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DIFFERENCE OF ACTIVITY BETWEEN CIS AND TRANS VITAMIN K_1

A COMPARISON OF THE ACTIVITY OF THE CIS- AND TRANS- ISOMERS OF VITAMIN K_1

by

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ABSTRACT

Vitamin K_1 has been separated by TLC into its $\Delta^{2',3'}$ - trans and cis isomers to a purity of better than 99% as indicated by TLC and NMR. The activities of the two isomers have been compared in vit. K deficient and coumarin anticoagulant pretreated rats. In vit. K deficient rats the cis isomer had 1.5% of the potency of the trans isomer with 5 percent fiducial limits at 1.1% and 1.9%. In coumarin pretreated rats the cis isomer had 11% of the potency of the trans isomer with 5 percent fiducial limits at 9% and 13%. Comparable results were obtained after addition of the two isomers to liver slices and microsomes from vit. K deficient rats.

Catalytic reduction of the 2',3'-double bond leaves the activity of the trans isomer unchanged, but increases that of the cis isomer to that of the trans isomer.

The results can be explained by the hypothesis that the mechanism of action of vit. K involves proton transport across a lipophilic membrane structure, and that the difference between the activity of the trans and cis isomers arises from the different spatial orientation of the phytyl side chain.

CONDENSE

La vitamine K_1 a été séparée par CCM (chromatographie sur couche mince) en ses isomères $\Delta^{2',3'}$-trans et cis, à un degré de pureté supérieur à 99%, comme cela a été démontré par CCM et RMN. L'activité de chaque isomère a été comparée chez des rats déficients en vitamine K ainsi que chez des rats préalablement traités avec des anticoagulants de type coumarinique. Chez les rats déficients en vitamine K l'activité de l'isomère cis n'est que 1.5% de celle de l'isomère trans, admettant comme limites de confiance 1.1% et 1.9%, avec 5% de certitude. Chez les rats préalablement traités avec des anticoagulants de type coumarinique, l'isomère cis n'a que 11% de l'activité de l'isomère trans, admettant comme limites de confiance 9% et 13%, avec 5% de certitude. Des résultats semblables ont été obtenus après l'addition de chaque isomère à des tranches de foie ainsi qu'à des fractions microsomiques provenant de rats déficients en vitamine K.

La réduction catalytique de la liaison double située à la position 2',3', ne change pas l'activité de l'isomère trans, mais augmente celle de l'isomère cis à la valeur de l'activité de l'isomère trans.

Les résultats peuvent être expliqués admettant l'hypothèse que la vitamine K participe au phénomème de déplacement de protons à travers une membrane lipophile, et que la différence entre l'activité de l'isomère trans et celle de l'isomère cis provient de la différente orientation spatiale du radical phytyl.

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INTRODUCTION

Mechanism of blood coagulation

The molecular basis of blood coagulation is the transformation of a soluble plasma protein, fibrinogen, to an insoluble protein, fibrin. This transformation is initiated by the action of an enzyme, thrombin, whereby two pairs of small polypeptides (fibrinopeptides A and B) are split from the fibrinogen molecule (1,2), thus removing most of the charges as well as changing the conformation of the fibrinogen molecule. The resulting fibrin monomer aggregates spontaneously to form an insoluble fibrin polymer. By the action of another enzyme, factor XIII (clot stabilizing factor) covalent bonds are formed between γ glutamyl and ε lysine residues of adjacent fibrin monomers (3,4). It is this last reaction which gives to the blood clot its elasticity and tensile strength.

Normally, blood does not coagulate because thrombin is present in an inactive form as prothombin or factor II. Under physiological conditions the activation of prothrombin to thrombin only occurs when coagulation is required to prevent loss of blood resulting from damage to the cardiovascular system. There are two mechanisms by which this activation can be initiated through the sequential activation of a number of plasma protein clotting factors (5,6).

In the first mechanism factor XII becomes activated on contact with damaged endothelium (7,8), the active factor XII then activates factor XI, which in turn activates factor IX (9). Active factor IX then forms a complex with factor VIII and factor X on the aqueous interphase of phospholipid derived from the simultaneous disintegration of platelets (platelet factor 3). The formation of this complex is also dependent

on the presence of calcium ion and results in the activation of factor X (10,11, 12,13). Active factor X forms a similar complex with factor V and factor II to activate factor II to thrombin (14,15,16,17,18).

In the second mechanism, tissue fragments enter the blood after injury and, in the presence of calcium ion, activate factor VII. The particular tissue component which activates factor VII, known as thromboplastin, is thought to be a phospholipid rich fragment derived from the plasma membrane or endoplasmic reticulum (19). Active factor VII in turn activates factor X (10). Active factor X interacts with factor V and factor II to form a complex held together by calcium ion on the aqueous interphase of thromboplastin. This leads to the transformation of factor II to thrombin.

The two mechanisms, therefore, converge in their final reaction and differ only in the manner by which factor X is activated and in the nature of the phospholipid involved. The first sequence of activation is called the intrinsic pathway or mechanism, since all of its components are derived from the plasma, while the second is called the extrinsic pathway or mechanism, because one of the components, thromboplastin, is derived from outside the plasma. Fig. 1 gives a summary of the sequence of reactions.

The sequential activation results in the amplification of the initial stimulus to produce a rapid rate of thrombin formation. For coagulation to occur, the rate of formation as well as the amount of thrombin formed are important. Free thrombin will be available for action on fibrinogen only if its rate of formation is greater than its rate of inactivation by antithrombin. On the other hand, this mechanism may also serve to

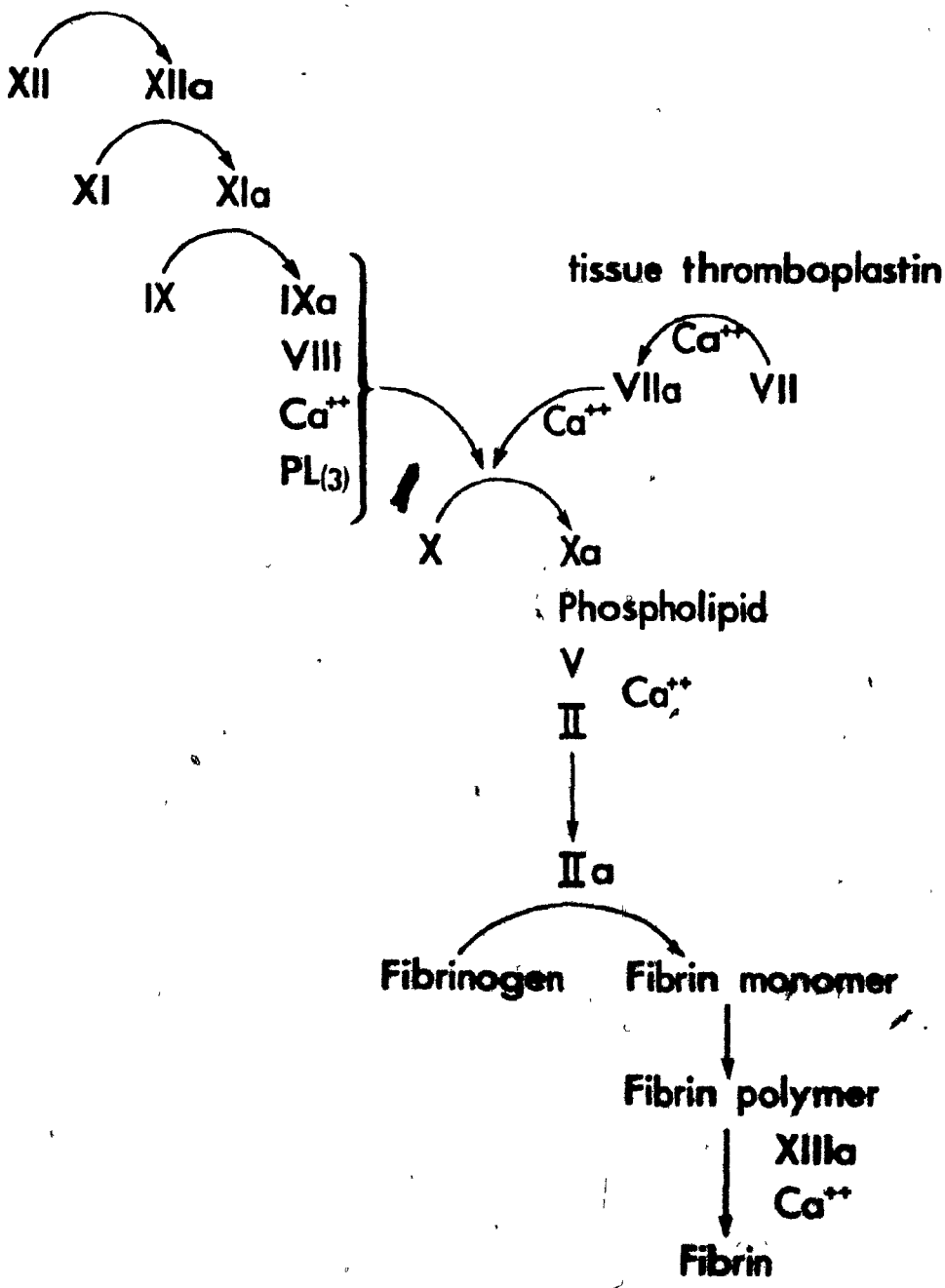


Fig. 1. Cascade mechanism of blood coagulation.

filter out weak initial stimuli under conditions where coagulation does not serve a physiological purpose. The sequential, cascade or waterfall-like activation of a series of plasma proteins appears to be a general mechanism by which plasma kinases like the plasminogen, the kallikrein, and the complement system are activated.

Until recently, most of the research in blood coagulation was descriptive or phenomenological. It was only after techniques for the isolation and purification of minor plasma proteins had been developed that the biochemical basis of the clotting mechanism could be studied. Four of the clotting factors (factors II, VII, IX and X) have been shown to be serine proteases (20,21,22). These four factors have certain common properties; they are present in plasma as inactive precursors, they have a high capacity to bind calcium ions and they can be removed from plasma by adsorption on barium sulfate. After activation they can also hydrolyse simple esters of basic amino acids, like arginine and lysine, and they are inhibited by diisopropyl fluoro phosphate (DFP). In addition, one of the fat soluble vitamins, vit. K, is required for their formation, since in its absence their activities in the plasma decrease. Partial amino acid sequence analysis indicates that the four clotting factors show a high degree of homology and it has been suggested that these proteases have evolved from a common ancestral gene by gene duplication and translocation (20).

The best characterized of these four factors is prothrombin because, compared to the others, it is present in plasma in relatively high concentrations (10-15 mg/100 ml) and, therefore, its isolation and

purification are comparatively easy. Prothrombin is a single chain glycoprotein with a molecular weight of 68,000 - 72,000 with an N-terminal alanine (23,24,25). It has recently been shown that the calcium binding capacity is localized at the N-terminal of the peptide and is due to the presence of a new tricarboxylic amino acid γ carboxyglutamic acid, of which there seem to be present from 10 to 12 units (26,27,28). During activation by active factor X (Xa), prothrombin is cleaved into two parts; they are prethrombin 2, which carries the masked active site, and fragment 1:2, which carries the calcium binding sites (29,30). Factor Xa also activates prethrombin to thrombin by cleavaging into an A and a B chain with simultaneous unmasking of the active site, the two chains being held together by a disulfide bond. The amino acid sequence of the B chain of thrombin, which contains the active site, shows a considerable homology with other serine proteases (28). The various steps of prothrombin activation are summarized in Fig. 2.

Active factor X, the enzyme responsible for the cleavage of prothrombin, is a two chain serine protease (mol. wt. 46,000) held together by a disulfide bond(s) and is derived from its inactive precursor, factor X, by either the intrinsic or extrinsic coagulation system. During activation a peptide is split from the N-terminal of the heavy chain to unmask the serine active center (10). The activation of factor X is, therefore, similar to that of the prothrombin with one important difference, the calcium binding sites which are on the light chain do not dissociate during the activation process.

Factor V is a high molecular weight (about 290,000) cofactor or

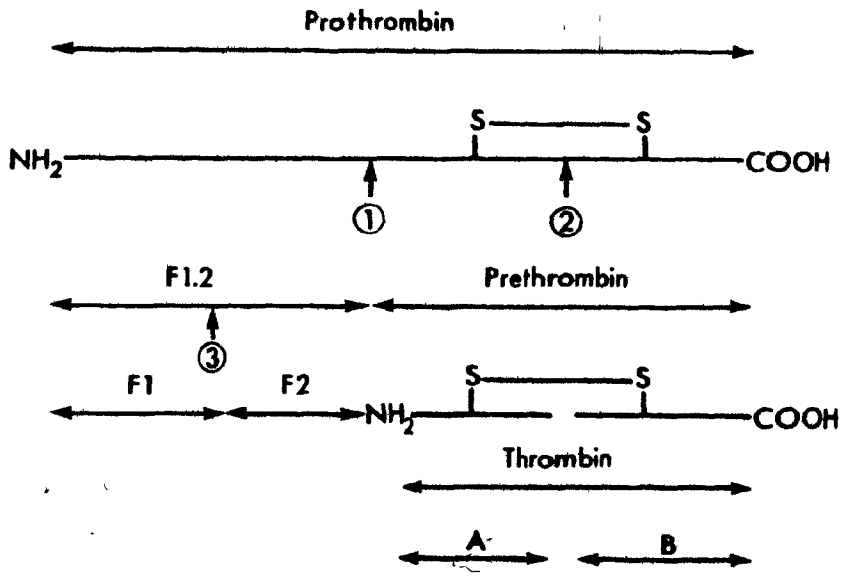


Fig. 2. Schematic outline of prothrombin activation.

1 and 2 sites of action of factor Xa on prothrombin.

3 site of action of factor II on F1.2 fragment.

regulatory protein which facilitates the interaction between factor Xa and prothrombin (15,17,29). The other components of the prothrombin-factor X activation complex are phospholipid and calcium ions. The function of the phospholipid is to provide a surface onto which the proteins are bound (15). Prothrombin and factor Xa are bound to the phospholipids through their calcium binding sites, while the interaction between factor V and phospholipids does not require calcium ion.

The activation of prothrombin is further complicated by the fact that the product of the activation, thrombin, can degrade prothrombin into prethrombin 1 and fragment 1 and can degrade fragment 1:2 into fragment 1 and fragment 2 (30). Whether these reactions occur under physiological conditions is at present not certain.

