationship between these compounds was indicated when Quick in 1937 observed that alfalfa prevented the anticoagulant effect of spoiled sweet clover in rabbits (91). Finally in 1942 Overmann showed that vitamin K reversed the hypoprothrombinemia caused by dicoumarol in rats (84).

At present it is known that vitamin K deficiency and indirect anticoagulants not only reduce plasma prothrombin but also plasma factors VII, IX, and X (34, 47, 49). Because of certain similar properties, such as absorption on barium sulphate, this group of plasma clotting factors is also known as the prothrombin complex. In contrast factors V, VIII, and fibrinogen are not affected by dicoumarol treatment or by a deficiency of vitamin K (14).

The anticoagulant effect of indirect anticoagulants can be readily reversed by vitamin K_1 and vitamin K_2. However, 2-methyl-1, 4-naphthoquinone (menadione, or vitamin K_3) has no effect in the fully anticoagulated animal. A possible explanation is that vitamin K_3 can be transformed in the body to a vitamin K_2-like compound by condensation with a polyisoprenoid side chain (74). While the rate of transformation is sufficient to overcome the deficiency in vitamin K deficient animals, it is not sufficient to reverse the clotting defect in coumarin treated animals.

THE NATURE OF THE ANTAGONISM BETWEEN VITAMIN K AND INDIRECT ANTICOAGULANTS

After a relationship between vitamin K and indirect anticoagulants was discovered, it was suggested that the nature of the antagonism might be of the competitive type. This suggestion was based on the assumption that the chemical structures of vitamin K and dicoumarol were sufficiently similar to give rise to this type of antagonism (85, 105). Both indirect anticoagulants and compounds with vitamin K-like activity possess two carbonyl groups as part of a ring structure. However, the two carbonyl groups of the 1,4-naphthoquinone structure of vitamin K-like compounds constitute a reversible quinone-hydroquinone oxidation-reduction system,



quinone hydroquinone

whereas, as mentioned before, the most prominent chemical reactivity of the 1,3-dicarbonyl configuration present in compounds with anticoagulant activity is their ability to exhibit keto-enol tautomerism.

While an exact definition of structural similarity is open to different interpretations, there appears little doubt that these two classes of compounds are different as regards chemical structure or reactivity (49).

Almquist (45) attempted to determine the antagonism from data published by Quick and Stefanini (92) who measured the response to graded doses of vitamin K_1 and vitamin K_3 in vitamin K deficient chicks in the absence and presence of dicoumarol. Since the presence of dicoumarol resulted in parallel displacement of the dose response curve, it was concluded that the antagonism was of the competitive type. However, only a single dose of dicoumarol was tested and other experimental details such as the duration of the experiment, leave this interpretation open to question.

Collentine and Quick (22) showed that the dog made vitamin K deficient by cholecystnephrostomy was much more susceptible than the normal dog to the anticoagulant activity of a small dose of dicoumarol. Furthermore, in such animals the increase of the plasma clotting factors by vitamin K_1 was prevented by the simultaneous administration of dicoumarol, incidentally demonstrating the immediate onset of action of coumarin drugs -- a fact which had not been shown previously because in normal animals there is a long induction period related to the slow disappearance of the clotting factors from the plasma. Although only one dose of vitamin K was used, Collentine and Quick concluded that the antagonism between dicoumarol and vitamin K was of the competitive type, apparently unaware that the criterion for competitive antagonism, constant inhibition with constant ratios of increasing doses of agonist and antagonist, was not demonstrated.

Babson and associates (8) incorporated constant ratios of vitamin K_1 and dicoumarol into the diet of rats and measured the prothrombin time when the rats had been on the diet for seven days. For any dose

ratio, the anticoagulant effect reached a maximum at low doses, but as the doses were increased while the ratio was held constant the anticoagulant effect disappeared. Therefore, at higher doses the effect of dicoumarol was always overcome by vitamin K_1, an observation not in agreement with a competitive antagonism. However, because of the duration of the experiment, the possibility can not be overlooked that the fat-soluble vitamin K_1 might have been stored more readily in the body so that at higher dose levels the effect of vitamin K might have predominated.

STATEMENT OF THE PROBLEM

Because of their clinical use, the nature of the antagonism between vitamin K_1 and indirect anticoagulants is not only of theoretical but also of practical interest. Although it has been stated frequently that the antagonism is of the competitive type, this has not been demonstrated experimentally (45, 85, 105). For example, the ready reversibility of the anticoagulant effect of coumarin drugs by vitamin K_1 and the experiments of Collentine and Quick (22) are only in agreement with, but are not sufficient evidence for such conclusions. On the other hand, the experiments of Babson (8) appear to indicate that the antagonism is not of the competitive type. It can therefore be concluded that the minimum requirement for a competitive type of antagonism, that is, constant inhibition of constant ratios of increasing doses of vitamin K_1 (agonist) and coumarin anticoagulants (antagonist) has not been demonstrated experimentally.

The indeterminate state of the problem can be traced partly to experimental conditions encountered when the antagonism is studied in normal animals. While indirect anticoagulants interfere with the formation of prothrombin, factor VII, factor IX, and factor X at the cellular level (34, 47, 49), the appearance of the anticoagulant effect depends on the rate of disappearance of the clotting factors from the plasma. Because of this, the onset of the anticoagulant effect is rather slow (12 to 24 hours), and other variables such as rate of metabolism and excretion may enter into the interpretation of the experiment. In addition, because of the non-linear relation between the plasma

concentration of clotting factors and coagulation time, the methods for the determination of these factors are not very sensitive to small variations about normal concentration (100%). Therefore, the initial changes brought about by coumarin anticoagulants cannot be determined with accuracy.

It was envisaged that these difficulties might be partly avoided by studying the effect of indirect anticoagulants on the increase of the plasma clotting factors by vitamin K in vitamin K deficient and coumarin treated animals. In particular, previous investigations had shown that when vitamin K_1 is brought into suspension with the aid of Tween 80 and administered intravenously, its speed of onset is appreciably increased (68). Under these experimental conditions one measures the relatively rapid increase of the plasma clotting factors by vitamin K over a period of two to two and one-half hours. During this interval the plasma concentration of the clotting factors can be assumed to be predominantly a function of the rate of secretion from the tissues into the plasma, while the rate of disappearance from the plasma can be assumed to be less important. Furthermore, because the plasma concentration of the clotting factors is low, the experimental measurements are made within a range where the methods show greater sensitivity.

The present investigation is an attempt to test, under these experimental conditions, increasing doses of constant ratios of agonist and antagonist, to determine more accurately the nature of the antagonism between vitamin K_1 and indirect anticoagulant drugs.

II. METHODS AND REAGENTS

Vitamin K Deficient Animals: Norwegian hooded male rats, 6 to 8 weeks old, weighing 150 to 200 grams, were selected from stock maintained since weaning on calf meal pellets. They were placed in square tubular cages to prevent coprophagy (79) and were fed a vitamin K deficient diet (69). To reduce the growth of bacterial flora, the cages, water-bottles, and feeding dishes were washed twice weekly, and the water was changed daily. Animals were screened once a week for development of vitamin K deficiency.

Anticoagulant Pretreated Animals: For anticoagulant pretreatment normal rats were given .25 mg warfarin (3-[α-acetonyl benzyl-]-4-hydroxycoumarin) / 100 gm body weight 24 hours before being used for experimental studies.

Blood Samples and Injections: Rats were lightly anaesthetized with diethyl ether for these procedures. Blood samples were taken from the lateral tail vein of the rat by drawing 0.1 ml blood into 0.9 ml veronal buffer containing 0.38% sodium citrate in a silicone coated 1.0 ml tuberculin syringe. The blood was transferred to a silicone coated test tube (10 x 75 mm.) and centrifuged at 2500 r.p.m. for 10 minutes. Injections of vitamin K and warfarin were administered into the tail vein at the time of the test.

Standard Curves: Pooled plasma of rats on a normal diet obtained as described above was serially diluted with veronal buffer and the individual clotting factors, prothrombin, factor VII, and Factor X determined. Standard curves were obtained when the log of the clotting time was plotted against the log of the concentration. Standard curves were checked frequently and new curves were prepared whenever new reagents

were prepared.

Clotting Methods: Prothrombin, factor VII, and factor X were determined on rat plasma obtained as described above without further dilution by established one-stage methods. All determinations were done in duplicate.

Factor VII Determination: (58) Into a silicone coated test tube 0.1 ml factor VII-free reagent plasma was pipetted. The tube was placed in a constant temperature water bath at 37°C and 0.1 ml of the test plasma was added. A stop watch was started as 0.1 ml thromboplastin solution was added. After a 30-second incubation period, the stop watch was reset to zero and started again simultaneously with the addition of 0.1 ml 0.025 M calcium chloride. The tube was observed carefully for the formation of a clot. The time required for clot formation was converted to per cent of normal plasma concentration by reference to the factor VII standard curve.

Prothrombin Determination: (58) Into a silicone coated test tube 0.1 ml prothrombin-free reagent plasma was pipetted. 0.1 ml test plasma was added and the mixture incubated 60 seconds at 37° with 0.1 ml thromboplastin. 0.1 ml 0.025 M calcium chloride was then added and the time required for clot formation noted. Results were expressed as per cent of normal plasma concentration by reference to the prothrombin standard curve.

Factor X Determination: (9) In a silicone coated test tube 0.1 ml factor X-free reagent plasma, 0.1 ml of the test plasma, and 0.1 ml Russell viper venom-cephalin reagent were incubated for 30 seconds at 37°C. A stop watch was started as 0.1 ml 0.025 M calcium chloride was added. The time required for clot formation was recorded and results

expressed as per cent of normal plasma concentration by reference to the factor X standard curve.

Vitamin K Deficient Diet: (69)

	Per Cent
Salt mix	4.0
D,L methionine	0.5
Wheat germ oil	0.5
Glycerol	2.0
Vitamin mixture	4.0
Sucrose	67.5
Soy Protein*	20.0
Cod liver oil	1.5

Vitamin Mixture

	Mg per kg diet
Thiamine hydrochloride	10.0 mg
Riboflavin	10.0
D,L calcium pantothenate	50.0
Pyridoxine hydrochloride	5.0
Nicotinic acid	20.0
Folic acid	1.0
Vitamin B_{12}	0.1
Choline chloride	1000.0
Biotin	0.1
Cerelose	48.9 g

*Soy protein obtained from the Drackett Company was extracted with alcohol before use. Other ingredients with the exception of sucrose were obtained from Nutritional Biochemicals Company.

The diet was prepared in 10 kilogram quantities and stored at $4^{o}C$ to prevent deterioration.

Thromboplastin: Rabbit brain thromboplastin was supplied by Warner Chilcott Laboratories. It was prepared for use by suspending 1.5 g of the dessicated brain extract in 100 ml 0.9% sodium chloride for 15 minutes at 50 to 52°C with occasional stirring. After incubation the sediment was filtered off through several layers of cheesecloth and the filtrate stored in ampules at -20°C.

Russell Viper Venom-Cephalin Reagent: (9) Russell viper venom (Stypven^{R}) was supplied by Burroughs Wellcome. Cephalin was prepared from calf brain according to the technique of Hjort (46) and Milstone (80). The Russell viper venom was dissolved in veronal buffer containing 0.07% cephalin to give a final concentration of 1/40,000.

Veronal Buffer:

Stock Solution: 9.714 g sodium acetate, 14.714 g veronal sodium dissolved in 500 ml distilled water.

Working solution: 250 ml stock solution, 200 ml 4.25% sodium chloride, 217 ml 0.1 N hydrochloric acid, 683 ml distilled water. Adjust the pH to 7.35.

Calcium chloride: 0.025 molar calcium chloride was used in all experiments.

Vitamin K_1: Vitamin K_1 was obtained through the courtesy of Merck Sharp & Dohme of Canada Ltd., and was made up as a clear aqueous suspension with Tween 80 (polyoxythylene sorbitan monocleate, Atlas Powder Company) (68).

Warfarin: (3-[α-acetonyl benzyl-]-4-hydroxycoumarin) was obtained commercially and a stock solution containing 20 mg/ml was prepared by dissolving the warfarin with an equivalent amount of sodium hydroxide, adjusting the pH to 7.5.

III. RESULTS

DEVELOPMENT OF VITAMIN K DEFICIENCY

Rats were fed a vitamin K deficient diet and kept in square tubular cages to prevent coprophagy. Table I shows the incidence of vitamin K deficiency as measured by the weekly determination of the plasma level of factor VII. Arbitrarily a plasma level of factor VII of less than fifteen per cent was considered to represent a sufficiently severe deficiency to make these animals useful for subsequent studies. While there were exceptions, most of the animals had to be kept on the diet for at least three weeks before the plasma concentration of factor VII fell below this level.

TABLE I

DEVELOPMENT OF VITAMIN K DEFICIENCY IN RATS ON A VITAMIN K FREE DIET ESTIMATED BY WEEKLY DETERMINATION OF PLASMA CONCENTRATION OF FACTOR VII*.

Weeks	1	2	3	4	5	6	7	8
Rats								
342	2.2							
418	2.6							
455	6.4							
405	18.6	1.6						
415	27.0	12.5	8.0					
427	36.0	3.6						
428	14.0	13.5	5.0					
429	16.0	13.0	7.0					
451	15.0	8.0						
331	100.0	55.0	5.2					
414	100.0	17.0	5.6					
426	80.0	29.0	2.8					
431	25.0	2.3	7.2					
447	100.0	36.0	3.0					
448	22.0	9.0	3.3					
432	34.0	6.4	13.0					
329	86.0	50.0	46.0	1.2				
330	19.5	20.0	5.6	1.0				
352	38.0	29.0	11.5	2.7				
443	100.0	20.0	12.0	2.1				
416	25.0	38.0	11.0	30.0	1.5			
417	35.0	13.0	32.0	33.0	2.7			
300	54.0	71.0	62.0	4.8	11.5	7.8		
343	100.0	58.0	23.0	50.0	9.6	1.2		
297	65.0	28.5	45.0	74.0	63.0	100.0	2.6	
425	100.0	30.0	18.5	28.0	23.0	15.0	4.5	
353	25.0	28.0	10.5	100.0	100.0	39.0	10.5	12.0
301	50.0	23.5	56.0	78.0	36.0	100.0	100.0	60.0

* Per cent of normal concentration.

INCREASE OF PLASMA LEVELS OF FACTOR VII, PROTHROMBIN, AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF VITAMIN K_1 IN VITAMIN K DEFICIENT RATS

To determine the effect of a small dose of vitamin K_1 on the vitamin K dependent clotting factors (prothrombin, factor VII, and factor X) in vitamin K deficient rats, 0.5 μg vitamin K_1/100 g of body weight was injected intravenously (Table II*, figure 2a). Twenty minutes after the injection the plasma concentration of prothrombin had increased from 4.4% to 9.6%, of factor VII from 13.5% to 19.0%, and of factor X from 15.5% to 21.0%. After 45 minutes the plasma concentrations of prothrombin, factors VII, and X were 40.0%, 28.5% and 38.0% and after 157 minutes 66.0%, 58.0% and 60.0%.

When the dose of vitamin K_1 was increased to 5.0 μg/100 g, the response was essentially the same (Table III, Figure 2b). Since the pattern of the response of all three factors was similar, only factor VII was determined in subsequent experiments.

* Table II to XVIII see appendix.

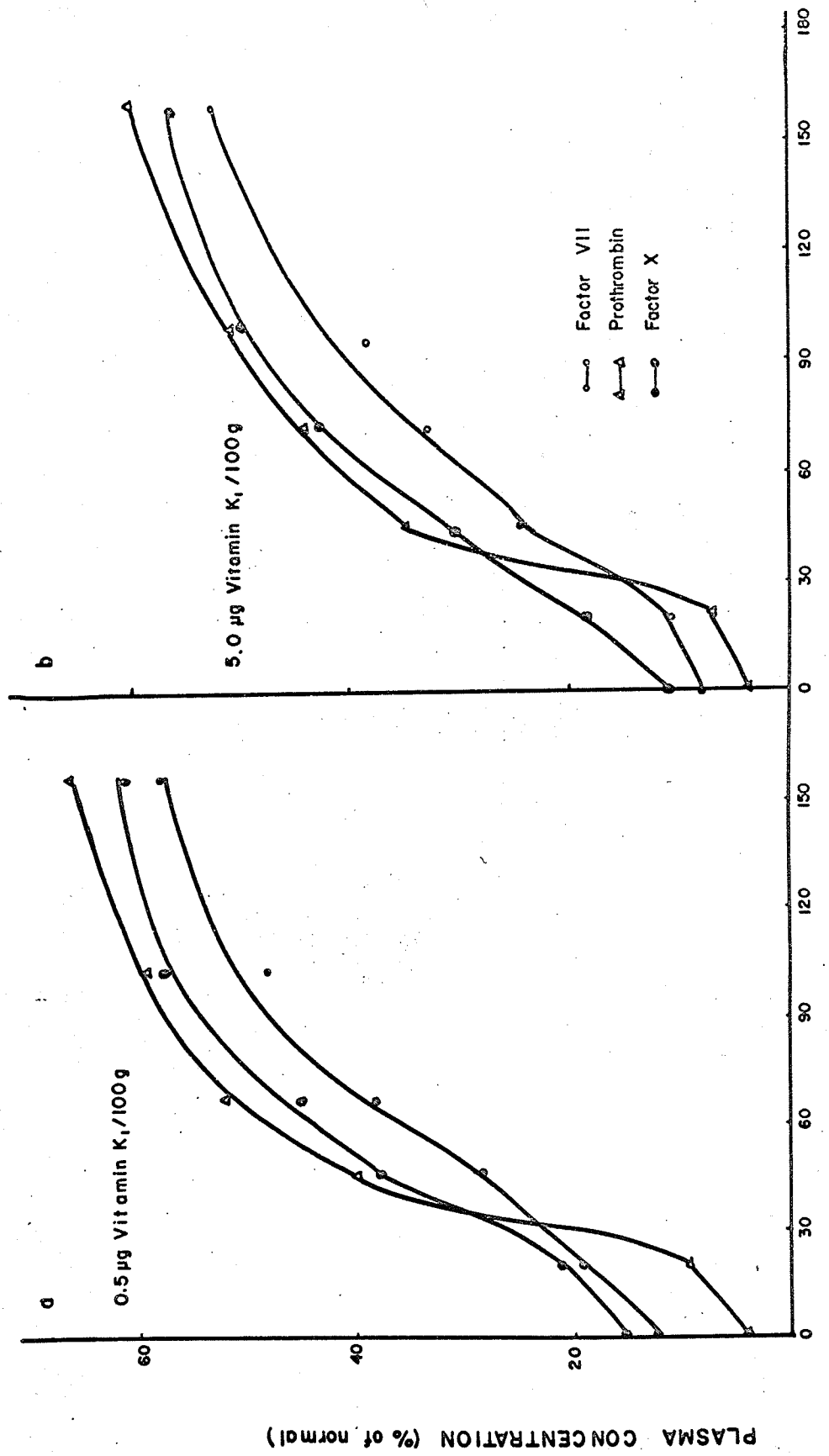


FIGURE 2

INCREASE OF PLASMA CLOTTING FACTORS (FACTOR VII, PROTHROMBIN, AND FACTOR X) AFTER INTRAVENOUS ADMINISTRATION OF (a) 0.5 µg VITAMIN K1/100 g AND (b) 5.0 µg VITAMIN K_1/100 g IN VITAMIN K DEFICIENT RATS.

INCREASE OF PLASMA LEVEL OF FACTOR VII BY GRADED DOSES OF VITAMIN K_1 IN DEFICIENT RATS

To find the dose range for which the response to vitamin K_1, as measured by the increase of factor VII, becomes proportional to the dose, graded doses were given to vitamin K deficient rats.

As shown in Table IV and Figure 3, a dose of 50, 40, 20, 10, 5, 2.5, and 0.5 μg/100 g of vitamin K_1 gave essentially the same response, while below 0.5 μg the response became proportional to the dose (Table V, Figure 4). It therefore appears that in the vitamin K deficient rat 0.5 μg vitamin K_1/100 g produced an optimal response.

The increase in factor VII was detected after 20-30 minutes. Such a rapid increase can only be obtained when vitamin K is suspended with the aid of Tween 80 and is given intravenously. A similar rapid increase after intravenous administration has been demonstrated previously in the coumarin anticoagulant treated rat (68).

It would have been desirable to establish the optimal dose of vitamin K_1 by constructing a complete dose response curve. Unfortunately, this could not be done because of the limited number of vitamin K deficient animals available. However the data are sufficient to demonstrate that the optimal dose in the vitamin K deficient animal is 0.5 μg/100 g and only this information was required for the design of subsequent experiments.