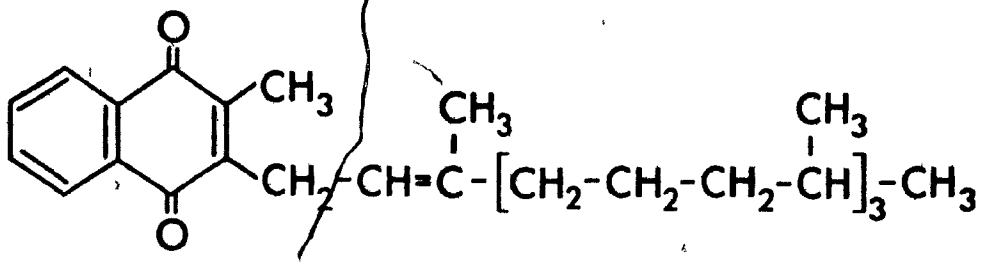
The molecular basis for the initial reaction of the intrinsic system, activation of factor XII on contact with collagen or damaged endothelium (contact activation), is only partly known; factor XIIa converts factor XI to an enzyme (Factor XIa) which activates factor IX (31). The complete structure of factor IX is not known, but appears to be similar to that of factor VII, factor X and prothrombin (20).

Vitamin K

The signs of vit. K deficiency were originally observed in 1929 by Dam (32,33) who noticed bleeding and slow clotting of the blood in chicks kept on an almost lipid free diet. Similar results were reported by McFarlane (34). Dam showed that the symptoms did not appear when the diet was supplemented with large amounts of cereals, hemp seeds, and similar vegetable sources (35), but not by any of the then known vitamins or dietary factors. Dam, therefore, assumed the existence of a new fat soluble vitamin necessary for normal blood coagulation which he called vit. K (in German Koagulation Vitamin, (36)). In 1936 Schönheyder at Dam's Institute was able to trace indirectly the coagulation defect to a lack of plasma prothrombin (37).

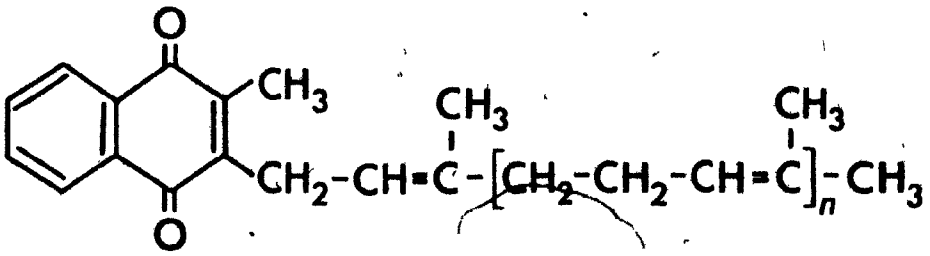
Almquist et al (38) showed that the vitamin can also be formed by bacterial putrification of fish meals, and that in addition coprophagy could serve as a source of the vitamin because of the formation of vit. K by the intestinal flora (39). With this information Dam in collaboration with the Swiss chemist Karrer undertook the isolation and chemical identification of the vitamin from alfalfa and reported the partial characterization of a yellow oil with high activity (40). In the same month, Doisy et al reported isolation of the same oil from alfalfa and of a crystalline substance with similar activity from putrefied fish meal. In May of 1939, Doisy's group identified the oily substance from alfalfa as vit. K_1 and the crystalline substance from putrefied sardine meal as vit. K_2 (41,42). Doisy's group established the structure of vit. K_1 as 2-methyl-3-phytyl-1, 4-naphthoquinone (I). This structure was confirmed by chemical synthesis in several labora-

tories (43,44,45).



I

The structure of vit. K_2 was shown by Doisy (46) to be 2-methyl-3-difarnesyl-1,4-naphthoquinone (II). Later studies by Isler et al showed that vit. K_2 was predominantly 2-methyl-3-digeranyl farnesyl-1, 4-naphthoquinone (47,48).



II, $n=5$
IIa, $n=6$

The two vitamins differ, therefore, only in the number of carbon atoms and the degree of unsaturation of the aliphatic side chain at the 3-position. The nomenclature of biological quinones has been standardised by the International Union of Biochemistry (IUB). According to this

nomenclature the official name of vit. K_1 is phylloquinone, and vit. K_2 menaquinone, with a subscript indicating the number of isoprenoid units of the side chain at the 3-position, for example vit. $K_{2(5)}$ or menaquinone$_{(5)}$, denotes vit. K_2 with 30 carbon atoms or with 6 isoprenoid units (49).

In man and most other animals a simple dietary vitamin deficiency does not occur. Even if it is not supplied in the diet, synthesis by intestinal flora can serve as a source of the vitamin. In some animals, such as chicks or rats, absorption from the intestine is insufficient and vit. K deficiency will develop after feeding a vit. K deficient diet and preventing coprophagy. Vit. K deficiency can occur as a result of failure of intestinal absorption, either because of the absence of bile, which is required for the saponification of the vitamin, or in certain malabsorption syndromes. The first demonstrations of the effect of vit. K in humans, were carried out in 1937 and 1938 (50,51) when oral ingestion of concentrates from alfalfa or even putrefied fish meal was used to treat patients with hemorrhagic diathesis due to jaundice. Vit. K deficiency can also be produced in animals by diverting the secretion of bile into the intestine (52,53). Inhibition of bacterial synthesis by antibiotics or sulfonamide drugs has been reported to produce vit. K deficiency (54,55). Germ-free animals also show vit. K deficiency (56). Newborns, particularly premature newborns, frequently show a bleeding tendency which has been attributed to vit. K deficiency because of insufficient bacterial synthesis.

In 1941 Campbell and Link isolated and identified the substance

responsible for the clotting defect produced by feeding animals with spoiled sweet clover, dicoumarol (3'3-methyl'bis'(4-hydroxy-coumarin)) (57,58). Dicoumarol was found to produce a coagulation defect identical with that of vit. K deficiency and, moreover, the defect could be reversed by vit. K_1 (59).

With a better understanding of the coagulation mechanism a more precise definition of the clotting defect produced by vit. K has become possible; it is now known that vit. K specifically lowers the plasma activity not only of prothrombin but also of factors VII, IX and X. At the same time, none of the other components of the clotting system like fibrinogen and factors V, VIII, XI and XII are directly affected. The effect of vit. K seems to be limited to the coagulation system, since no other symptoms or defect have been directly related to K deficiency.

Mechanism of action of vitamin K

Addition of vitamin K to blood from normal or vit. K deficient animals was found to have no effect on coagulation (60). Repeated attempts to demonstrate the presence of vit. K in the prothrombin molecule were likewise unsuccessful or inconclusive (61). An intracellular site of action was indicated by the experiment of Lupton (62) who demonstrated that the perfusion of blood from coumarin anticoagulant treated animals through livers from normal animals increased the prothrombin level, while by perfusion through livers from coumarin anticoagulant pretreated animals had no effect.

The first suggestion of a possible mechanism of action of vit. K at the molecular level was made by Martius in 1954 (63), when he reported that mitochondria from vit. K deficient chicks showed decreased P/O ratios. Martius concluded that vit. K was a component of the terminal electron transport chain that was necessary for the coupling of electron transport and ATP formation (oxidative-phosphorylation). Additional, but indirect, support for this explanation was provided by the finding that addition of dicoumarol to mitochondria from normal animals uncoupled oxidative-phosphorylation. On the other hand, mitochondria from coumarin anticoagulant treated animals showed normal ratios (64). Martius et al published a series of additional papers to substantiate and extended their findings (65) and for several years the concept that vit. K was involved in oxidative-phosphorylation was generally accepted and incorporated into biochemical text books.

Several investigators tried to study the possible mechanism by which vit. K could participate in high energy bond (ATP) formation in model

systems. While many of these experiments added to our knowledge of the chemical reactivity of the vit. K molecule, they have been found to be of no biological significance. For example, Wieland (66) proposed the phosphorylated hydroquinone of vit. K as the active intermediate and obtained experimental evidence that oxidation of hydroquinone by iodine, in the presence of ADP and inorganic phosphate, can result in ATP formation.

Lederer et al (67) assumed cyclization of vit. K to a phosphorylated chromane because of the presence of a double bond in the 2',3'-position of the phytyl side chain. Matschiner isolated the 2,3-epoxide of vit. K_1 from liver (68) and postulated that its formation from and retransformation to vit. K_1 is part of the mechanism of action of the vitamin (69,70,71,72). However, at present it is not certain that the epoxidation reaction plays a functional role or is simply a metabolic transformation of the vitamin (73).

Gradually, evidence accumulated that contradicted Martius' findings when a number of investigators (74,75,76) were unable to confirm that mitochondria isolated from vitamin K deficient animals have a lower P/O ratio than those from normal animals. Mitochondria from animals treated with the 2-chloro analogue of vit. K_1 (2-chloro-3-phytyl-1, 4-naphthoquinone), a competitive antagonist with a high anticoagulant activity, also have a normal P/O ratio (75).

It appears that the original results of Martius were obtained with animals that were in the terminal stage of hemorrhage, so that the mitochondria were probably damaged by lysosomal enzymes liberated during severe hepatic anoxia due to poor blood flood through the liver. In

retrospect, it seems remarkable, in view of the specificity of the effect of vit. K deficiency in intact animals, that this concept was ever seriously reconsidered because interference with a process as essential as oxidative-phosphorylation would be expected to produce more generalized effects.

The question of how a fat soluble vitamin could specifically affect the synthesis of four plasma proteins, therefore, remained unsolved.

By 1964 the general outline of protein synthesis had become known and a different approach to this question became possible. Olson (77,78) reported that Actinomycin D, an inhibitor of DNA-dependent messenger RNA synthesis, inhibited the response of vit. K in vit. K deficient chicks and dicoumarol pretreated rats; he concluded that vit. K acted by derepressing a repressor protein which regulates synthesis of the messenger RNA for the four clotting factors that make up the prothrombin complex. Other investigators were unable to confirm these findings. Johnson (79), Suttie (80) and Hill (81) found that Actinomycin D did not inhibit the response in vit. K deficient and coumarin anticoagulant pretreated rats. Similarly, experiments using isolated organs or tissue preparations did not confirm Olson's results (80).

Results similar to those of Olson were obtained in other laboratories with Puromycin and Actidione an inhibitor of protein synthesis at the stage of peptide formation at the ribosomal level. However, the experimental design of these experiments was difficult to evaluate because of the systemic toxicity of these inhibitors. For

example, by using liver slices and homogenates from coumarin pretreated or vit. K deficient rats it was possible to show that Puromycin did not block the response to vit. K added "in vitro" under conditions where protein synthesis was inhibited 98%, as measured by incorporation of C^{14} labelled amino acid (82). From these results it can be concluded that the response to vit. K does not depend directly on "de novo" protein synthesis, but that the vitamin must act on an intermediate beyond the level of polypeptide formation. Furthermore, in both vit. K deficient and coumarin anticoagulant pretreated animals, this intermediate must be present in amounts sufficient to obtain a measurable response by vit. K in the absence of "de novo" protein synthesis.

Hemker (83) concluded, from an analysis of the kinetics of the coagulation time (thrombo test) of normal plasma, plasma from patients with chronic liver diseases and plasma from patients treated with coumarin anticoagulants, that after treatment with anticoagulants the plasma contained a protein precursor which acted as a competitive inhibitor of prothrombin conversion. This protein was later called by Hemker (84) PIVKA (Protein Induced by Vitamin K Absence or Antagonists).

More direct evidence for the presence of such a functionally inactive variant of prothrombin was obtained in 1968 by Josso (85) and Ganrot and Nilehn (86) with a monospecific antibody against human prothrombin. Josso found that the plasma of patients on anticoagulant therapy with low prothrombin activity (32% average) indicated higher prothrombin levels when tested against the antibody. Similar results were obtained by Garnot and Nilehn. These findings were interpreted to indicate the presence of an inactive, prothrombin-like protein which was possibly,

but not necessarily, a precursor of prothrombin. Unlike prothrombin, this precursor was not removed from plasma by adsorption on $BaSO_4$ and its electrophoretic mobility was not retarded by calcium ion (87,88,89). These observations indicated that it differed from prothrombin in its inability to bind calcium ions, a reaction necessary for physiological prothrombin activation. In contrast, agents that can activate prothrombin in the absence of calcium ions and other cofactors like staphylocoagulase, or prothrombin specific venoms (85,90) will also activate the "inactive" prothrombin. This clearly demonstrated that the thrombin portion of the abnormal protein is intact. In various immunological tests the abnormal prothrombin gave a reaction of complete immunological identity. A similar substance was identified and isolated, in amount and purity sufficient for identification, from bovine plasma after anticoagulant treatment. The purified, "inactive" prothrombin gave the same amino acid, carbohydrate and end group analysis as prothrombin (90,91), but from a comparison of the amino acid sequence analysis and finger printing, (26, 89, 92) it was found that it differs by the absence of γ carboxyglutamic residues in the nonthrombin moiety (F.1). From this it was concluded that the carboxylated residues are essential for calcium binding and that carboxylation of specific glutamyl residues takes place after ribosomal formation of the polypeptide chain. These findings were confirmed by Howard & Nelsestuen (94) and Girardot, Delaney & Johnson (95). The carboxylation seems to be vit. K-dependent, so that in the absence of the vitamin there appears in the plasma a non-carboxylated inactive intermediate. Similarly, functionally inactive variants have been demonstrated for the other vit. K-dependent factors of the prothrombin complex (96,97,98) and Bucher (99) reported the presence of γ carboxyglutamic residues in bovine factors IX and X.

In support of this explanation, vit. K-dependent incorporation of C^{14} labelled CO_2 by liver microsomes from vit. K-deficient rats has been reported from a number of laboratories (95,100,101,102,103,104,105). Initially Suttie (100) reported that the incorporation was ATP-dependent, but in later publications no energy requirement could be demonstrated; either vit. K_1 or the hydroquinone of vit. K_1 was sufficient to catalyze incorporation (106). Likewise, a requirement for biotin, the coenzyme for similar known carboxylation reactions, could not be demonstrated (107). Even more surprising was the finding that simple glutamyl containing polypeptides could be carboxylated by this cell-free system in the presence of vit. K or its hydroquinone (105).