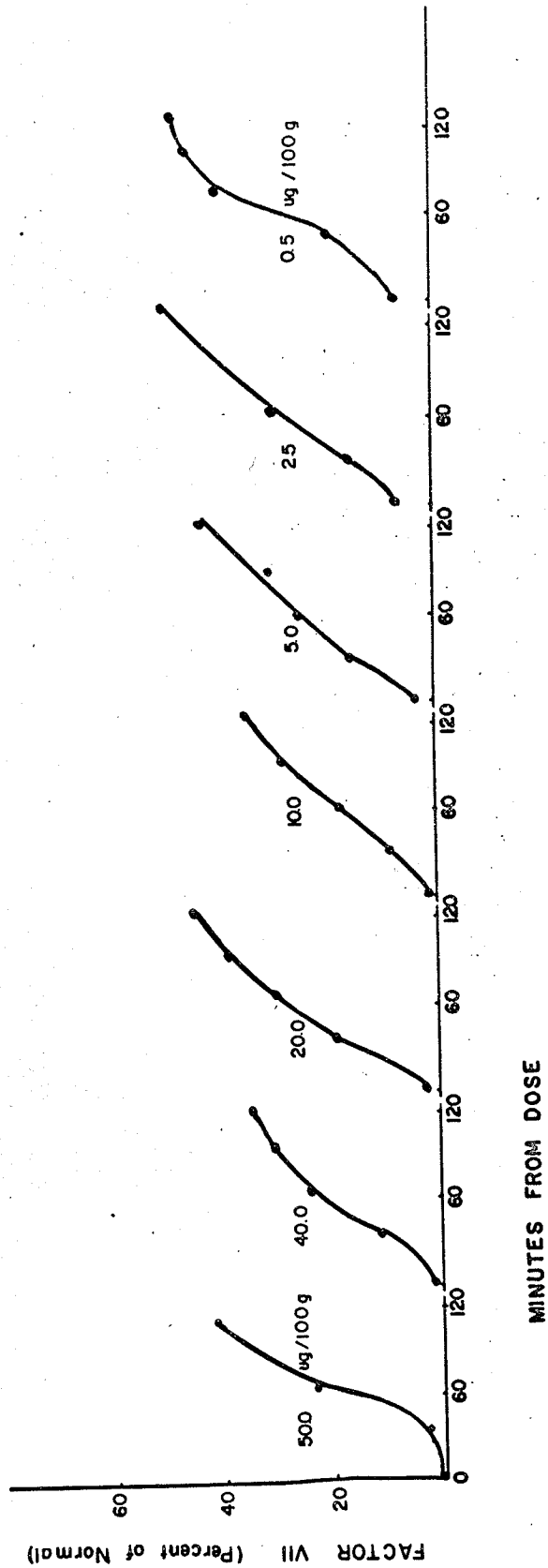


FIGURE 3

INCREASE OF PLASMA LEVEL OF FACTOR VII AFTER INTRAVENOUS ADMINISTRATION OF SUPEROPTIMAL GRADED DOSES OF VITAMIN K_1 IN VITAMIN K DEFICIENT RATS.

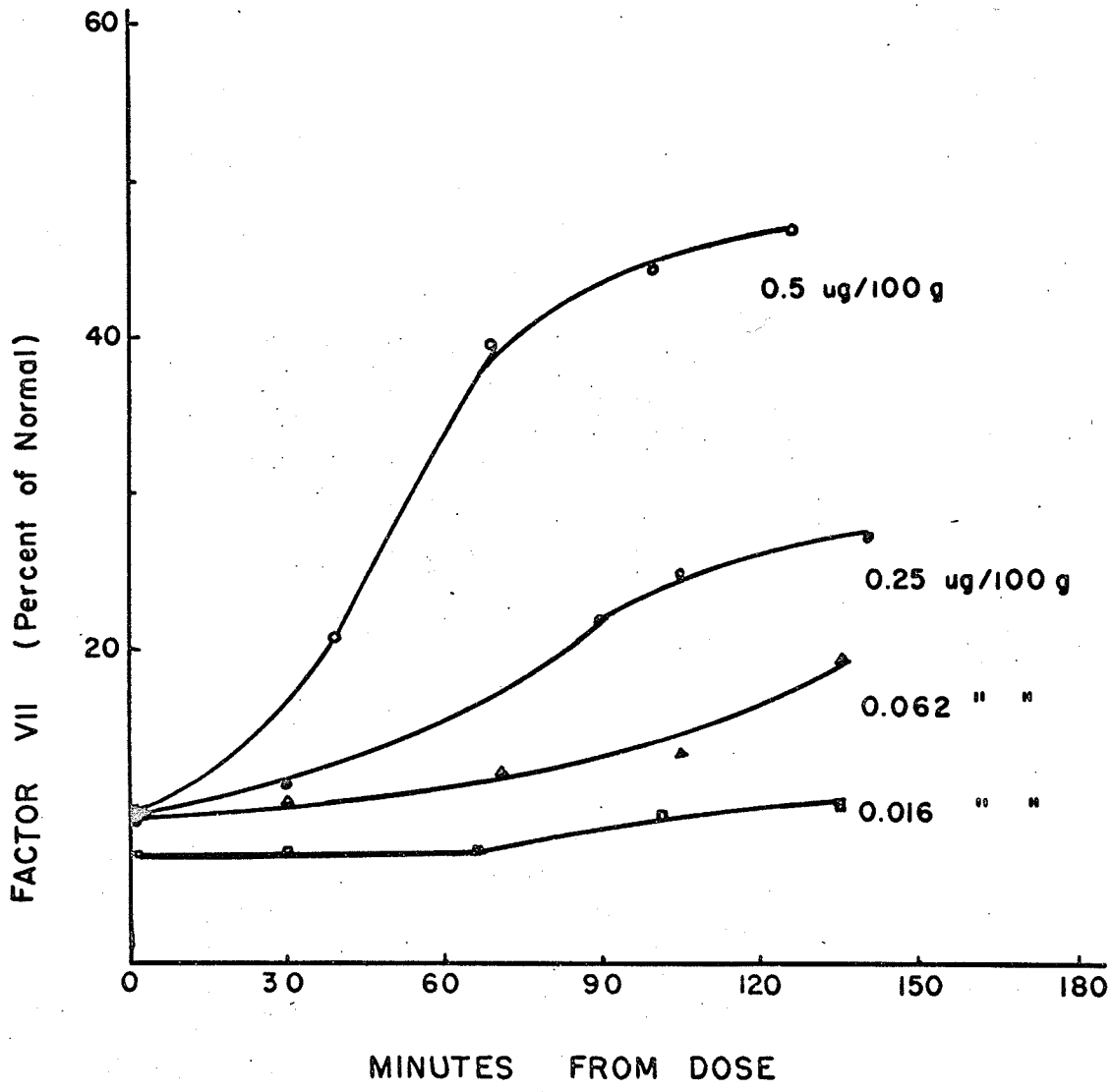


FIGURE 4

INCREASE OF PLASMA LEVEL OF FACTOR VII AFTER INTRAVENOUS ADMINISTRATION OF SUBOPTIMAL DOSES OF VITAMIN K_1 IN VITAMIN K DEFICIENT RATS.

EFFECT OF SIMULTANEOUSLY INJECTED WARFARIN ON THE INCREASE OF FACTOR VII BY VITAMIN K_1 IN VITAMIN K DEFICIENT RATS

To test the effect of an indirect anticoagulant on the increase of the plasma level of factor VII by vitamin K_1 in vitamin K deficient rats, an optimal dose of vitamin K_1, 0.5 μg/100 g, was given together with increasing doses of warfarin.

When 5.0 μg warfarin/100 g was injected together with 0.5 μg vitamin K_1/100 g the response to vitamin K was slightly inhibited (Table VI, Figure 5). When the dose of warfarin was increased to 10 μg/100 g, the inhibition was increased and when the dose of warfarin was 25.0μg/100 g or 50.0 μg/100 g, the inhibition was complete. When 5.0 μg warfarin/100 g was given alone to a vitamin K deficient rat, there was a small decrease of the plasma concentration of factor VII over the next 165 minutes, from 13.0% to 10.0%.

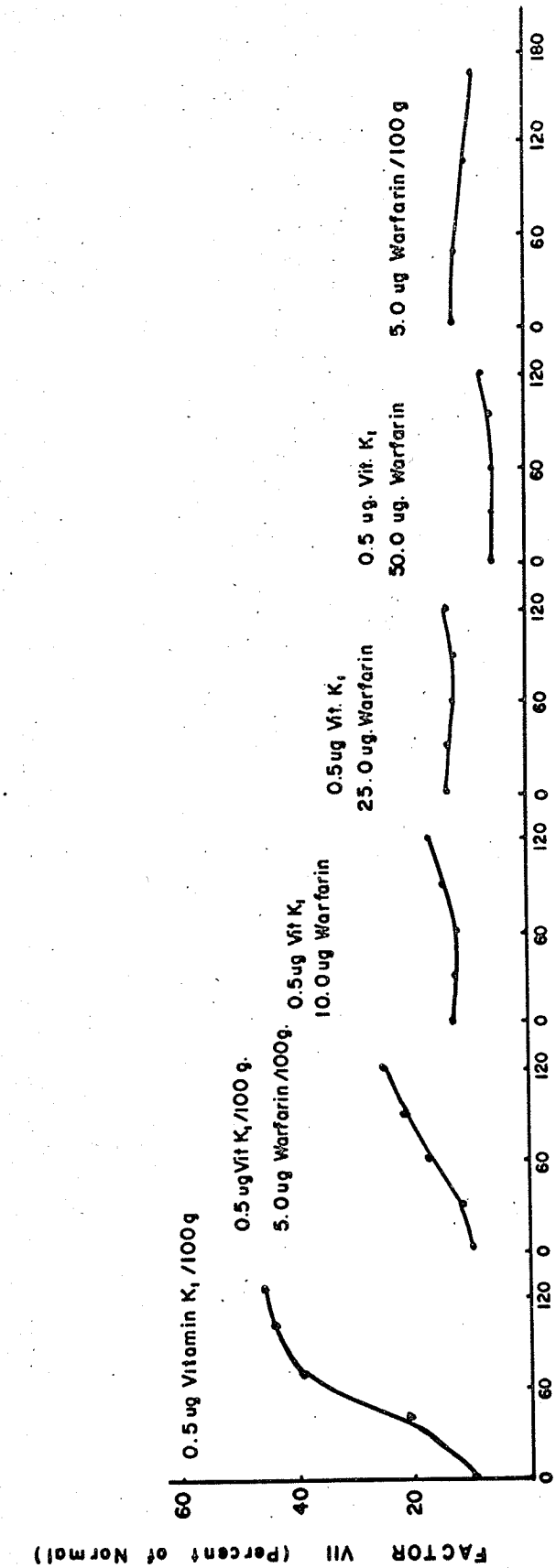


FIGURE 5

EFFECT OF INCREASING DOSES OF WARFARIN ADMINISTERED SIMULTANEOUSLY WITH 0.5 µg VITAMIN K_1/100 g ON THE INCREASE OF THE PLASMA LEVEL OF FACTOR VII IN VITAMIN K DEFICIENT RATS.