By comparison with similar known biochemical carboxylations, for example the formation of malonyl coenzyme A from acetyl coenzyme A in fatty acid synthesis, there seems to be little doubt that such a reaction requires not only the activation of the CO_2 to be incorporated, but also the activation of the carboxylic acid to be carboxylated. The failure to demonstrate an energy requirement is at present difficult to understand. It is true that certain γ keto acids like pyruvic acid can be reductively carboxylated, but the equilibrium of this reaction is in favour of decarboxylation. Such keto acids probably react in their tautomeric enol form where the π bond is sufficiently activated for carboxylation to take place by a mechanism that does not require ATP or biotin but requires NADPH. A similar mechanism would be unlikely for a carboxylic acid like glutamic acid. There seems to be little doubt that the formation of calcium binding sites by carboxylation of specific glutamyl residue is involved in the formation of prothrombin,

but whether or not this reaction is directly dependent on vit. K and, if so, by what mechanism still remains to be established.

Chemical structure and vitamin K activity

In 1939, the structure of vit. K was determined and a method for its synthesis worked out. It therefore became possible to prepare and test analogues of the vitamin. One of the aims of such studies has been to establish the structural requirements for vit. K-like activity, in the expectation that this might provide information about its possible mechanism of action.

In trying to evaluate and summarize the results of experiments carried out in various laboratories for more than 30 years, it is necessary to emphasize the differences in the experimental conditions. These include the species and condition of the animal used to test for vit. K-like activity. Some investigators estimated activity as the ability to correct the coagulation defect in vit. K deficient animals (chicks and rats), while others measured activity as the ability to reverse or antagonize the effect of coumarin anticoagulant drugs (rats, rabbits, dogs). It has been found that the relative activity of vit. K analogues may be different in the two tests, the first of which measures the dietary activity, while the second measures the antidotal activity. Since vit. K is a fat soluble substance, completely insoluble in water, the method used for solubilization, particularly for the preparation of aqueous suspensions suitable for intravenous injection, is important. In addition, the route of administration also affects activity. It has been found that not only the potency, but also the speed of onset and duration of action depend on these factors. The method used to measure the activity, the coagulation

test, also varies greatly. For example some investigators used the whole blood clotting time, others the one stage prothrombin time or one of the modifications. Results have been reported as the change of clotting time in seconds, the increase of plasma level of the clotting factors as percent of their normal plasma concentration, but often, when compounds with slight activity were tested, the activity has not been quantitated at all.

With many analogues, the maximal effect that can be obtained will be less, often significantly less, than that produced by the optimal dose of vit. K_1. This can be explained by the hypothesis that in terms of drug receptor interaction, the overall activity of a drug depends on its ability to combine with a complementary receptor site (i.e. its affinity) and the ability of the drug receptor combination to produce an effect (i.e. its intrinsic activity). Unlike analogues that have both high affinity and high intrinsic activity and therefore can produce a maximal response, partial agonists-antagonists possess relatively high affinity but low intrinsic activity, so that, even at full receptor occupancy, they do not produce the maximal response of which the system is capable. When given together with a small dose of an agonist, there are sufficient receptors available for combination with both and the response is additive, but when given with a large dose of an agonist, they compete for receptor occupancy with the more active agonist and hence reduce its effect (108). When only a single dose of an analogue with low activity is tested partial agonism may be missed. To-date, only one example of such a partial agonist-antagonist has been fully demonstrated experimentally for a vit. K_1 analogue, although it probably occurs very often (109).

To simplify the discussion of the structural requirements for activity, the vit. K molecule can be arbitrarily considered to be made up of several distinct chemical parts (Fig. 3).

1. Naphthoquinone nucleus

The naphthoquinone nucleus appears to be an important, but not absolutely essential requirement for activity. Increase of its size by substitution of methyl or methoxy groups in the 5, 6 or 7 position decreases or abolishes activity (110). Partial hydrogenation results in a marked reduction of activity (111). Replacement of the para-naphthoquinone by a benzoquinone structure results in either a large decrease or complete loss of activity (109). For example, 2,5,6 - trimethyl-3-phytyl-1,4-benzoquinone shows partial vit. K-like activity (III). That is, it behaves as a partial agonist-antagonist. The 2,5 and 5,6-dimethyl analogues show no activity. It is also of interest to note that the phytol analogue of coenzyme Q (IV) the para-benzoquinone found in mitochondria, is completely devoid of vit. K-like activity (109).

2. Para-quinone structure

A para-quinone structure seems to be an absolute requirement for vit. K-like activity. Certain esters of the hydroquinone of vit. K_1 such as acetate, sulfate, and phosphate, show activity, undoubtedly because they can be hydrolysed and oxidized "in vivo" to a quinone. On the other hand, the dimethyl ether of vit. K_1 which should be more resistant to hydrolysis, shows no activity (112). The only exception seems to be a report by Fieser (111) that an isomeric ketonic by-product (2-methyl-2-phytyl-3,3 dihydro-1, 4-naphthoquinone) (V) obtained in the synthesis of vit. K shows 2% of the activity of vit. K_1. When

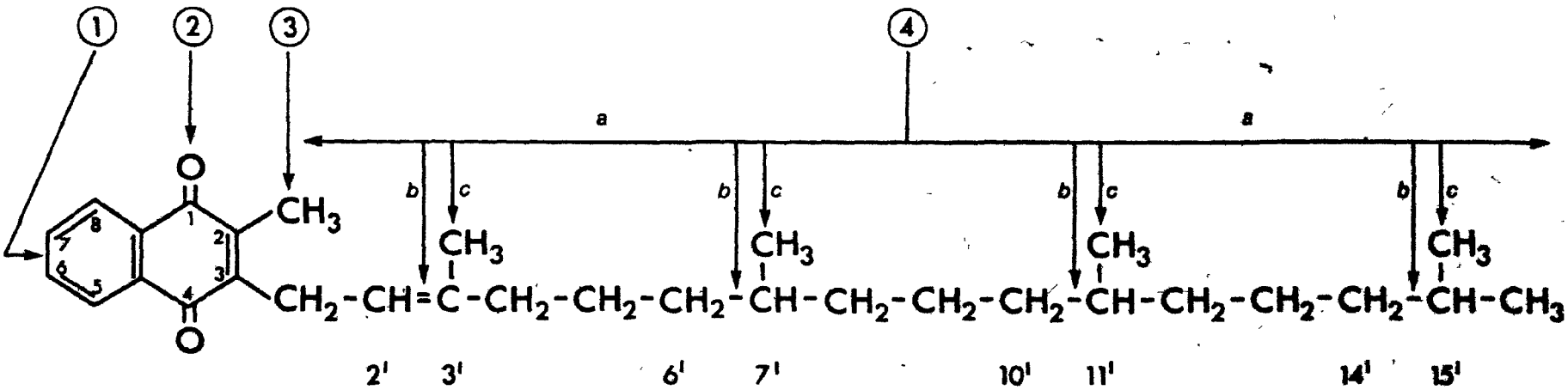
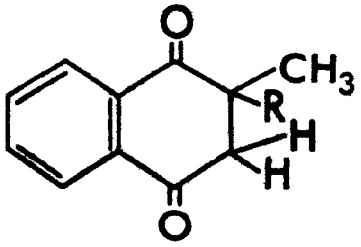


Fig. 3. Schematic presentation of the structure activity relation of vit. K analogues by reference to vit. K_1.



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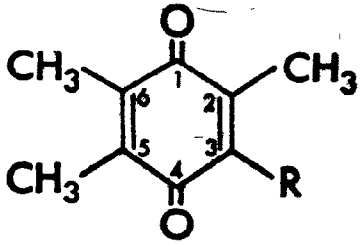

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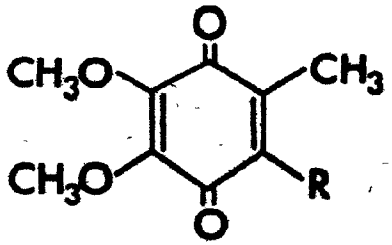

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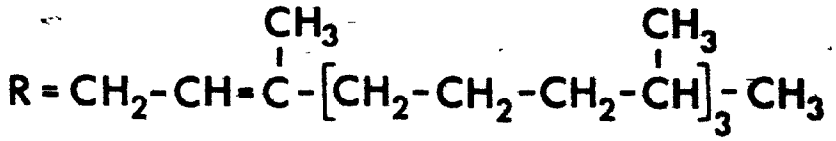


a sample of this compound, purified by TLC, was tested recently in our laboratory it was found to be completely inactive (112).

3. Substitution at the 2-position

The requirements for the substituents at the 2-position are found to be relatively specific. In the naturally occurring vit. K_1 and K_2 the substituent is a methyl group. Replacement by a hydrogen atom or an ethyl group results in a marked reduction of activity, and these compounds behave as partial agonists-antagonists (111,113). A further increase in the size of the substituent to a propyl group; 3 carbon atoms; gives an analogue with very slight and transient activity, and still larger groups such as butyl; 4 carbon atoms; and amyl; 5 carbon atoms; give analogues which are completely devoid of activity (113).

Replacement of the methyl group by a methoxy group gives an analogue that has 25% of the full activity of vit. K_1 in vit. K deficient and in coumarin anticoagulant pretreated rats (113). Larger alkoxy groups, like ethoxy; 2 carbon atoms; or propoxy; 3 carbon atoms; show only slight activity and do not produce the full effect of vit. K_1 while still larger groups like butoxy; 4 carbon atoms; are completely inactive (113).

Replacement of the methyl group of vit. K_1 by a chlorine or bromine atom gives competitive reversible antagonists (114, 115). When given to normal animals, these compounds produce an anticoagulant effect by decreasing the plasma activity of the vit. K dependent clotting factors and this defect can be reversed by vit. K_1. When given simultaneously with vit. K_1 to vit. K deficient or coumarin anticoagulant pretreated animals, depending on the relative doses, they partially or completely

inhibit the response to vit. K_1. When a ratio of vit. K_1 and the chloro analogue producing partial inhibition is kept constant, but the doses are increased, the degree of inhibition remains constant. Hence the inhibition must be of the classical competitive kind. Replacement of the 2-methyl group of the benzoquinone (2,5,6-trimethyl-3-phytyl-1, 4-benzoquinone) by a chlorine or bromine atom likewise results in a change from vit. K to antivit. K activity (109).

4. The lipophilic side chain at the 3-position

(a) In naturally occurring vit. K_1 and K_2 these are polyisoprenoid side chains. In vit. K_1 (I), the side chain is a phytyl group of 4 isoprenoid units or 20 carbon atoms with only one double bond in the 2',3'-position. In vit. K_2 (II), the length of the side chain can vary from 6 to 9 isoprenoid units or 30 to 45 carbon atoms and each isoprenoid unit has a double bond. Isler et al (116) have synthesized and tested the vit. K_1 and vit. K_2 analogues from 5 to 35 carbon atoms. The analogues with 5 carbon atoms show only slight transient activity. Highest activity in the vit. K_1 series was obtained with 20 carbon atoms, that is with the naturally occurring vit. K_1. In the vit. K_2 series, the highest activity was obtained with 25 carbon atoms. All of these compounds produce the maximal effect as vit. K_1, that is they are agonists, but they do show significant differences in their duration of action.

(b) Because of the presence of olefinic double bonds, vit. K_1, vit. K_2 and their analogues can exist in the cis and trans configuration. Matschiner from a comparison of the activity of impure preparations of the trans and cis isomers of vit. K_1 came to the conclusion that the

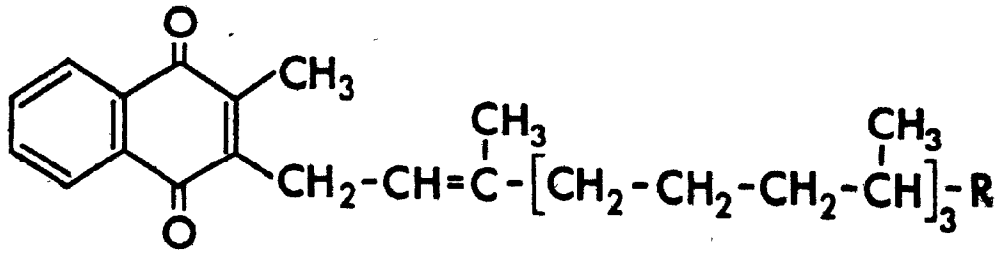
cis isomer lacks biological activity (117).

In the vit. K_2 series, the mono 6',7'-cis isomer shows less activity than the 6',7'-trans isomer, but the cis isomers of the more distal double bonds (10',11' and 14',15') have the same biological activity as the all trans isomer; the cis trans isomers of the 2',3'-double bond in the vit. K_2 series have not been prepared.

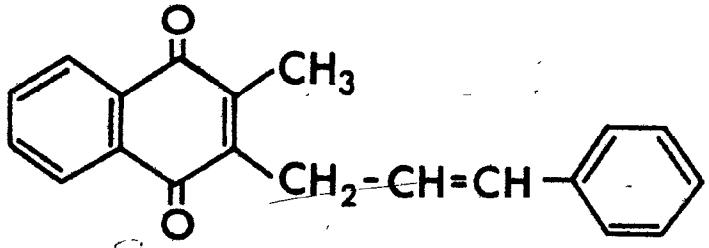
The effect of the double bond in the 2',3'-position on biological activity was already investigated by Karrer and by Fieser in 1940 (111, 118). Using the chick assay Karrer reported that the 2',3'-dihydro analogue of vit. K_1 had 1/24 while Fieser reported 1/8 of the activity of vit. K_1.

(c) Replacement of the polyisoprenoid side chains by unbranched aliphatic side chains (i.e. removal of the methyl groups) reduces, but does not abolish, activity provided the side chain contains more than 5 carbon atoms (119,120). Introduction of the carboxy group at the terminal carbon of the side chain greatly reduces activity (VI); if the carboxy group is esterified (VII) or reduced to an alcohol (VIII) the activity is increased (112,121). More complex aliphatic aromatic side chains like cinnamyl (IX) have also been tested and are reported to possess activity, but other, still more bulky side chains for exchange in compound (X) result in a complete loss of activity (122).

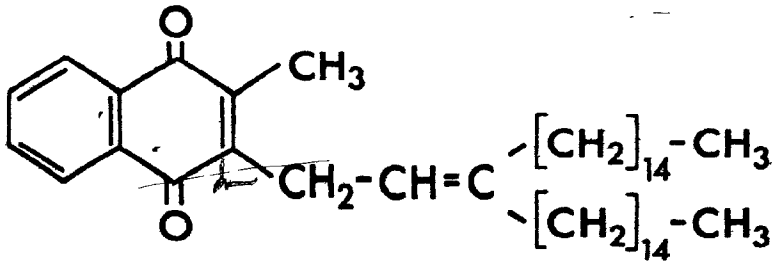
In our laboratory a series of vit. K_1 analogues in which the methyl (XI) group of the side chain at the 3'-position has been replaced by a hydrogen atom (XII), an ethyl group; 2 carbon atoms; (XIII), a propyl group; 3 carbon atoms; (XIV) and a butyl group; 4 carbon atoms; (XV)



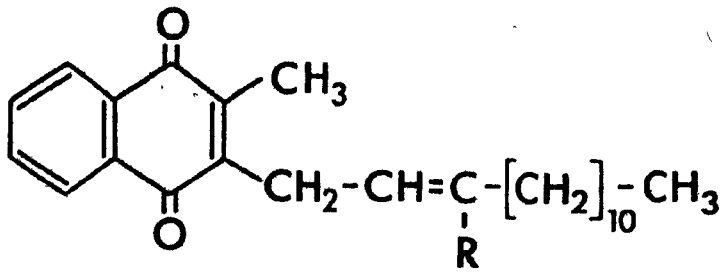
VI R = CO_2H
VII = CO_2CH_3
VIII = CH_2OH



IX



X



XII	R = H
XI	CH_3
XIII	C_2H_5
XIV	C_3H_7
XV	C_4H_9

has been prepared recently and is currently being tested. The results so far obtained indicate that replacement by a hydrogen atom and by an ethyl group retains activity, while replacement by the larger propyl or butyl group abolishes activity (112). The exact quantitative comparison of the activity of these compounds, and in particular the difference between their cis and trans isomers, is currently in progress.

What general conclusions can be drawn from these results?

(1) The para-quinone structure, the most reactive function of the vit. K molecule, appears to be an absolute requirement for activity. Since this function can be reversibly reduced and oxidized, it seems likely that the vitamin functions as part of a reversible redox system. The change from vit. K to antivit. K activity, after replacement of the 2-methyl group by a more electronegative chlorine or bromine atom, would be in agreement with this explanation since such a replacement will change the redox potential to a more positive value.

(2) The overall size of the naphthoquinone nucleus has an effect on activity, any increase or decrease in the size either reduces or abolishes activity. The size of the substituent at the 2-position seems to be particularly critical, an increase or decrease by only one carbon atom results already in a very marked decrease of the activity. The only exception to this generalization is replacement by the methoxy group, which surprisingly gives an analogue with maximal vit. K effect, although this group has a larger size than the ethyl group. At present, no satisfactory explanation for this discrepancy can be given. The results are in agreement with the explanation that the interaction or combination of the naphthoquinone part of the vit. K molecule with a complementary

site of an enzyme or similar structure is involved in the mechanism of action. The change from vit. K-like to antivit. K activity of the classical competitive reversible type associated with the replacement of the 2-methyl group by a chlorine or bromine atom supports this explanation. Since a chlorine or bromine atom is similar in size to a methyl group, these analogues can interact at the receptor or the complementary site but the combination lacks activity.

(3) From the data which is presently available it is difficult to define completely the structural requirements of the lipophilic side chain at the 3-position. The primary function of this group seems to be a non-specific increase in the lipid solubility of the vit. K molecule. For example analogues with side chains of less than 10 carbon atoms show little or no activity. In addition, the double bond in the 2',3'-position is another chemically reactive center because in a reductive acidic medium it can cyclize to chromane or chromene. The partial activity, as reported by two laboratories, of the 2',3'-dihydro derivatives makes it unlikely that cyclization is part of the biological mechanism. Because of a discrepancy in the data it would be desirable to reinvestigate this compound in greater detail. On the other hand, the reported lack of activity of the cis isomer of vit. K and the loss of activity resulting from the replacement of the methyl group at the 3'-position by a propyl group clearly indicate that in addition to the effect on solubility the side chain must have a more direct effect on activity. The remaining analogues, having a more bulky side chain, are too heterogeneous to allow any general conclusion. A more systematic investigation of the effects of modifications of the side chain of the

vit. K molecule on biological activity, including a reinvestigation of some of the previous experiments by the more sensitive and accurate techniques currently available, seems to be desirable. The present work was undertaken as part of such an investigation.

METHODS

Coumarin anticoagulant pretreated animals

Rats were given, by stomach tube, a single dose of 200 µg/100 g of body weight of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) 18-24 hours before being used for experimental studies. This dose was sufficient to lower the activity of factor VII to less than 5% of normal.

Vitamin K deficient animals

The production of vit. K deficiency in the rat seems to depend on a number of factors, since intestinal bacterial synthesis of the vitamin and coprophagy can serve as an adequate source in the absence of the vitamin from the diet. It would appear that in addition to preventing coprophagy by keeping the rats in special cages and administering poorly absorbed sulfonamide (succinyl sulfathiazine) to reduce intestinal flora, the presence of glycerol in the diet may be important because it reduces the absorption of fat soluble vitamins in general. The vit. K deficient diet seems to produce specifically vit. K deficiency because, unlike other fat soluble vitamins, vit. K is not stored in the body, so that the deficiency develops before the tissue stores of the other vitamins become depleted.

Rats were made vit. K deficient by a modification of the procedure of Mameesh and Johnson (123). The animals were fed a vit. K deficient diet and kept in square tubular cages to prevent coprophagy (124). To reduce bacterial contamination, which can serve as a source of the vitamin, water bottles were changed daily and sodium benzoate (0.08%) was added to the drinking water. Cages were sterilized twice a week. Only animals with a plasma level of factor VII of less than 5% of normal

were used for the experiments. On the average, the rats had to be kept on the diet from 10 to 15 days to lower the concentration of factor VII to this level.

Drawing of blood samples and giving intravenous injections

Rats were lightly anaesthetized with diethyl ether and the tail vein was dilated by warming in water at approximately 50°C and gentle massaging. An aliquot of 0.1 ml of blood was drawn into 0.9 ml of rat sample solution using a 27 or 28 gauge needle and a 1.0 ml tuberculin syringe. The sample was immediately transferred to a polystyrene tube (11 x 63 mm) and centrifuged at 2500 rpm for 10 min. at room temperature. If the determinations were not done immediately, the samples were kept on ice. The plasma was used without further dilution for the determination of factor VII. Injections were likewise given by tail vein puncture. Routinely, blood was drawn to check that the needle was in the vein before starting the injection.

Determination of factor VII

The method is a modification of the procedure of Koller et al (125); 0.1 ml of plasma was pipetted into a plastic test tube (100 x 17 mm) followed by 0.1 ml of factor VII-free reagent and 0.1 ml of thromboplastin. After incubation for 30 sec. at 37°C, the reaction was started by the addition of 0.1 ml of $CaCl_2$ (0.025 M), taking as the end point the time required to initiate the formation of a fibrin clot. The concentration of factor VII is expressed as percent of its concentration in normal rat plasma by reference to a standard curve.

Determination of factor II (prothrombin)

The plasma concentration of prothrombin was determined by the one-stage method of Koller et al (125); 0.1 ml of plasma was pipetted into

a plastic test tube (100 x 17 mm), followed by 0.1 ml of prothrombin-free reagent plasma and 0.1 ml of thromboplastin. After incubation for 60 sec. at 37°C, the reaction was started by the addition of 0.1 ml of $CaCl_2$ (0.025 M), taking as the end point the time required to initiate the formation of a fibrin clot. The concentration of prothrombin is expressed as percent of its concentration in normal rat plasma by reference to a standard curve.

Factor VII standard curve

Plasma samples, obtained as described above from three normal rats, were pooled for the determination. The undiluted sample was taken as 100% of normal and serial dilutions of 50, 25, 12.5, 6.25, 3.125 and 2% were made up with working solution. The relation between clotting time in seconds and factor VII concentration was found to be linear between 100% and 2% when plotted on double log graph paper. It should be pointed out that, because of this relationship, the sensitivity of the method is not uniform over the entire range of concentration, but increases with lower concentrations. For example, concentrations of 100% and 80% give clotting times of 35.5 and 37.8 sec. respectively, while 50% and 5% give clotting times of 42 and 88 sec. Since most of the measurements reported in this investigation are below 80%, the more sensitive part of the standard curve was used. A new standard curve has to be prepared for every new preparation of factor VII free reagent or thromboplastin. A representative standard curve is shown in Fig. 4.

Factor II standard curve

The standard curve was prepared with serial dilutions of pooled normal rat plasma as described for the preparation of the factor VII

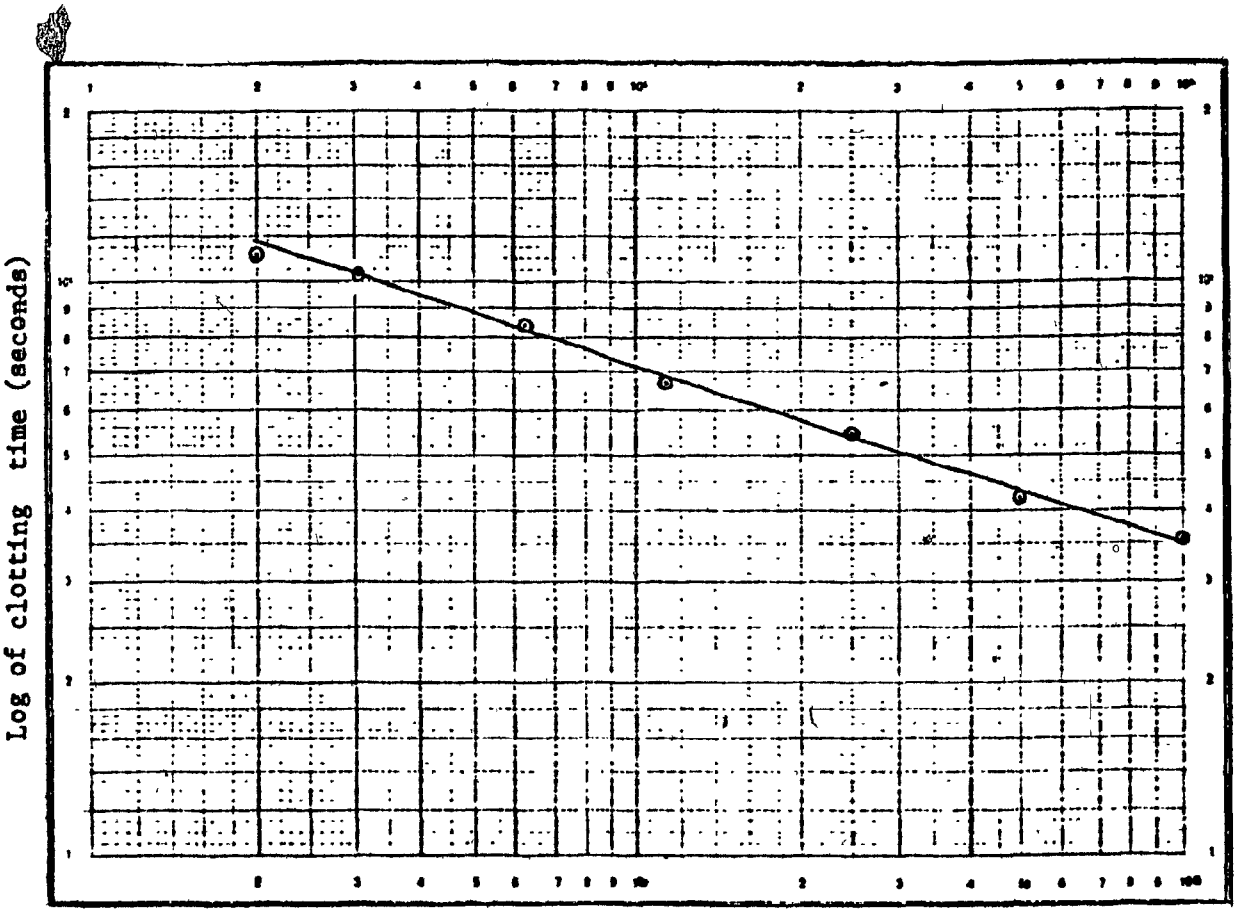


Fig. 4. Standard curve for determination of factor VII.

standard curve. The relation between clotting time in seconds and factor II concentration was found to be linear between 100% and 10% when plotted on double log graph paper. The standard curve was required to monitor the concentration of factor II in the preparation of the factor VII-free reagent.

Purification of vit K_1

Synthetic vit K_1, which is a mixture of the cis and trans isomers in a ratio of approximately 2 to 8, was obtained from commercial sources and first purified by column chromatography on Florisil (magnesia silica gel 60-100 Mesh, Fluka AE, Buchs SE Switzerland).

Approximately 1 gram of vit. K_1 was placed on the column (30 x 2 cm) and the sample was then developed with 98% petroleum ether (38.0° - 53.3°G): 2% diethyl ether. The vit. K_1 was recovered by evaporation of the solvent in a rotory evaporator under vacuum at 50°C and stored in the dark in a desiccator under a high vacuum. The cis and trans isomers were separated by thin layer chromatography (TLC) on precoated plastic sheets (20 x 20 polygram 0.25 mm silica gel without gypsum with fluorescent indicator UV_{254} (Macherey-Nalgel & Co. MN) obtained from Brinkmann Instruments (Canada) Ltd.). Vit. K_1 was dissolved in diethyl ether (200 mg in 2 ml) and approximately 100µl of this solution, containing approximately 5 mg of vit. K_1 was applied as a narrow streak on each sheet at 3 cm from the bottom and 2 cm from each edge. The sheets were developed with 90% hexane: 10% butyl ether in all glass tanks (4 x 12 x 9"). The tanks were lined with filter paper to provide a more homogeneous atmosphere of solvent vapors. The chromatograms were visualized by brief exposure under an ultraviolet light (mineralight UVS. 11). The racemate could

be resolved into two bands with R_f values of 0.51 and 0.43 for the cis and trans isomers respectively (Fig. 5).