EFFECT OF INCREASING DOSES OF CONSTANT RATIOS OF VITAMIN K_1 AND WARFARIN IN VITAMIN K DEFICIENT RATS

To study the nature of the antagonism between vitamin K_1 and warfarin, demonstrated in the previous series of experiments, constant ratios of increasing doses of vitamin K_1 and warfarin were tested. As shown before, simultaneous administration of 5.0 μg warfarin/100 g partially inhibited the response to 0.5 μg vitamin K_1/100 g. Keeping this ratio of vitamin K_1 to warfarin (1 : 10) constant and increasing the doses 5-fold (2.5 μg vitamin K_1 and 25 μg warfarin/100 g), the increase in the plasma level of factor VII by vitamin K_1 was nearly completely inhibited (Tables VII, VIII, Figure 6a). When the doses were increased 10-fold (5.0 μg vitamin K_1 and 50.0 μg warfarin/100 g) the inhibition became complete, but a 100-fold increase (500 μg vitamin K_1 and 5,000 μg warfarin/100 g) resulted in a complete disappearance of inhibition.

Using a ratio of vitamin K_1 to warfarin of 1 to 20, 10 μg of warfarin produced nearly complete inhibition of the increase of factor VII by 0.5 μg vitamin K_1/100 g. A 10 and 20-fold increase of the doses resulted in complete inhibition, but after a 40-fold increase the inhibition decreased and there was a small response (Tables IX, X, Figure 6b). Finally, after a 100-fold and 1,000-fold increase of the doses, the inhibition by warfarin disappeared completely.

With a ratio of vitamin K_1 to warfarin of 1 to 50, the inhibition remained constant when the doses were increased 10-fold, but disappeared after a 100 and 1,000-fold increase (Tables XI, XII, Figure 6c). Similar

results were obtained when the ratio of vitamin K_1 to warfarin was 1 to 100 (Table XIII, Figure 6d) and 1 to 500, (Table XIV, Figure 6e), i.e., the inhibition disappeared after the doses had been increased 100-fold. Therefore for any ratio tested the inhibition disappeared completely when the doses were increased at least a 100-fold. Since the lowest dose of vitamin K_1 for all ratios was 0.5 μg/100 g, this means that the inhibition disappeared when the dose of vitamin K_1 was 50.0 μg/100 g regardless of the dose of warfarin administered simultaneously.

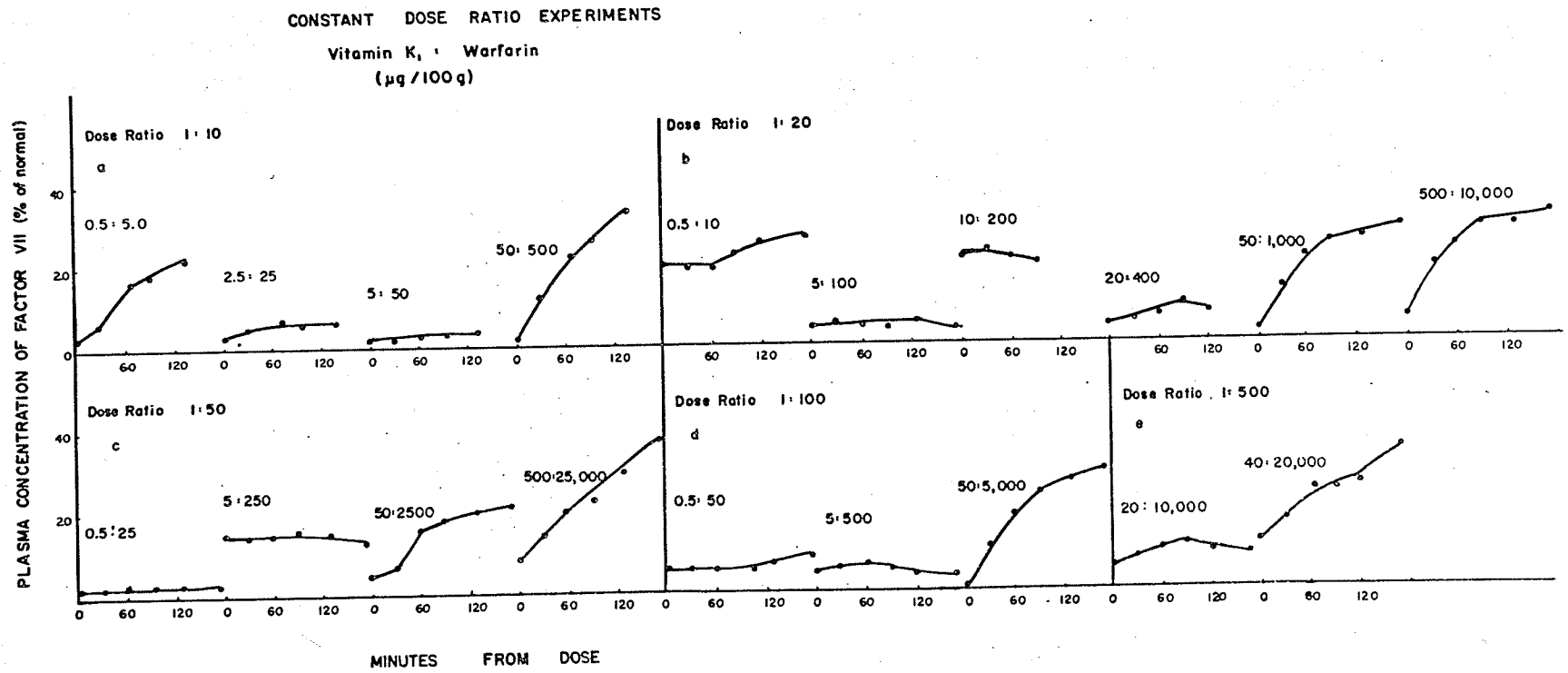


FIGURE 6

EFFECT OF INCREASING DOSES OF CONSTANT RATIOS OF VITAMIN K_1 AND WARFARIN ON THE INCREASE OF THE PLASMA LEVEL OF FACTOR VII IN VITAMIN K DEFICIENT RATS.

INCREASE OF PLASMA LEVEL OF FACTOR VII BY GRADED DOSES OF VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS

When normal rats were given 0.25 mg warfarin/100 g of body weight by stomach tube, the plasma concentration of factor VII decreased after 18 to 20 hours to about 1 to 2% of normal, while the concentrations of prothrombin and factor X were usually between 5 and 15%. To find the doses of vitamin K_1 required to increase the plasma level of factor VII, graded doses of vitamin K_1 were injected intravenously (Table XV, Figure 7).

When 60.0 µg vitamin K_1/100 g were given, the plasma level of factor VII increased from 1.0% at the time of the injection to 19.2% after 40 minutes, to 32.0% after 68 minutes, and reached 36.0% after 120 minutes.

When the dose of vitamin K_1 was 40.0 µg/100 g, the plasma level of factor VII increased from 1.4% to 18.8% after 38 minutes, to 33.8% after 70 minutes, and was 41.0% after 125 minutes.

Thirty-five minutes after 20.0 µg vitamin K_1/100 g the plasma level of factor VII had increased from 1.0% to 7.0%, after 70 minutes it was 11.0%, after 107 minutes 16.0%, and after 137 minutes it was 18.0%. The increase of factor VII after 20.0 µg vitamin K_1/100 g is therefore approximately one-half that of the 40.0 µg dose.

With a dose of 10.0 µg/100 g the plasma level of factor VII after 57 minutes had increased from an initial value of 1.1% to 4.2%, while after 147 minutes it was 8.0%. The total increase in the concentration of factor VII was about 1/5 that of the increase given by a 40.0 µg dose. When the dose of vitamin K_1 was 5.0 µg/100 g the increase in the plasma level of factor VII was negligible. The range in which the increase of

factor VII was proportional to the dose was approximately between 10.0 and 40.0 μg vitamin K_1/100 g body weight. Therefore in rats pretreated with 0.25 mg warfarin/100 g, a dose of vitamin K_1 of 40.0 μg/100 g produced an optimal increase of factor VII. This dose of vitamin K_1 is approximately 80 times greater than the dose of vitamin K_1 required to overcome vitamin K deficiency in rats.