The bands corresponding to the two isomers were cut out and eluted separately with diethyl ether in a Soxhlet extraction apparatus for 3 hr. Usually, in one preparation 24 sheets were developed. To eliminate any particles carried over from the extraction, the ether solution was filtered through a sintered glass funnel with slight suction and the solvent evaporated in a rotory evaporator at 50°C. The residual oils were transferred to conical tubes and the remaining solvent blown off under a stream of dry nitrogen. For further purification, each isomer was rechromatographed and isolated by the same procedure. If necessary the purification was repeated until the two isomers were obtained at a purity better than 99%. Care was taken during all manipulations to prevent photocatalyzed isomerization, particularly of the more labile cis isomer, by carrying out all procedures in subdued light and all solvents were distilled immediately before use over lithium aluminium hydride to eliminate peroxide. The chromatographic purity of the preparations was estimated by TLC, Fig. 6 shows that the lowest concentration of vit. K_1 that can be detected by TLC is 0.2 µg. When 50 µg of the purified cis or trans isomer was chromatographed, no contamination by the other isomer can be detected. The purity of the preparations must, therefore, be better than 99%. To test for the possibility that low concentrations of the contaminating isomer may not separate when chromatographed with a high concentration of the other isomer, mixtures of the two isomers were chromatographed. Fig. 7 shows that when 100 µg of the trans, 99 µg of the trans and 1 µg of the cis, 98 µg of the trans and 2 µg of the cis,

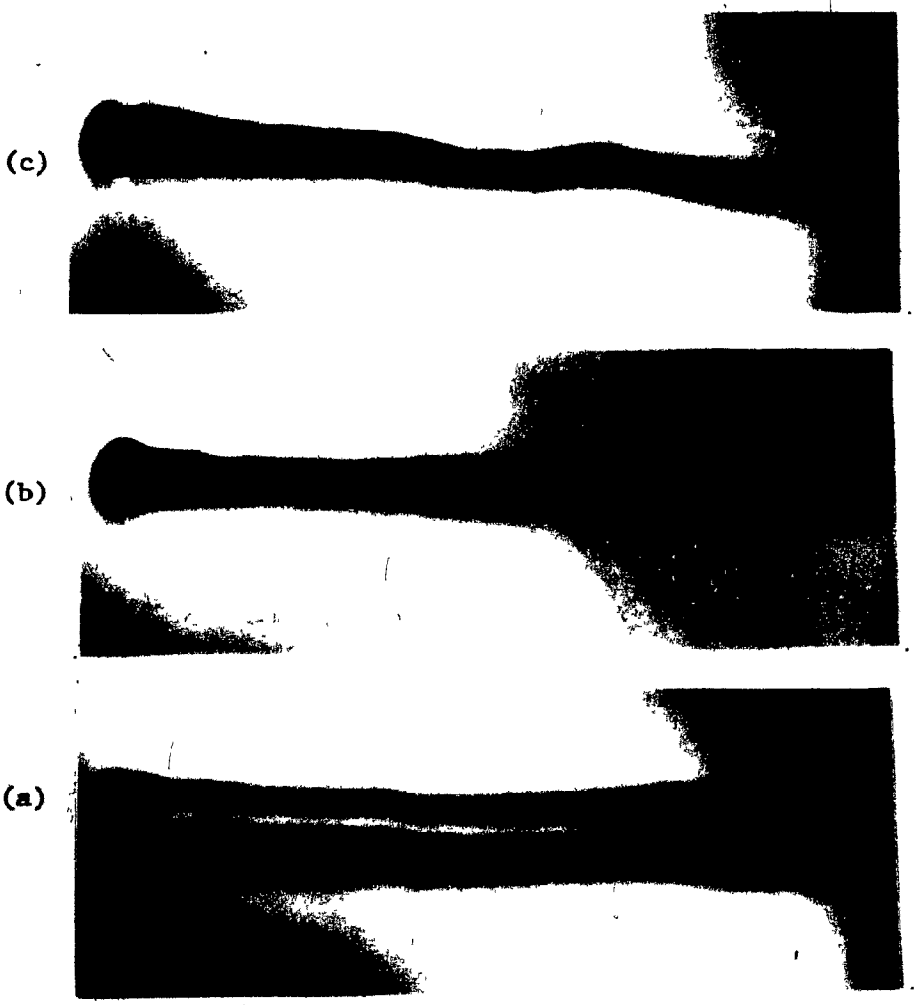
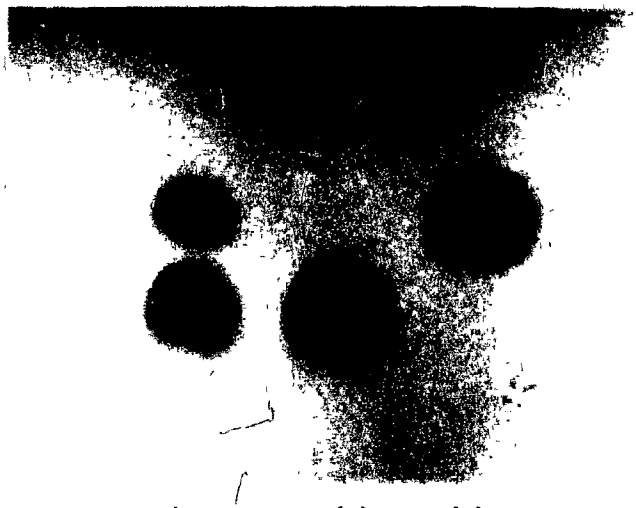


Fig. 5. Separation of vit. K_1 racemic into its cis and trans isomers. (a) 5 mg vit. K_1 racemic developed on silica gel (0.25 mm) with fluorescent indicator UV_{254} (precoated plastic sheets 20 x 20 cm) with 90 % n-hexane: 10% n-butylether. (b) and (c) rechromatography of the trans and cis isomers after separation.

(b)

0.2 µg 0.4 µg 0.8 µg 1.6 µg



(a)

(a) (b) (c)

Fig. 6. Determination of the purity of the cis and trans isomers of vit. K_1.

(a) a: 50 µg vit. K_1 racemic, b: 50 µg trans vit. K_1, c: 50 µg cis vit. K_1

(b) Increasing concentrations of trans vit. K_1 were applied to determine the lowest concentration of vit. K_1 that can be detected after chromatography, which was found to be between 0.2 to 0.4 µg.

Fig. 7. Chromatography of mixture of the trans and cis isomers of vit. K_1.

(a) 100 µg trans vit. K_1 isomer.

(b) 99 µg trans vit. K_1: 1 µg cis vit. K_1 isomer.

(c) 98 µg trans vit. K_1: 2 µg cis vit. K_1 isomer.

(d) 96 µg trans vit. K_1: 4 µg cis vit. K_1 isomer.

(e) 92 µg trans vit. K_1: 8 µg cis vit. K_1 isomer.

96 µg of the trans and 4 µg of the cis, or 92 µg of the trans and 8 µg of the cis were chromatographed there was a distinct separation of the two isomers at all concentrations.

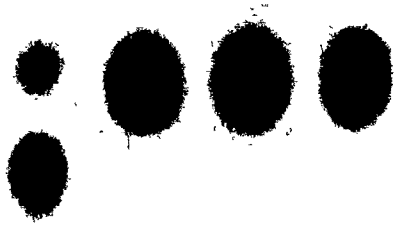
Reduction of vit. K_1 to 2',3'-dihydrovitamin K_1

The reduction was carried out on a semi-micro scale in an open system by the method of Cheronis & Koeck (126); 200 ml of absolute ethyl alcohol and 80 to 200 mg of vit. K_1 (cis or trans), with four times its weight of 10% palladium on charcoal, were placed in the reaction vessel. Hydrogen was passed from a tank into the reaction mixture through a sintered gas dispenser (pore size 10 to 15 microns) while the mixture was agitated by a magnetic stirrer. A number of palladium on charcoal catalysts from different sources were tried. It was found that the catalysts differed greatly in their activity. Some produced extensive reduction of the aromatic part, others cyclization of the phytyl side chain to the chromane. Best results were obtained with an aged 10% palladium on charcoal catalyst obtained from Nutritional Biochemicals Corporation. For reproducibility the same lot of catalyst was used for all reductions.

The course of the reduction of the trans isomer could be readily followed by removing a small sample for TLC because the R_f value of the dihydro derivative (0.43 to 0.49 respectively) was sufficiently different. This was not possible for the cis isomer, since the dihydro vit. K_1 has nearly the same R_f value (0.49 and 0.51) as the cis isomer. The reduction had, therefore, to be arbitrarily discontinued after a fixed time. Best results were obtained when the reduction was carried out for 90 min. Shorter times resulted in incomplete reduction and longer times in extensive reduction of the naphthalene ring. After completion,

the reaction mixture was filtered by gravity through a fluted filter paper (Ra grade 202, size 15 cm) to eliminate most of the catalyst and the alcohol evaporated in a rotory evaporator at 60°C in vacuo. The residue was purified by TLC until the 2',3'-dihydro derivative of vit. K_1 was obtained as a single band.

The purity of the products from the reduction of the cis and trans isomers was tested by TLC. The reduction products gave a single spot (Fig. 8). While the R_f value of the trans isomer and the 2',3'-dihydro vit. K_1 are different, the dihydro derivative has the same R_f value as the cis isomer. This made it difficult not only to follow its reduction but likewise its purification by TLC. The ultraviolet and infrared spectra of both isomers and the dihydro derivatives showed no distinct difference, but their nuclear magnetic resonance spectra (NMR) are different and can be used for identification and estimation of purity. The cis and trans isomers showed identical NMR spectra (Fig. 9) except for a chemical shift due to the protron of the methylene group (CH_2) in the 1'-position. The CH_2 of the cis appears at lower field than the CH_2 of the trans, whereas the 3'CH_3 of the cis appears at higher field than the 3'CH_3 of the trans isomer. The NMR spectra confirm the high purity of the compounds already demonstrated by TLC. After reduction (Fig. 10), the olefinic double bond of each isomer disappears and changes are seen (upfield shifts) for the CH_2 of the quinoid and methyl groups of the former olefinic structure. From the spectra, the reduced trans isomer appears to be reasonably pure. In contrast, the spectra of the reduced cis isomer indicated the presence of some unreduced compound and possibly impurities due to reduction of the naphthalene ring. This can be traced



(a) (b) (c) (d)

Fig. 8. Chromatography of the 2',3'-dihydro vit. K_1.

(a) vit. K_1 racemic shown for comparison.

(b) 2',3'-dihydro vit. K_1 from the reduction of vit. K_1 racemic.

(c) 2',3'-dihydro vit. K_1 from the reduction of the trans isomer of vit. K_1.

(d) 2',3'-dihydro vit. K_1 from the reduction of the cis isomer of vit. K_1.

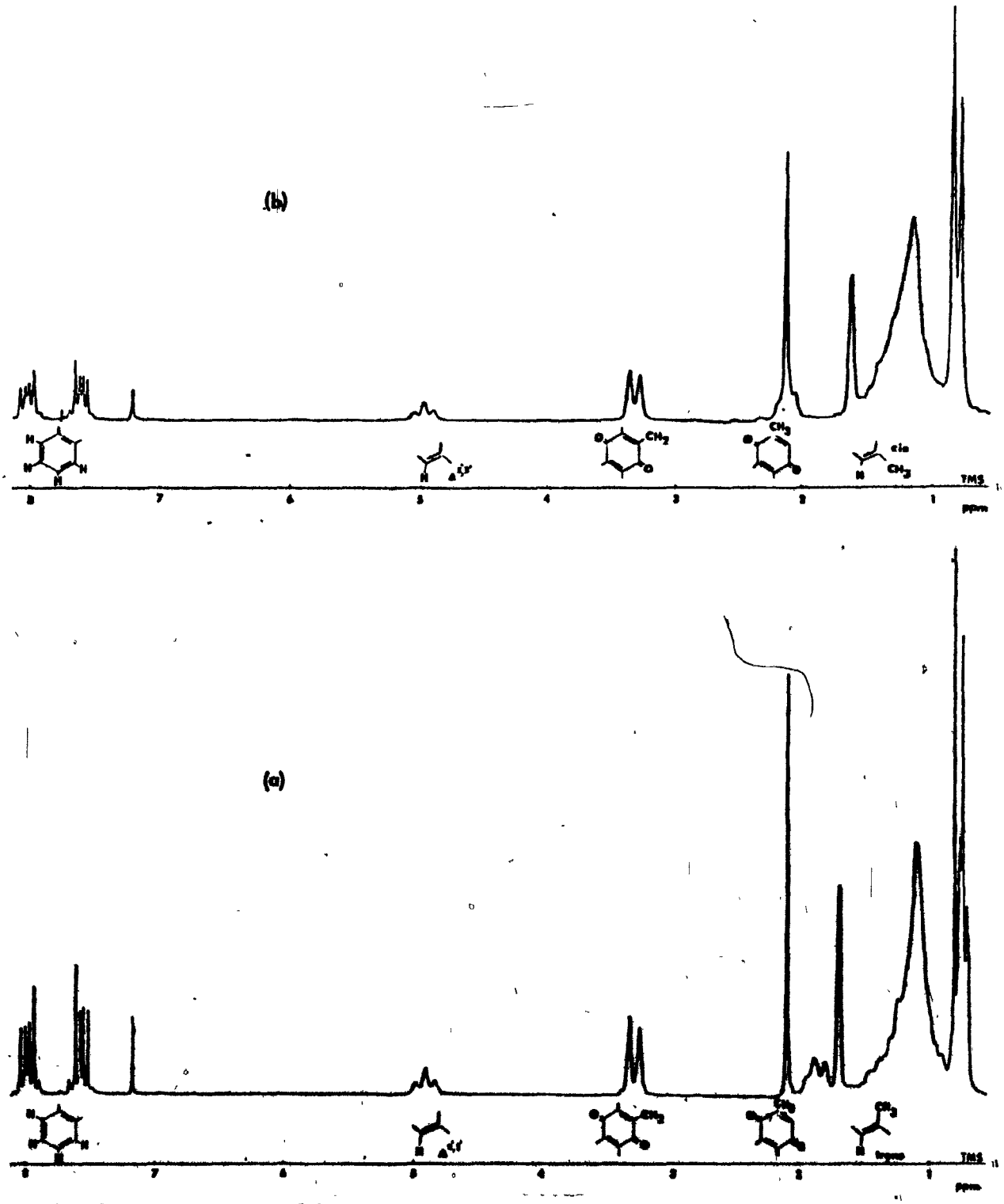


Fig. 9. NMR spectra (a) trans vit. K_1 isomer, (b) cis vit. K_1 isomer. The NMR spectra were determined and interpreted by Drs. A.S. Perlin and G. Hamar of the Chemistry Department, McGill University. Bruker WH-90 FT NMR spectrometer. Solvent $CDCL_3$ referenced to internal TMS.

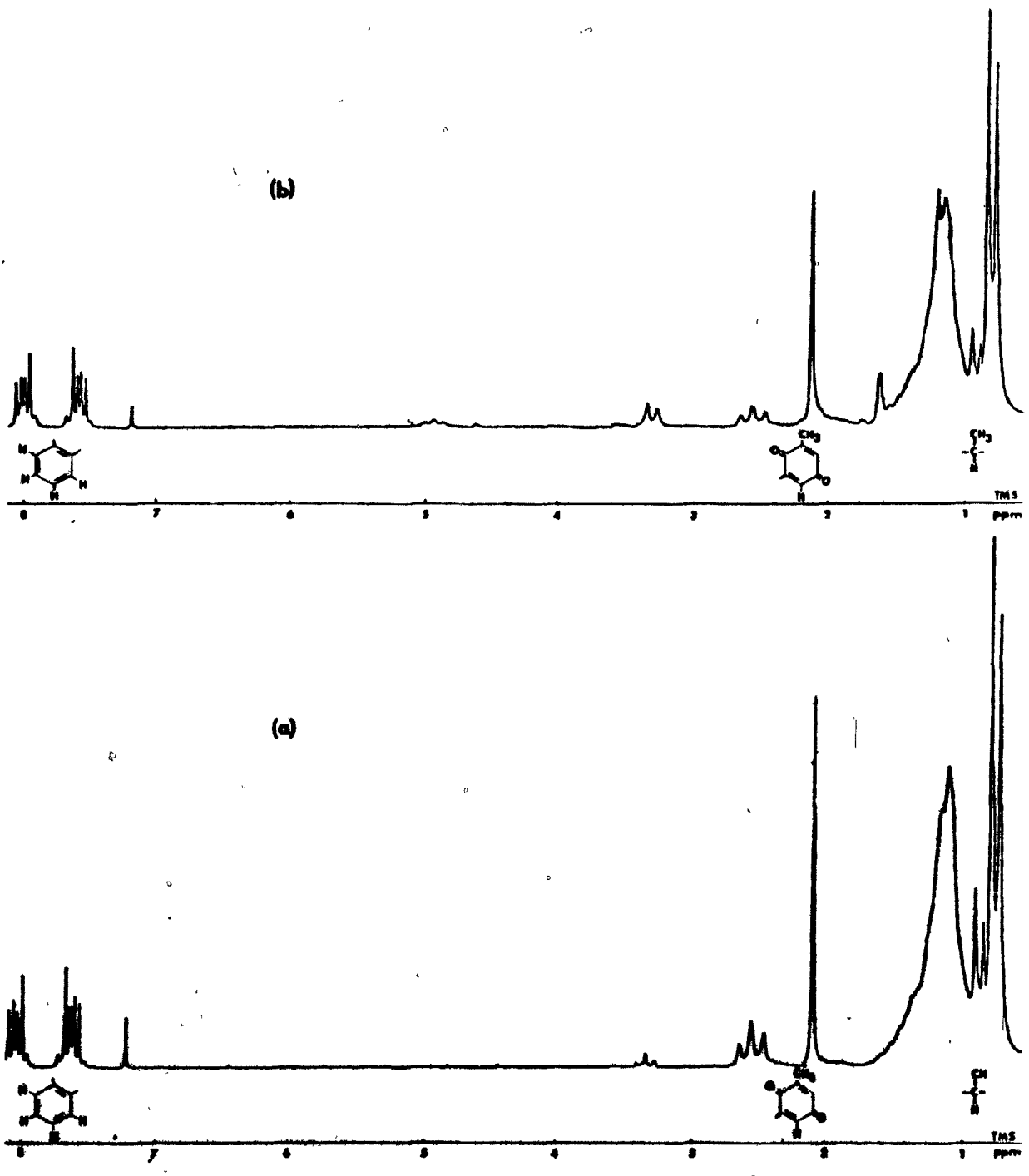


Fig. 10. NMR spectra (a) 2',3'-dihydro vit. K_1 from reduction of trans vit. K_1, (b) 2',3'-dihydro vit. K_1 from reduction of cis vit. K_1.

to the difficulty encountered in the purification of the reduction product of the cis isomer by TLC.

Solubilization of Vit. K_1

Vit. K_1 was solubilized (suspended) with the aid of a non ionic detergent Tween 80, by a standardized procedure worked out in our laboratory. A small quantity of vit. K_1 on the tip of a small glass rod (approximately the amount to be used) was transferred to a 12 ml graduated conical test tube containing 3 drops of Tween 80 (polyoxyethylene sorbitan mono oleate). The vitamin and the non ionic detergent were mixed with a glass rod to a homogeneous paste. Water was added drop-wise, the mixture being reworked carefully into a homogeneous paste after each addition until the vitamin-detergent paste gradually went into solution. If care is taken to add the water initially very slowly, a completely clear solution is obtained. Careful solubilization of the vit. K_1 is important, because it has not only an effect on the potency but also on the onset and duration of action (127). The concentration of the solution was determined from a standard curve by measuring the optical density at 270 mμ. The solutions were made up daily, since it was found that in solution, the cis isomer on storage for about 10 hr. even in the dark, was transformed to slight extent to the trans isomer. However immediately after preparation, no trans isomer could be detected.

Vit. K_1 standard curve

Approximately 5 mg of vit. K_1 was weighed out to the third decimal on an analytical balance (Sartorius-Werke GMBH Gottingen type 240 4 FB No. 154577) by placing a small amount of the vitamin, an oil, on the tip of a tarred glass rod. The rod was transferred to a 12 ml graduated

conical test tube containing 0.1 ml of Tween 80, the vitamin was solubilized as described, and the solution made up to a final concentration of 1 mg/ml in a 10 ml glass stopped graduated cylinder. Serial dilutions of 50, 25, 12.5, 6.25 and 3.125 µg/ml were made up with distilled water, and the optical density at 270 mµ read in a Zeiss spectrophotometer (PMQ II). The aqueous solutions of vit. K_1 prepared with Tween 80, although perfectly clear, are not true solutions but highly dispersed suspensions which still produce some light scattering (Tyndall effect) which depends on the concentration of the nonionic detergent. To compensate for this effect each concentration of vit. K_1 has to be read against a blank containing the same final concentration of Tween 80 as the sample.

It is of interest to note that the ultraviolet spectra of vit. K_1, solubilized with Tween 80, is nearly identical to that of a vit. K_1 solution in ethyl alcohol (Fig. 11). It has been shown (128) that the chromophore for the ultraviolet absorption of vit. K is the naphthoquinone structure. From the spectra of the aqueous suspension of vit. K_1 it appears that the lipophilic phytyl side chain is responsible for the attachment of the molecule to the micelle of the detergent, while the more polar naphthoquinone moiety that carries the chromophore is oriented into the aqueous phase.

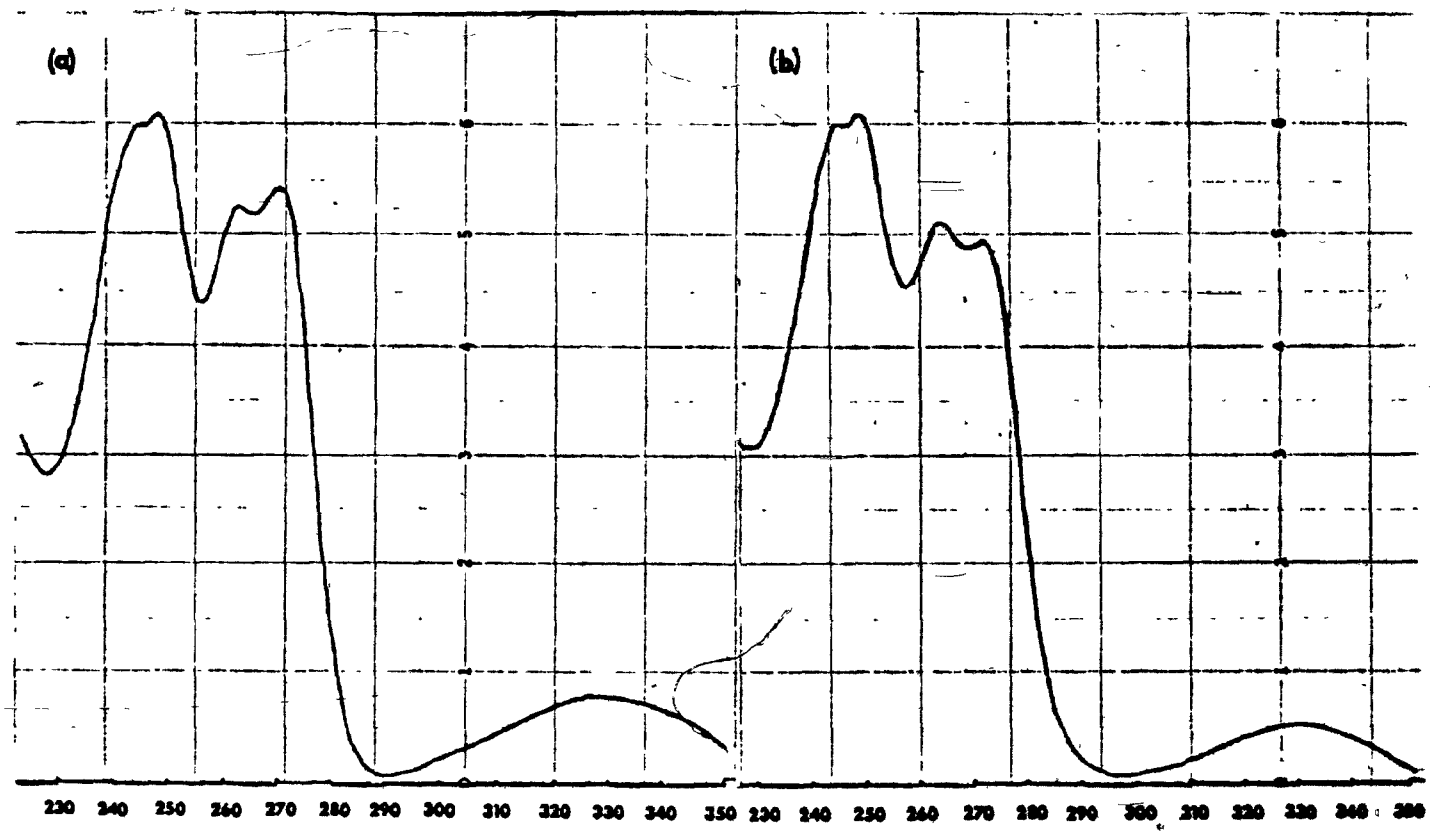


Fig. 11. Utraviolet absorption spectrum of vit. K_1 (trans); (a) in ethanol, (b) solubilized in water with Tween 80. Scale rate 0 - 1, scan rate 50 nm/min. Varian Techtron spectrophotometer model 635.

REAGENTS

Thromboplastin

Rabbit brain thromboplastin was obtained from Modern Laboratories Limited Toronto. 1.5 g of thromboplastin was suspended in 100 ml of 0.9% sodium chloride preheated to 50°C and gently stirred for 15 min. at 50-52°C. The suspension was filtered through several layers of cheese cloth and the filtrate stored in siliconized glass tubes (70 x 10 mm) at -20°C. The reagent was kept for at least 4 days at this temperature before being used.

Factor VII-free reagent

Fresh bovine blood obtained at the slaughter house was immediately transferred to a 2 liter plastic bottle containing sodium oxalate (100 ml 0.1 M sodium oxalate for 900 ml of blood). The blood was centrifuged at 2000 r.p.m. for 30 min. at room temperature (International Model U.V. centrifuge head #266) in 250 ml siliconized glass bottles. The oxalated plasma was collected with a plastic bulb baster and recentrifuged for 15 min. at the same speed to remove any residual suspended erythrocytes. Factor VII-free reagent was prepared by the method of Koller et al (125). The filter (Ertel Engineering Corp. Kingston, New York) was set up with two asbestos filter pads, the upper with a 20% and the lower with a 30% asbestos content (filters No. 5 and No. 7, Filtrox and Co., St. Gallen, Switzerland). Fifty ml of saline were first placed in the reservoir and filtered under slight pressure. One liter of oxalated bovine plasma was then placed into the reservoir and the pressure was adjusted to permit a flow rate of 1 to 2 drops per second. The first 200 ml of filtrate was discarded and the filtrate collected

in 30 ml fractions in plastic beakers. The factor VII concentration of every fraction and the prothrombin concentration of every fifth fraction were determined. Fractions having a prothrombin concentration of at least 30% of normal and a factor VII concentration of less than 1% of normal were pooled and kept in small siliconized test tubes in 1 or 2 ml lots and stored at -20°C.

Factor II-free reagent

The reagent was prepared by mixing equal parts of barium sulfate adsorbed oxalated beef plasma and human serum. The plasma serves as the source of factor V and the serum of factors VII and X (125).

(a) To 200 ml of oxalated beef plasma was added 10 g of barium sulfate, the mixture was stirred gently for 20 min. at room temperature and the barium sulfate separated by centrifugation at 2000 r.p.m. for 15 min. One ml of the adsorbed plasma was mixed with 1 ml of serum (see below). This mixture should give a blank value of at least 60 sec; if the time is shorter the adsorption is repeated, the quantity of barium sulfate used for the second adsorption depending on the difference from the desired blank value. It may be necessary to repeat the adsorption until a blank value of greater than 60 sec. is obtained.

(b) Oxalated human serum; the blood was allowed to clot in 10 ml aliquots in plain 12 x 2.5 cm glass test tubes containing 0.1 ml thromboplastin. After incubation at 37°C for 1 hr, the clotted blood was left at room temperature overnight and the serum collected. To 180 ml of serum was added 20 ml of sodium oxalate (0.1 M), and the precipitate removed by centrifugation at room temperature at 700 g x 20 min. The

reagent was prepared by mixing equal volumes of the adsorbed plasma and serum and should give a blank value greater than 60 sec. The reagent was stored in aliquots of 1 or 2 ml at -20°C.

Veronal buffer

(a) Veronal stock solution: 5.85 g sodium acetate, 14.71 g sodium Veronal (sodium barbiturate) dissolved in 500 ml of deionized water.

(b) Veronal working solution: 92.5 ml Veronal stock solution, 74 ml 4.25% (W/V) sodium chloride, 80.5 ml of 0.1 N HCL, 253 ml water, and the pH adjusted to 7.35.