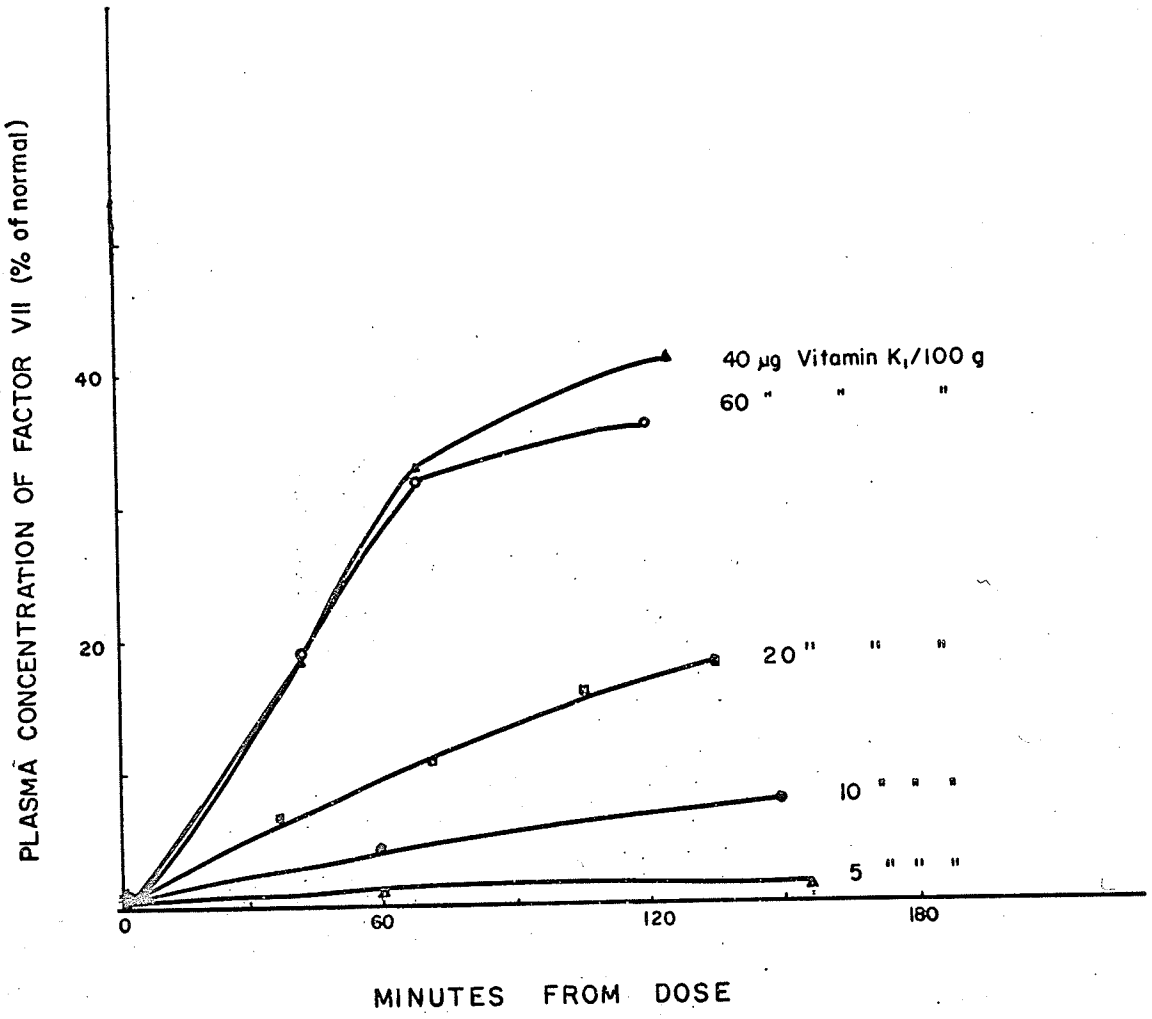


FIGURE 7

INCREASE OF PLASMA LEVEL OF FACTOR VII AFTER INTRAVENOUS ADMINISTRATION OF GRADED DOSES OF VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS

INCREASE OF PLASMA LEVELS OF FACTOR VII, PROTHROMBIN, AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS

Twenty-four hours after oral administration of 0.25 mg warfarin/100 g, an optimal dose of 40.0 μg vitamin K_1/100 g was injected and the increase in the plasma level of the clotting factors, factor VII, prothrombin, and factor X followed. An increase in the plasma concentration of each factor was detected after 15 minutes when factor VII increased from 1.4% to 2.4%, prothrombin from 19.5% to 23.0%, and factor X from 2.0% to 3.5%. After 30 minutes factor VII was 18.8%, prothrombin 35.0% and factor X 16.0%, while after 60 minutes factor VII was 33.8%, prothrombin 46.0% and factor X 23.0% of normal. At the end of the experimental period factor VII had reached 41.0%, prothrombin 55.0%, and factor X 34.0%. The total increase in concentration of each factor and the rate of increase were similar (Table XVI, Figure 8).

The results of an identical experiment are shown in Table XVII.

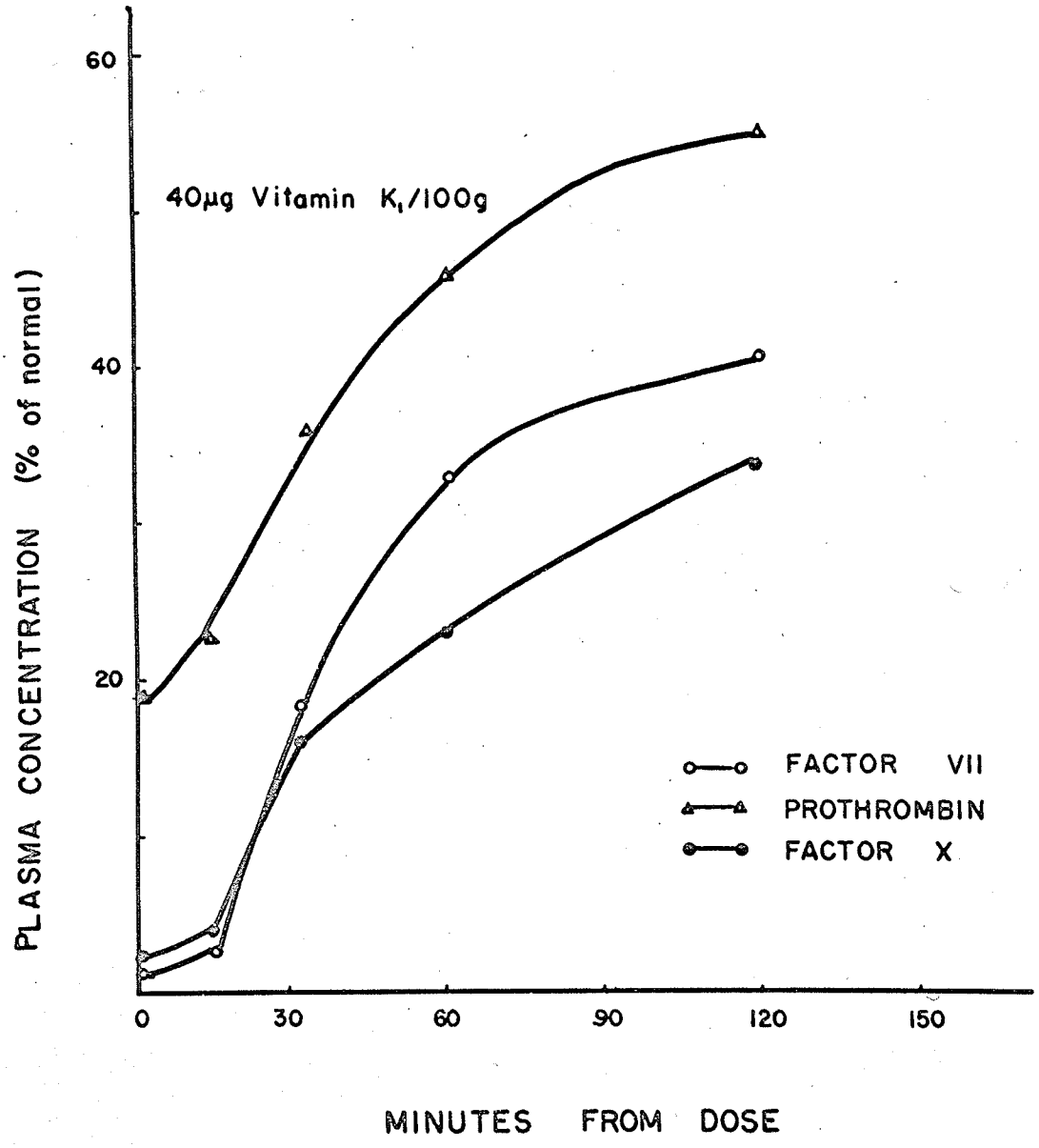


FIGURE 8

INCREASE OF PLASMA CLOTTING FACTORS (FACTOR VII, PROTHROMBIN, AND FACTOR X) AFTER INTRAVENOUS ADMINISTRATION OF 40.0 μg VITAMIN K_1/100 g IN THE ANTICOAGULANT PRETREATED RAT.

EFFECT OF SIMULTANEOUSLY INJECTED WARFARIN ON THE INCREASE OF FACTOR VII BY VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS

To investigate the effect of simultaneously administered warfarin on the activity of vitamin K_1 in rats pretreated with 0.25 mg warfarin/100 g orally, the highest tolerated dose of warfarin, 20.0 mg/100 g, was injected simultaneously with an optimal dose of 40.0 μg vitamin K_1/100 g (Table XVIII, Figure 9). The effect of 40.0 μg vitamin K_1/100 g given alone is shown for comparison. After 35 minutes the increase in the plasma concentration of factor VII by 40.0 μg vitamin K_1/100 g in the presence of warfarin was not reduced when compared to 40.0 μg vitamin K_1 given alone. Similarly after 70 minutes there was no inhibition of the activity of vitamin K_1 by warfarin. At the end of the experimental period there was no marked difference in the increase of factor VII by this dose of vitamin K_1 in the presence or absence of warfarin.

When the dose of vitamin K_1 was reduced to 20.0 μg/100 g, 20 mg/100 g again had no effect on the response.

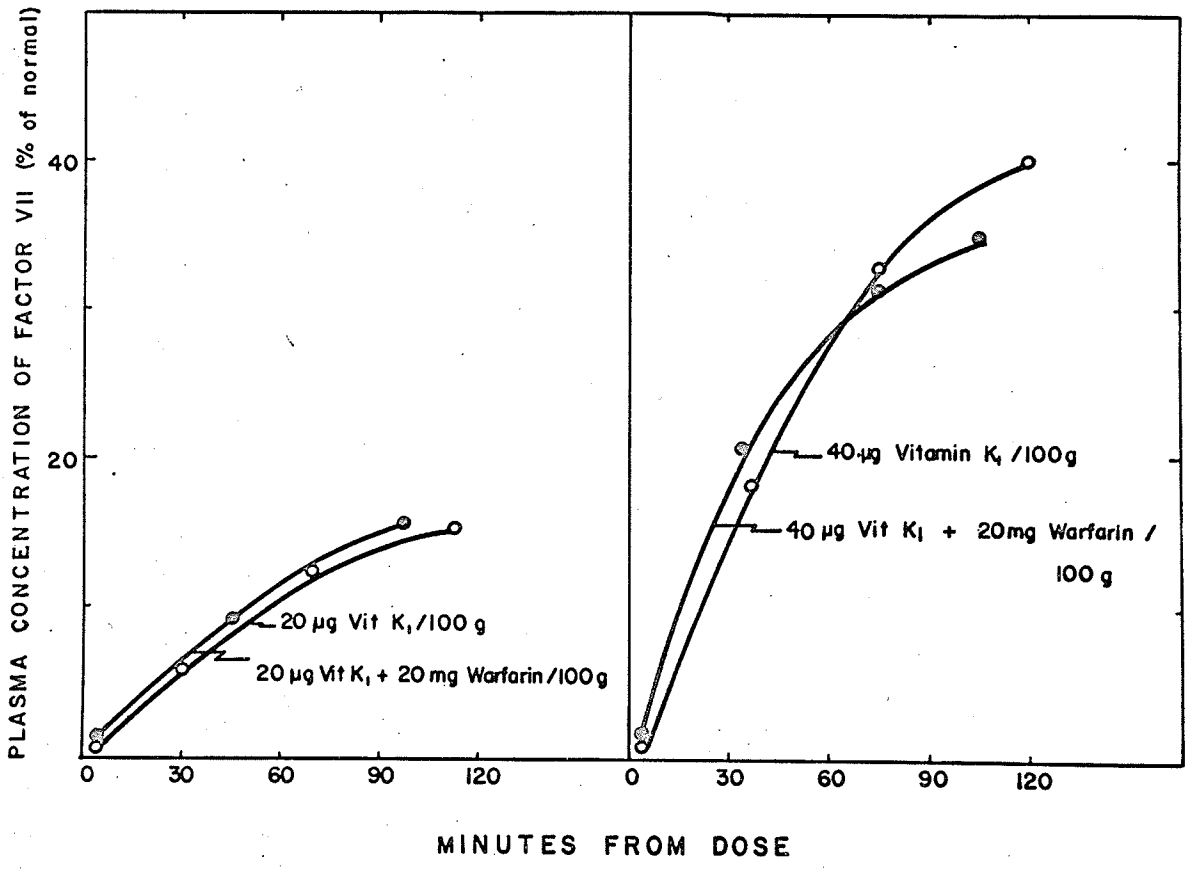


FIGURE 9

EFFECT OF SIMULTANEOUS ADMINISTRATION OF WARFARIN ON THE INCREASE OF THE PLASMA LEVEL OF FACTOR VII BY VITAMIN K_1 IN ANTICOAGULANT PRE-TREATED RATS.

IV. DISCUSSION

The experimental results obtained in the present investigation show that the antagonism between vitamin K_1 and coumarin anticoagulants is not of the classical competitive reversible type. In the coumarin pretreated animal simultaneous administration of warfarin at the highest dose tolerated had no effect on the activity of an optimal dose of vitamin K_1 as measured by the increase of the plasma level of factor VII. In contrast, when warfarin was administered with an optimal dose of vitamin K_1 in vitamin K deficient animals, depending on the dose of warfarin, the response was either partly or completely inhibited. When the various ratios of vitamin K_1 to warfarin were kept constant, but the doses increased, the inhibition increased and became complete for those ratios which produced partial inhibition, but when the doses were increased further the inhibition disappeared. Similarly for ratios that produced complete inhibition, an increase of the doses resulted in disappearance of the inhibition. Inspection of the data shows that for all the ratios tested the inhibition disappeared when the dose of vitamin K_1 was approximately 40 to 50 μg/100 g. The disappearance of the inhibition was therefore independent of the dose of simultaneously administered anticoagulant. Furthermore, the dose of vitamin K necessary to overcome the inhibition by simultaneously administered warfarin in vitamin K deficient animals was of the same order of magnitude as that necessary to reverse the anticoagulant effect in the warfarin pretreated animal.

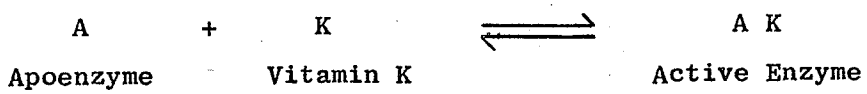
A tentative explanation of these findings can be given if it is assumed that coumarin anticoagulants block the normal mode or site of action of vitamin K_1 by an antagonism that is not of the competitive

reversible type, but is either of the competitive irreversible (non-equilibrium) or non-competitive type. However, at large doses, vitamin K_1 can act by an alternate site or mechanism that is not affected by coumarin anticoagulants and thus bypass the inhibition. The above explanation depends on the assumption that for all ratios tested the relative concentrations of vitamin K_1 and warfarin at the site of action remain constant when the doses are increased. To assume that these results could arise from a change of the ratio of the relative concentrations at the site of action would require that for low doses the concentration of warfarin increases faster than that of vitamin K_1, but reaches a limited value, while the concentration of vitamin K_1 continues to increase. This is considered to be unlikely and therefore the first explanation, constancy of the relative concentration at the site of action with increasing doses of constant ratios, is given preference. The fact that the dose of vitamin K_1 required to overcome the anticoagulant effect in the warfarin pretreated animal is of the same order of magnitude as that required to overcome the effect of simultaneously administered coumarin anticoagulant in the vitamin K deficient animal is also in agreement with this explanation. Results obtained from further studies on the mode of action of vitamin K_1 in this laboratory are also in agreement with and can be explained by an extension of the above scheme.

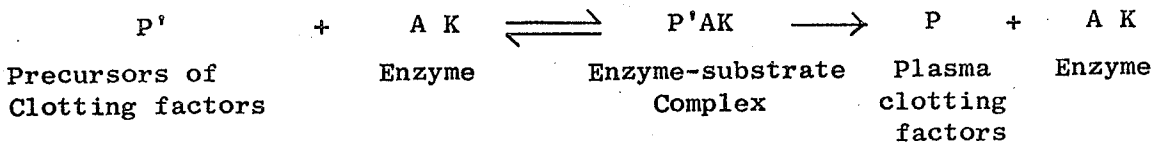
The present findings are supported by the results of Collentine and Quick (22), who in 1922 showed that simultaneous administration of dicoumarol blocked the response to vitamin K_1 in dogs made vitamin K deficient by cholecystnephrostomy. In this publication Quick drew attention to the rapid reappearance of prothrombin following intravenous

administration and postulated that since the reappearance is so rapid, vitamin K may be required as a cofactor or coenzyme of an enzyme system involved in the synthesis of prothrombin. This hypothesis can be restated as follows:

(a) Vitamin K first combines reversibly with a specific apoenzyme to form the active apoenzyme-coenzyme complex.



(b) The enzyme then transforms the precursors of the clotting factors via the formation of an enzyme-substrate complex into the clotting factors.



Quick assumed that dicoumarol and vitamin K were competing for the active site of the apoenzyme. However, no attempt was made to demonstrate competitive antagonism by the use of increasing doses of constant ratios of vitamin K_1 and dicoumarol.

These experiments were really the first unequivocal demonstration that coumarin anticoagulants antagonized directly the effect of vitamin K_1, and furthermore, they also demonstrated that the slow onset of the anticoagulant effect arises entirely from the slow rate of disappearance of the clotting factors present in the plasma at the time of administration and not from the transformation of the anticoagulants. However, the

significance of this observation was not appreciated and, in fact, this important paper appears to have been neglected by many workers who have reviewed the literature of the subject. While the mechanism of action of coumarin anticoagulants proposed by Quick has turned out to be not correct, structural analogues of vitamin K_1 have been synthesized in this laboratory which do act by this mechanism (64, 66).

In 1956 Babson and associates (8) tried to determine the nature of the antagonism by feeding constant ratios of vitamin K_1 and dicoumarol to normal rats. For any ratio the anticoagulant effect increased to a maximum, but as the doses were increased further, the effect of the dicoumarol was completely blocked and the prothrombin time returned to normal. The results indicated the antagonism was not of the competitive type. However, with such a long term experiment, other factors such as different rates of absorption, storage, and excretion, etc., may have had an effect on the response, and this makes it difficult to evaluate the significance of this experiment.

In 1953 and 1954, Martius and Nitz-Litzow (70, 71, 72) reported in a series of papers that mitochondria from vitamin K deficient chicks showed decreased P:O ratios (oxidative phosphorylation), and also that addition of dicoumarol to mitochondria from normal animals decreased P:O ratios. Martius therefore concluded that vitamin K was a component of the terminal electron transport chain necessary for the coupling of oxidation and phosphorylation, and that coumarin anticoagulants acted <u>in vivo</u> as uncoupling agents at this site. These findings introduced an entirely new approach to the study of the mode of action of

vitamin K_1 and coumarin anticoagulants at the cellular level. However, it now appears that the experimental findings and the conclusions based on them are open to serious doubt. Several workers have failed to observe decreased oxidative phosphorylation in mitochondria from vitamin K deficient animals (12, 86). Mitochondria from animals pretreated with anticoagulant doses of a coumarin anticoagulant or with a competitive antagonist of vitamin K have been shown also to give normal P:O ratios (42, 87). Furthermore, the presence of vitamin K_1 in mitochondria has not been demonstrated. Instead a benzoquinone, ubiquinone or coenzyme Q, has been found to be present in mitochondria. This quinone lacks vitamin K-like activity (23, 65). Furthermore, it is difficult to visualize, on one hand, why a defect as general as an interference with oxidative phosphorylation should be limited to such a specific effect as a decrease of a limited number of clotting factors. On the other hand, since there are many alternate sites for the generation of high energy phosphate bonds, which could possibly be required for the formation of the plasma clotting factors, it is equally difficult to visualize why the organism should be unable to supply the energy requirements for this transformation which is certainly only an insignificant part of its total energy requirement.

Martius tried to explain these contradictions by assuming that the anticoagulant effect arose from the high turn over rate of these particular proteins (73). It is however doubtful that this explanation can be correct since no other changes have ever been noted when anticoagulants are used in the long term treatment of patients. Nor can

the uncoupling action of coumarin anticoagulants when added in vitro be considered conclusive evidence for a similar action in vivo.

There is considerable evidence demonstrating that coumarin anticoagulants inhibit numerous enzyme systems in vitro, but enzyme activity from tissues of animals pretreated with large doses of coumarin anticoagulants is normal (42). Similar objections can be made to the demonstration of so-called quinone reductases, i.e., enzymes capable of reducing or oxidizing para-quinones, which are inhibited by coumarin anticoagulants (74, 75). These enzymes show little or no activity for vitamin K_1 but act on vitamin K_3, which as has already been mentioned, is not a naturally occurring vitamin. Furthermore this group of enzymes show little substrate specificity and can also reduce other oxidation-reduction acceptors of appropriate potential that are not related in chemical structure to vitamin K_1. It is therefore by no means certain that the specific substrate of these enzymes is vitamin K_1. Nor can inhibition by coumarin anticoagulants, in view of their general inhibitory activity, be considered evidence that vitamin K_1 is the specific substrate of these enzymes. According to Martius (73, 74) and Ernster (33, 37) vitamin K is not a component of the terminal electron transport system in the pigeon. However, addition of coumarin anticoagulants to mitochondria from normal pigeons uncoupled oxidative phosphorylation.