(c) Sodium citrate stock solution (3.8%): 3.8 g sodium citrate in 100 ml of deionized water.

(d) Rat sample solution (Anticoagulant solution for drawing blood samples): 89 ml of Veronal working solution, 1 ml of 3.8% sodium citrate stock solution.

Calcium chloride solution

(a) Stock solution (0.1 M): 11.0994 g $CaCl_2$ dissolved in one liter deionized water.

(b) Working solution: 25 ml 0.1 M Stock solution was made up to 100 ml with deionized water and stored in the refrigerator.

Warfarin solution

0.1 g of Warfarin was suspended in deionized water and dissolved by the addition of a few drops of sodium hydroxide to give a 1 mg/ml stock solution with a pH of approximately 7.5.

Vit. K deficient diet

The vit. K deficient diet is a modification of the one described for M.S. Mameesh and B. Connor Johnson (123).

	g/kg diet
Sucrose	495
Vitamin mix.	100
*Casein	300
Methionine	7.5
salt mix.	40
wheat germ oil	6.5
glycerol	30
cod liver oil	17.5
succinyl sulfathiazine	5
Na benzoate	1

Vitamin Mixture	mg/kg diet
Thiamine hydrochloride	10.0
Riboflavin	10.0
D, L Calcium pantothenate	50.0
Pyridoxine hydrochloride	5.0
Nicotinic acid	20.0
Folic acid	1.0
Vit. B_{12}	0.1
Choline chloride	1000.0
Biotin	0.1
sugar	48980.0

* Casein, essentially vitamin-free, obtained from Sigma Chemical Company was extracted by stirring two times with hot alcohol for 6 hr.

All other chemicals were obtained from commercial sources and whenever possible were at least of reagent grade.

RESULTS

The cis and trans isomers of vit. K_1 were first tested in vit. K deficient rats. A blood sample was taken at zero time by tail vein puncture, the needle left in place, and the injection given immediately. To follow the response, blood samples were then taken from each animal after 30, 60, 90 and 150 min. for the determination of the plasma level of factor VII. Preliminary experiments were carried out to find the range at which the relation between the dose and the response would be linear when the responses are plotted against the log of the doses. The final comparison between both isomers was planned as a 6 point symmetrical parallel line assay (129,130), so that each isomer was tested at 3 dose levels. Groups of 5 animals received 0.0312, 0.0625 or 0.125 µg/100 g of body weight of the trans and 1.6, 3.2 or 6.4 µg/100 g of body weight of the cis isomer respectively. Fig. 12 shows the time course of the responses.

Not only do the two isomers differ in their potency, but it appears that they differ also in their onset of action with the trans isomer having a faster onset. From Fig. 13 it can be seen that when the means of the responses are plotted against the log of the doses, the two dose response curves are parallel after 150 min.

The responses after 150 min. were therefore used for the estimation (Table I). The analysis is summarized in Table II. The differences due to the various preparations (term 1) and doses (term 2, regression) are highly significant when tested against the general error (term 7). The third term, is a measure of the deviation from parallelism of the two response curves and is not statistically significant at the 5 percent

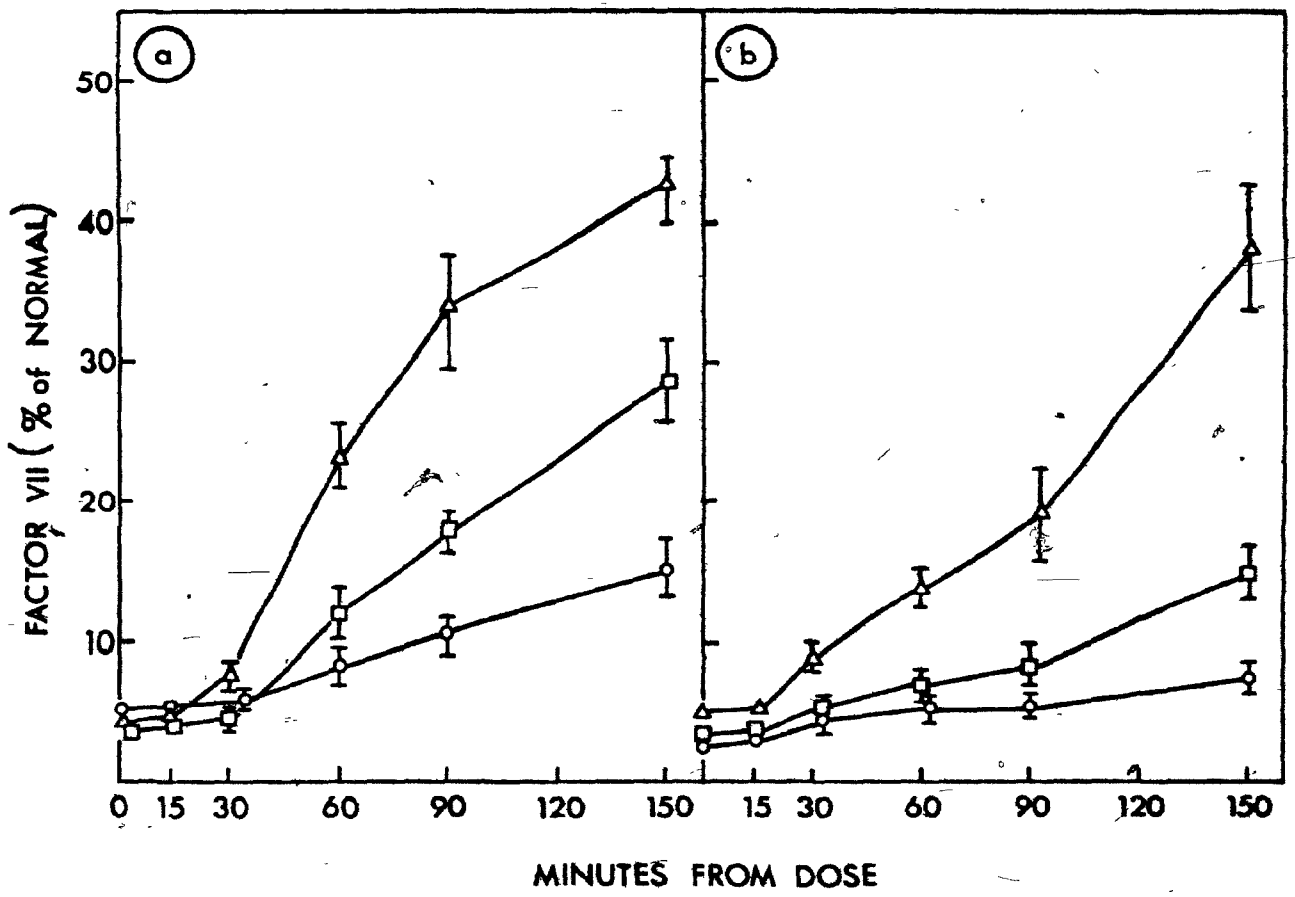


Fig. 12. Time course of response to graded doses of the trans and cis isomers of vit. K_1 in vit. K deficient rats. (a) the trans isomer, o 0.0312 µg/100 g, ▫ 0.0625 µg/100 g, Δ 0.125 µg/100g. (b) the cis isomer of vit. K_1, o 1.6 µg/100 g, ▫ 3.2 µg/100 g, Δ 6.4 µg/100 g. (All results are the mean of at least 5 different experiments).

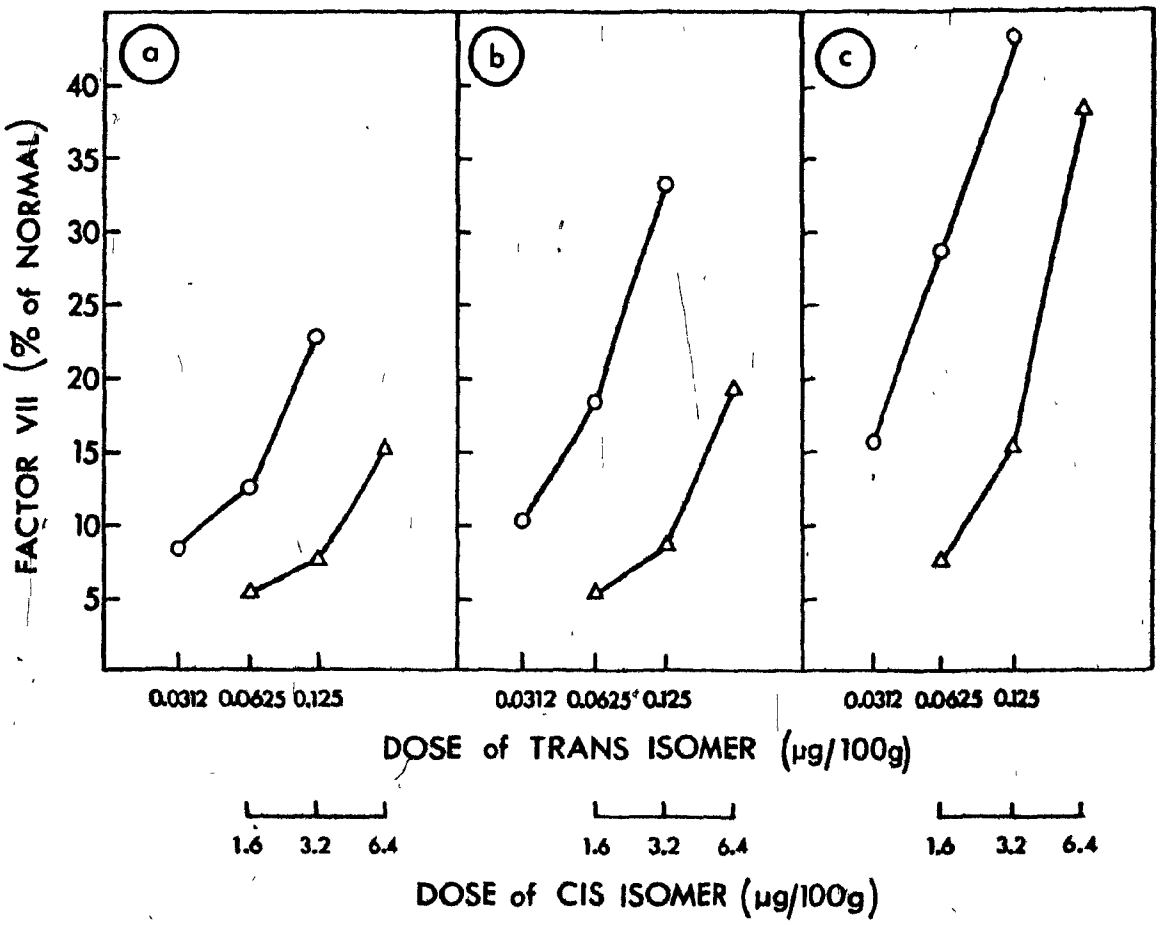


Fig. 13. Means of responses to graded doses of the trans and cis isomers of vit. K_1 in vit. K deficient rats; (a) after 60 min., (b) after 90 min., (c) after 150 min. (○ the trans isomer, Δ the cis isomer). The dose scale is logarithmic, but is graduated in true doses.

	Dose of trans vit. K_1			Dose of cis vit. K_1		
	0.0312 µg	0.0625 µg	0.125 µg	1.6 µg	3.2 µg	6.4 µg
	14.5	34	48.3	14.3	22.5	48.2
	20	31.2	45.5	4.3	18.5	51.8
	20.5	20.5	34.2	6.6	15.4	38.5
	9.2	36.2	38.4	7.8	10	33
	14.2	22.5	48.5	5.9	10.8	21.7
Total	78.4	144.40	214.9	38.9	77.2	193.2
Mean	15.68	28.88	42.98	7.78	15.44	38.64

Table I. Increase of the plasma level of factor VII in vit. K deficient rats 150 min. after administration of 0.0312, 0.0625 and 0.125 µg of the trans and 1.6, 3.2 and 6.4 µg/100 g of body weight of the cis isomers of vit. K_1.

Nature of variation		df	sum of squares	mean square	F	P
(1)	Preparations	1	549.552	549.552		
(2)	Regression	1	4228.23	4228.23		
(3)	Deviation from Parallelism	1	15.49	15.49	0.31	>0.05
(4)	Curvature	1	112.61	112.61	2.25	>0.05
(5)	Diff. of Curvature	1	89.3	89.3	1.79	>0.05
(6)	Between doses	5	4995.54			
(7)	Error	24	1200.02	50		
	TOTAL	29	6195.56			

Table II. Analysis of variance of the data from Table I.

(Details of calculation are given in the appendix)

level of probability ($P > 0.05$). The relative activity of the two isomers can therefore be estimated by this method. The remaining terms (4, curvature; 5 difference from curvature) are an estimate of the deviation from linearity and are not statistically significant ($P > 0.05$). The slope and the intercept for the dose response curves of the two isomers were calculated. Fig. 14 shows the curves obtained with these parameters when the response is plotted against the log of the doses.

In terms of relative potency (R), in vit. K deficient rats, 1 µg of the cis isomer has the same activity as 0.015 µg of the trans isomer, with 5 percent fiducial limits at 0.011 µg and 0.019 µg. Expressed differently, the cis isomer has approximately 1.5% of the potency of the trans isomer with 5 percent fiducial limits at 1.1% and 1.9%.