Although the possible role of vitamin K_1 in oxidative phosphorylation has continued to draw considerable attention among workers interested in electron transport and oxidative phosphorylation (21, 60, 104), such a role of vitamin K_1 has by no means been established, nor does the

experimental evidence available indicate how such a function could be related to the requirement of vitamin K_1 for blood coagulation.

Based on these findings Lasch and Roka (59) attempted to study the effect of mitochondria on the formation of plasma clotting factors and reported that mitochondria formed prothrombin in the presence of suitable substrates such as aged serum. Several workers have been unable to confirm these experiments and there appears little doubt that the findings were due to the action of mitochondrial cathepsins on plasma and serum proteins, while others must have arisen from technical errors in Lasch and Roka's determinations of the clotting factors (10, 11, 63, 88).

Pool et al., (88, 89) showed that liver slices synthesized plasma clotting factors in vitro and attempted to use this system for a study of the antagonism between coumarin anticoagulants and vitamin K_1. It was found that slices from coumarin pretreated animals or the addition of coumarin anticoagulants to normal slices inhibited plasma clotting factor synthesis. However, while in slices from coumarin treated animals protein synthesis as measured by incorporation of radio-active amino acids was normal, except for formation of plasma clotting factors, addition of coumarin anticoagulants to normal slices inhibited not only plasma clotting factor synthesis but all protein synthesis. Attempts to reverse the effect of anticoagulants in either slices from coumarin pretreated animals or after addition to normal slices, by vitamin K_1 failed. This can perhaps be attributed to failure to solubilize the fat soluble vitamin. Pool and Borchgrevink (89) discussed various possibilities and concluded that there was no satisfactory explanation for this discrepancy between the effect of coumarin anticoagulants in vivo

and *in vitro*.

While the present results do not give any precise information of the mode of action of vitamin K_1, they clearly demonstrate the non-competitive nature of the antagonism between coumarin anticoagulants and vitamin K. However, although the antagonism is of the non-competitive type, the inhibition can always be overcome by large doses of vitamin K because of the existence of an alternate pathway or mechanism. From a review of the literature it can be seen that the existence of such an alternate pathway has not been recognized previously. Indeed, the ready reversibility of the anticoagulant effect by vitamin K_1 has generally been taken to indicate that the antagonism is of the competitive type. In any further study of this problem it will be necessary to take these two factors, the non-competitive nature of the antagonism and the existence of an alternate pathway, into consideration.

Further work in our laboratory on the mode of action of vitamin K has been in agreement with the explanation proposed here, and indicates that the normal site of action presents a specific transport site for the transfer of vitamin K which is inhibited by coumarin anticoagulants, while the alternate mechanism involves passage by diffusion at larger doses (67).

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A P P E N D I X

TABLE II

INCREASE OF PLASMA LEVELS OF FACTOR VII, PROTHROMBIN, AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF 0.5 μg VITAMIN K_1/100 g IN THE VITAMIN K DEFICIENT RAT.

Time	Factor VII			Prothrombin			Factor X		
minutes	seconds		percent	seconds		percent	seconds		percent
0	77.2	77.8	13.5	56.2	56.2	4.4	18.2	18.6	15.5
20	68.4	68.7	19.0	54.2	55.4	9.6	16.6	18.4	21.0
45	57.6	58.2	28.5	36.0	37.8	40.0	14.4	15.0	38.0
67	51.4	51.4	38.0	32.8	35.6	52.0	14.4	14.8	45.0
102	47.7	46.2	48.0	32.8	32.8	59.0	13.4	13.6	58.0
157	41.1	42.6	58.0	31.0	32.0	66.0	13.4	13.8	60.0

(Figure 2a)

TABLE III

INCREASE OF PLASMA LEVELS OF FACTOR VII, PROTHROMBIN AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF 5.0 μg VITAMIN K_1/100 g IN THE VITAMIN K DEFICIENT RAT.

Time	Factor VII			Prothrombin			Factor X		
minutes	seconds		percent	seconds		percent	seconds		percent
0	98.4	98.4	8.2	70.6	72.2	4.0	20.6	20.2	11.5
20	89.8	88.4	11.0	58.0	61.0	7.2	17.8	18.2	19.0
45	58.0	60.6	25.0	39.8	37.0	35.0	15.7	15.9	31.0
70	54.0	56.6	32.0	34.0	35.6	44.0	14.2	14.8	43.0
100	52.4	52.6	36.0	34.2	35.2	51.0	13.8	13.9	51.0
160	45.2	44.6	53.0	31.4	32.4	60.0	13.5	13.6	56.0

(Figure 2b)

TABLE IV

INCREASE OF PLASMA LEVEL OF FACTOR VII BY SUPEROPTIMAL GRADED DOSES OF VITAMIN K_1 IN VITAMIN K DEFICIENT RATS

Rat	Vitamin K_1 Dose µg/100 g	Time minutes	Factor VII seconds		Factor VII percent
296	50.0	0	199.5	200.3	3.1
		36	91.3	95.6	14.5
		65	72.2	71.4	25.0
		108	54.2	56.4	42.0
450	40.0	0	214.0	208.4	2.8
		37	93.6	87.8	15.8
		65	70.6	69.6	26.0
		94	64.4	62.8	31.0
		120	60.4	61.6	35.0
476	20.0	0	199.0	203.8	3.0
		35	82.6	79.8	19.5
		63	62.6	64.6	32.0
		92	57.8		38.0
		121	52.8	53.0	46.0
468	10.0	0	294.0	289.0	1.5
		30	117.8	119.8	9.0
		62	83.4	83.4	18.5
		94	66.2	67.6	28.5
		124	62.7	60.0	35.0
470	5.0	0	155.6	151.4	5.3
		30	90.4	88.6	16.0
		60	71.2	71.0	25.5
		93	65.8	66.0	29.2
		121	53.6	53.8	45.0
134	2.5	0	160.8	154.4	8.0
		33	112.6	116.6	16.0
		62	81.0	83.6	30.0
		142	64.4	62.8	50.0
455	0.5	0	172.0	168.0	9.9
		20	138.0	140.2	13.0
		40	93.5	85.7	21.1
		70	61.0	58.4	39.6
		100	57.0	56.8	44.6
		125	56.4	54.6	47.0

(Figure 3)

Table V

INCREASE OF PLASMA LEVEL OF FACTOR VII BY SUBOPTIMAL GRADED DOSES OF VITAMIN K_1 IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 Dose µg/100 g	Time minutes	Factor VII seconds		Factor VII percent
455	0.5	0	172.0	168.0	9.9
		40	93.5	85.7	21.1
		70	61.0	58.4	39.6
		100	57.0	56.8	44.6
		125	56.4	54.6	47.0
417	0.25	0	200.2	200.4	9.5
		30	184.4	178.4	11.2
		88	102.2		22.0
		105	91.0	87.0	25.5
		140	88.8	89.4	27.0
415	0.062	0	147.0	148.6	9.4
		29	143.4	138.8	10.5
		70	132.2	127.6	12.0
		105	121.6	120.5	13.6
		135	96.6	96.0	19.8
342	0.016	0	170.6	176.6	7.2
		30	167.0	163.4	7.7
		66	165.4	168.2	7.8
		101	144.4	148.4	9.4
		135	140.2	144.2	10.0

(Figure 4)

TABLE VI

EFFECT OF INCREASING DOSES OF WARFARIN ADMINISTERED SIMULTANEOUSLY WITH 0.5 μg VITAMIN K_1/100 g ON THE INCREASE OF THE PLASMA LEVEL OF FACTOR VII IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 μg/100 g	Warfarin μg/100g	Time minutes	Factor VII seconds		percent
455	0.5		0	172.0	168.0	9.9
			40	93.5	87.5	21.1
			70	61.0	58.4	39.6
			100	57.0	56.8	44.6
			125	56.4	54.6	47.0
297	0.5	5.0	0	242.8	250.0	9.4
			30	205.7	214.2	11.5
			63	145.6	140.8	17.0
			90	116.8	121.6	21.0
			120	99.6	102.6	25.0
331	0.5	10.0	0	122.0	128.0	13.0
			30	126.2	132.8	12.5
			60	128.4	136.4	12.2
			90	112.0	115.6	15.0
			120	103.0	99.4	17.0
330	0.5	25.0	0	159.6	170.0	14.5
			30	164.6	168.0	14.5
			60	183.8	190.0	12.5
			90	182.0	186.2	12.4
			120	176.8	177.4	13.5
296	0.5	50.0	0	364.6		6.4
			30	362.2		6.4
			60	379.4		6.0
			100	379.8		6.0
			130	293.6		7.8
183		5.0	0	93.5	92.0	13.0
			50	92.6	92.2	13.0
			114	96.6	98.0	11.7
			165	105.6	104.3	10.0

(Figure 5)

TABLE VII

EFFECT OF CONSTANT DOSE RATIO OF VITAMIN K_1 AND WARFARIN (1 : 10) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 µg/100 g	: Warfarin µg/100 g	Time minutes	Factor VII seconds		percent
477	0.5	5.0	0	203.0	206.3	3.0
			30	142.6	144.8	6.1
			69	85.4	88.9	16.5
			96	83.6	82.3	17.5
			135	76.2	73.8	22.5
448	2.5	25.0	0	195.0	197.0	3.25
			30	161.4	164.5	4.6
			75	131.2	133.8	7.2
			94	149.8	147.8	5.7
			140	138.8	139.8	6.5
425	5.0	50.0	0	234.8	239.2	2.2
			30	214.0	212.2	2.7
			65	182.2	188.0	3.7
			96	179.2	182.6	3.9
			135	176.8	178.0	4.0
432	50.0	500.0	0	212.0	210.8	2.8
			30	106.4	100.8	12.0
			67	76.2	74.8	22.5
			96	71.2	69.6	26.0
			139	63.6	61.8	33.0

(Figure 6a)

TABLE VIII

EFFECT OF CONSTANT DOSE RATIO OF VITAMIN K_1 AND WARFARIN (1 : 10) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 µg/100 g	: Warfarin ug/100 g	Time minutes	Factor VII seconds		percent
297	0.5	5.0	0	242.8	250.0	9.4
			30	205.7	214.2	11.5
			63	145.6	140.8	17.0
			90	116.8	121.6	21.0
			120	99.6	102.6	25.0
406	2.5	25.0	0	100.0	102.4	12.5
			30	100.4	100.6	12.5
			60	103.6	98.8	12.8
			92	102.4	104.6	12.0
			123	95.4	94.2	14.0
432	5.0	50.0	0	169.2	173.8	4.2
			30	175.8	173.8	4.1
			60	145.0	150.8	5.7
			90	144.0	146.4	6.0
			120	141.6	143.8	6.2
433	50.0	500.0	0	144.2	142.6	6.1
			30	110.4	116.0	10.1
			63	76.2	77.5	21.5
			90	65.4	65.4	29.5
			120	58.6	56.8	38.5

TABLE IX

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1 : 20) IN VITAMIN DEFICIENT RATS.

Rat	Vitamin K_1 : Warfarin μg/100 g	Time minutes	Factor VII seconds		percent
331	0.5 : 10.0	0	122.0	128.0	20.0
		30	126.2	132.8	19.0
		60	128.4	136.4	18.5
		90	112.0	115.6	22.0
		120	103.0	99.4	25.0
		190	96.4	100.4	26.0
405	5.0 : 100.0	0	240.4	237.0	4.4
		30	217.4	214.8	5.2
		60	244.8	238.8	4.4
		90	230.0		4.0
		125	217.8		5.2
		190	287.0		3.3
296	10.0 : 200.0	0	94.4	91.6	21.0
		30	85.0	88.8	23.5
		60	95.2	96.4	20.5
		88	96.8	98.4	19.5
353	20.0 : 400.0	0	230.8	233.8	4.3
		32	197.4	192.2	5.7
		58	187.6	193.2	6.0
		87	154.6	146.8	9.2
		120	166.6	165.0	7.6
330	50.0 : 1,000.0	0	283.2	280.8	3.3
		30	126.2	129.0	12.5
		60	97.0	93.6	20.0
		95	87.5	82.6	23.0
		128	81.6	82.6	25.5
		190	74.4	72.0	29.0
351	500.0 : 10,000.0	0	214.8	219.2	5.2
		30	106.4	102.2	17.5
		60	92.4	85.4	22.0
		95	79.0	77.8	27.0
		130	81.2	80.6	26.0
		190	69.2	74.6	31.5

(Figure 6b)

TABLE X

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1 : 20) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 : Warfarin µg/100 g	Time minutes	Factor VII seconds		Factor VII percent
311	0.5 : 10.0	0	156.0	154.5	6.6
		30	148.0	147.4	7.5
		60	138.5	138.0	8.5
		90	112.0	114.0	12.0
		120	89.0	89.6	18.0
406	5.0 : 100.0	0	270.0	268.6	2.7
		30	239.8	240.6	3.3
		60	248.0	250.0	3.1
		95	234.0	236.0	3.4
		125	224.0	226.0	3.7
329	50.0 : 1,000.0	0	166.8	168.0	6.2
		30	90.0	91.8	17.0
		60	69.0	69.8	27.5
		90	66.0	68.0	28.5
		120	66.2	66.0	30.0
350	500.0 : 10,000.0	0	250.2	252.2	3.0
		30	91.2	92.4	17.5
		60	72.0	72.6	26.0
		90	67.6	68.2	29.0
		120	63.8	64.4	31.0

TABLE XI

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1 : 50) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 : Warfarin µg/100 g	Time minutes	Factor VII seconds		Factor VII percent
331	0.5 : 25.0	0	395.0	392.0	2.0
		30	326.6	327.2	2.6
		60	299.0	299.0	3.1
		93	291.2	294.6	3.2
		127	272.0	276.6	3.6
		180	286.8	284.2	3.3
342	5.0 : 250.0	0	109.8	112.6	15.5
		30	112.2	116.4	15.0
		60	112.8	115.4	15.0
		92	111.2	107.0	16.0
		128	114.8	115.6	15.0
		180	128.4	129.4	12.0
344	50.0 : 2,500.0	0	235.6	239.6	4.5
		30	159.0	154.8	8.8
		60	108.4	110.6	16.0
		87	100.6	102.6	18.0
		123	94.8	92.2	20.5
		180	90.8		21.5
353	500.0 : 25,000.0	0	165.0	157.6	8.4
		27	119.0	119.8	14.0
		60	89.8	97.0	20.0
		89	89.4	89.2	22.5
		123	74.2	74.3	30.0
		180	65.6	64.0	37.5

(Figure 6c)

TABLE XII

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1:50) IN IN VITAMIN K DEFICIENT RATS

Rat	Vitamin K_1 : Warfarin μg/100 g	Time minutes	Factor VII seconds		percent
299	0.5 : 25.0	0	202.0	208.0	5.2
		30	199.3	190.8	5.7
		60	184.2	180.2	6.3
		130	195.4	191.0	5.9
343	5.0 : 250.0	0	94.6	95.4	16.5
		30	91.6	92.2	17.0
		60	89.0	90.0	18.0
		92	93.6	94.6	16.5
		125	95.2	96.6	16.0
296	50.0 : 2,500.0	0	215.0	224.0	4.7
		30	120.0	110.0	14.5
		60	85.2	87.0	24.0
		130	67.4	69.2	37.0

TABLE XIII

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1 : 100) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 µg/100 g	: Warfarin µg/100 g	Time minutes	Factor VII seconds		percent
296	0.5	50.0	0	364.6	350.5	6.4
			30	363.2		6.2
			60	379.4		6.0
			100	379.8		6.0
			130	293.6		7.8
			180	279.0	270.0	8.4
			260	377.0	382.0	6.0
330	5.0	500.0	0	211.0	214.6	4.9
			38	187.6	181.0	6.4
			60	169.8	175.4	7.2
			90	182.0	187.0	6.3
			120	243.0	244.1	3.9
			190	243.8	238.4	4.3
350	50.0	5,000.0	0	409.0	419.0	1.5
			35	140.2	124.4	11.0
			62	100.6	98.2	18.0
			90	84.4	88.4	24.0
			120	78.4	81.6	27.5
			190	77.4	78.2	29.0

(Figure 6d)

TABLE XIV

EFFECT OF CONSTANT DOSE RATIO VITAMIN K_1 AND WARFARIN (1 : 500) IN VITAMIN K DEFICIENT RATS.

Rat	Vitamin K_1 µg/100 g	: Warfarin µg/100 g	Time minutes	Factor VII seconds		percent
330	0.5	250.0	0	187.8	186.4	6.2
			33	185.4	187.0	6.2
			64	191.4	186.8	6.1
			92	200.2	200.8	5.4
			125	205.8	200.0	5.3
296	20.0	10,000.0	0	208.6	205.0	5.2
			31	163.0		6.0
			63	137.8		10.5
			93	135.4	136.6	11.0
			121	152.8	145.2	9.3
			198	153.4	155.6	8.6
351	40.0	20,000.0	0	130.6	130.2	11.5
			32	109.4	105.2	16.5
			65	93.2	93.0	24.0
			95	91.4	94.0	24.0
			123	83.6	84.8	25.0
			187	69.8	69.2	35.5

(Figure 6e)

TABLE XV

INCREASE OF PLASMA LEVEL OF FACTOR VII BY GRADED DOSES OF VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS.

Rat	Vitamin K_1 µg/100 g	Time minutes	Factor VII seconds		percent
401	60.0	0	543.0	548.0	1.0
		40	96.4	99.0	19.2
		68	74.6	74.4	32.0
		120	69.8	67.2	36.0
398	40.0	0	424.0	442.0	1.4
		38	98.8	101.8	18.8
		70	70.4	72.0	33.8
		125	63.4	63.6	41.0
375	20.0	0	606.0	594.0	1.0
		37	175.2	180.0	7.0
		70	131.2	138.8	11.0
		107	111.8	107.8	16.0
		137	103.8	107.0	18.0
		190	85.6	86.6	25.0
309	10.0	0	296.6	301.2	1.1
		57	167.9	166.3	4.2
		147	130.8	126.8	8.0
345	5.0	0	315.0	310.0	0.9
		65	299.4	290.0	0.9
		159	258.8	254.2	1.0
		250	319.2	319.4	0.9

(Figure 7)

TABLE XVI

INCREASE OF FACTOR VII, PROTHROMBIN, AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF 40.0 μg VITAMIN K_1/100 g IN THE ANTICOAGULANT PRETREATED RAT.

Time	Factor VII			Prothrombin			Factor X		
minutes	seconds		percent	seconds		percent	seconds		percent
0	424.0	442.0	1.4	48.0	47.6	19.5	24.8	23.4	2.0
15	285.0	286.0	2.4	43.8	43.8	23.0	21.6	21.4	3.5
30	98.8	101.8	18.8	40.8	39.8	36.0	16.0	16.4	16.0
60	70.4	72.0	33.8	36.4	36.2	46.0	14.8	15.0	23.0
120	63.4	63.6	41.0	34.0	35.0	55.0	13.8	13.6	34.0

(Figure 8)

TABLE XVII

INCREASE OF FACTOR VII, PROTHROMBIN, AND FACTOR X AFTER A SINGLE INTRAVENOUS DOSE OF 40.0 μg VITAMIN K_1/100 g IN THE ANTICOAGULANT PRETREATED RAT.

Time	Factor VII			Prothrombin			Factor VII		
minutes	seconds		percent	seconds		percent	seconds		percent
0	435.0		1.0	46.0	46.6	19.5	24.8	23.4	2.0
30	118.0		9.0	43.8		23.0	18.4		7.2
60	72.0	73.8	26.0	37.8	38.8	35.0	14.8	15.0	23.0
128	58.8	60.6	36.0	34.7	36.2	44.0	14.0	14.8	27.0

TABLE XVIII

EFFECT OF SIMULTANEOUS ADMINISTRATION OF WARFARIN ON THE INCREASE OF FACTOR VII BY VITAMIN K_1 IN ANTICOAGULANT PRETREATED RATS.

Rat	Vitamin K_1 : μg/100 g	Warfarin μg/100 g	Time minutes	Factor VII seconds		percent
366	40.0	20.0	0	385.0	395.0	1.6
			35	96.5	91.2	21.0
			70	73.8	74.1	32.0
			112	68.0	69.0	35.0
398	40.0		0	424.0	442.0	1.4
			38	98.8	101.8	18.8
			70	70.4	72.0	33.8
			125	63.4	63.6	41.0
376	20.0	20.0	0	619.4	641.4	1.0
			45	151.4	135.8	9.0
			105	128.2	127.0	15.0
421	20.0		0	657.0	660.0	1.0
			30	176.4	185.8	6.6
			72	124.2	116.8	13.5
			120	108.6	109.6	16.2
377	20.0	20.0	0	545.0		1.0
			40	107.6	108.4	16.2
			100	98.6	100.4	18.0
			160	113.2	111.8	16.5

(Figure 9)