A similar experimental design was used to estimate the potency of both isomers in coumarin anticoagulant pretreated animals. Preliminary experiments indicated a dose dependent response between 10, 20 and 40 µg/100 g of body weight of the trans and 80, 160 and 320 µg/100 g of body weight of the cis isomers. In coumarin anticoagulant pretreated animals the cis isomer showed an even slower onset of action than in vit. K deficient animals. While for the trans isomer a distinct response could be detected after 15 min., for the cis isomer a response could only be detected after 45 min. (Fig. 15). To try to select the data for a comparison, the means of the responses to the trans isomer after 60 min. and the cis isomer after 150 and 300 min. were plotted against the log of the doses (Fig. 16). The dose response curves best approach parallelism when the responses to the trans isomer after 60 min. and to the cis isomer after 300 min. are compared. The responses at these times (Table III)

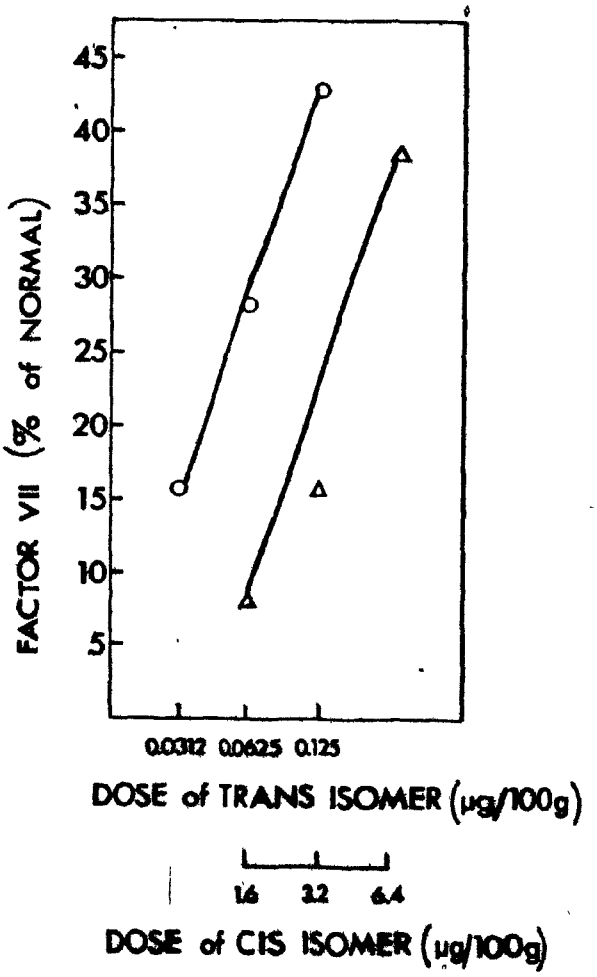


Fig. 14. Dose response curves for graded doses of the trans and cis isomers of vit. K_1 in vit. K deficient rats; O the trans isomer, Δ the cis isomer curves are calculated from data of Table I; details of calculation are given in the appendix. The dose scale is logarithmic, but is graduated in true doses.

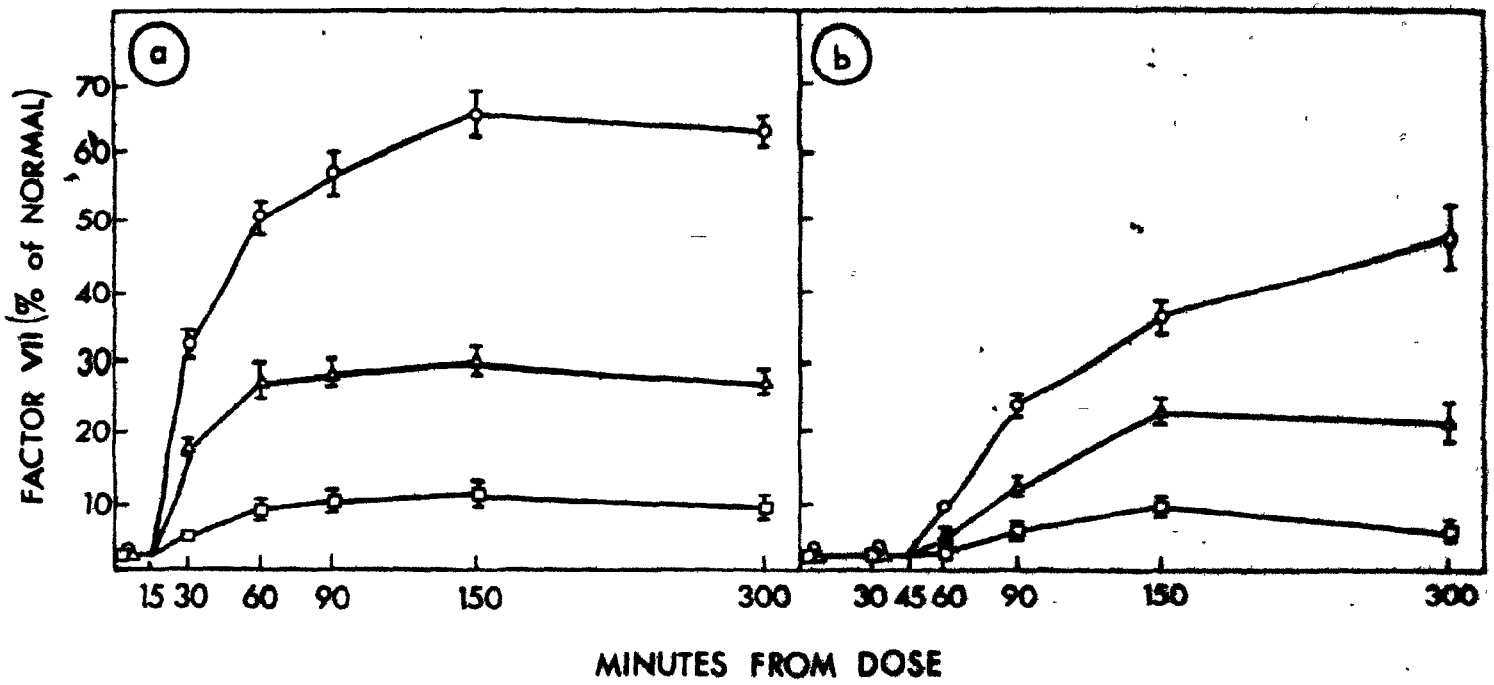


Fig. 15. Time course of response to graded doses of the trans and cis isomers of vit. K_1 in coumarin anticoagulant pretreated rats; (a) the trans isomer, ▫ 10 µg/100 g, ▲ 20 µg/100 g, ○ 40 µg/100 g. (b) The cis isomer, ▫ 80 µg/100 g, △ 160 µg/100 g, ● 320 µg/100 g. (all results are the mean of at least 5 different experiments).

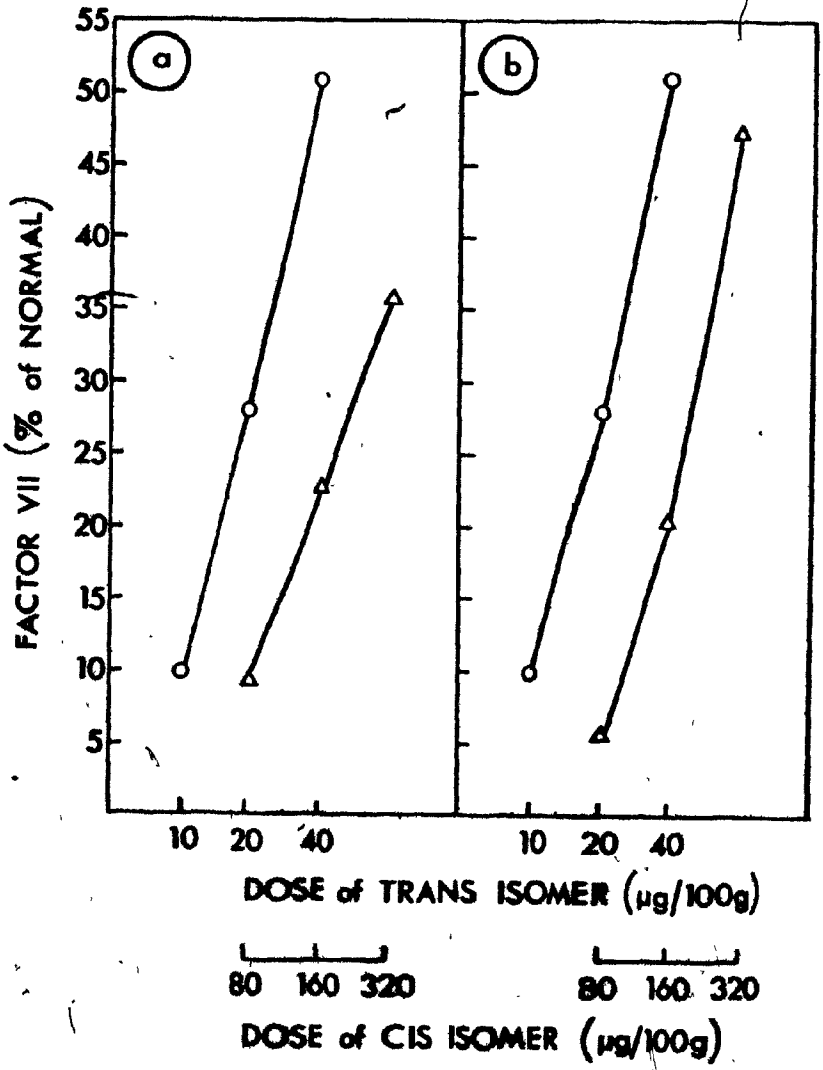


Fig. 16. Means of responses to graded doses of the trans and cis isomers of vit. K_1 in coumarin anticoagulant pretreated rats; (a) after 90 min., (b) after 150 min. (○ the trans isomer, △ the cis isomer). The dose scale is logarithmic, but is graduated in true doses.

were therefore used for the analysis (Table IV). As in the vit. K deficient rats, the differences between preparations (term 1) and between doses (term 2) are highly significant when tested against the general error (term 7). The deviation from parallelism (term 3) is not statistically significant ($P > 0.05$). The potency of both preparations can therefore be estimated by this method. Of the remaining terms the mean square for curvature (term 4) is just significant at the 5 percent level of probability, the required value of F is 4.26 and the calculated value is 4.83. The large mean square for curvature, which is a measure of deviation from linearity, probably arises from the different onsets of action of the two preparations and the arbitrary selection of the times for the comparison. In particular, for the two lower doses of the cis isomer the peaks of the responses already seem to occur after 150 min, while for the larger dose the response still increases after 150 min. (Fig. 15). This difference seems to be the major contributor to deviation from linearity. In contrast, the response curve for the cis isomer after 150 min. showed less deviation from linearity but showed poor approach to parallelism with that of the trans isomer (Fig. 16). Fig. 17 shows the dose response curves with the slopes and intercepts calculated from the data of the analysis.

In terms of relative potency (R), in coumarin anticoagulant pretreated rats, 1 µg of the cis isomer has the same activity as 0.11 µg of the trans isomer, with 5 percent fiducial limits at 0.09 µg and 0.13 µg or expressed differently, the cis isomer has approximately the 11% of the potency of the trans isomer with 5 percent fiducial limits at 9% and 13%.

In an attempt to obtain further evidence about the functional role of

	Dose of trans vit. K_1			Dose of cis vit. K_1		
	10 µg	20 µg	40 µg	80 µg	160 µg	320 µg
	13.2	30.5	58	7.4	24	40
	9.6	26.5	50	5.5	24	46.5
	8.4	28.7	55	5.4	19.2	50
	7.2	28.6	48	5.2	19.5	50
	10.1	25	42.5	4.5	19	49
Total	48.5	139.3	253.5	28	106.7	235.5
Mean	9.7	27.86	50.7	5.6	21.34	47.10

Table III. Increase of the plasma level of factor VII in coumarin anticoagulant pretreated rats 60 min. after administration of 10, 20 and 40 µg of the trans and 300 min. after administration of 80, 160 and 320 µg/100 g of body weight of the cis isomer of vit. K_1.

Nature of variation		df	sum of squares	mean square	F	P
(1)	Preparations	1	168.51	168.51		
(2)	Regression	1	8507.81	8507.81		
(3)	Deviation from Parallelism	1	0.31	0.31	0.02	>0.05
(4)	Curvature	1	90.04	63.04	4.83	<0.05
(5)	Diff. of Curvature	1	11.88	3.6	0.37	>0.05
(6)	Between doses	5	8778.55			
(7)	Error	24	295.18	13.04		
	TOTAL	29	9073.73			

Table IV. Analysis of variance of the data from Table III.

(Details of calculation are given in the appendix)

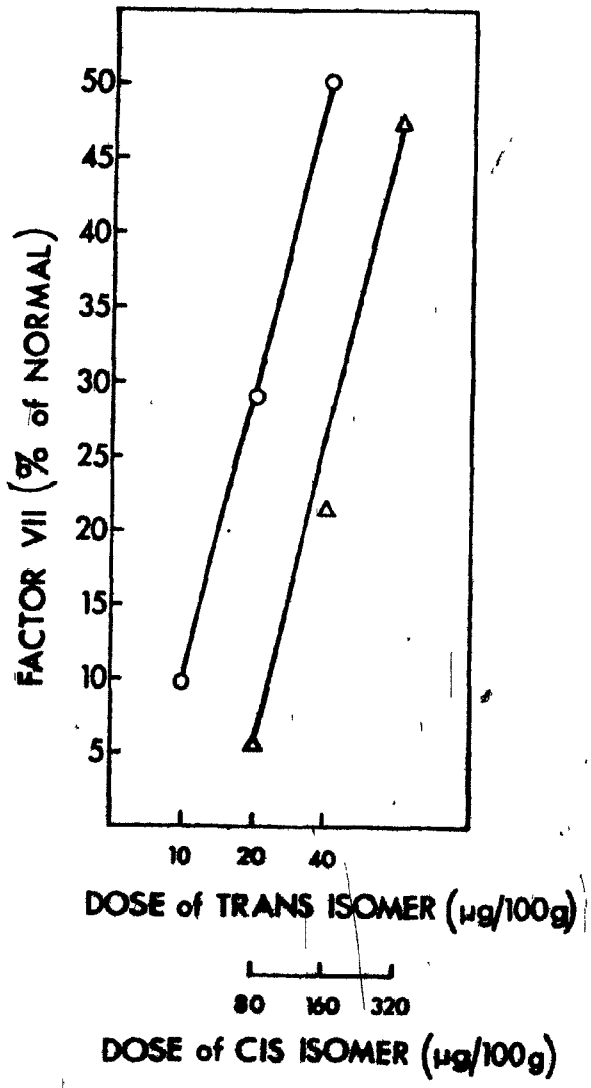


Fig. 17. Dose response curves for graded doses of the trans and cis isomers of vit. K_1 in coumarin anticoagulant pretreated rats (○ the trans isomer, ▲ the cis isomer). Curves are calculated from data of Table III, details of calculation are given in the appendix. The dose scale is logarithmic, but is graduated in true doses.

the double bond, the trans and cis isomers were reduced catalytically to 2',3'-dihydro vit. K_1. The activities of the reduced isomers were compared in coumarin anticoagulant pretreated animals at a dose level of 20 and 40 μg/100 g of body weight with that of the trans isomer (Fig. 18). The reduced product of either the cis or the trans isomer showed the same activity and onset of action as the more active trans isomer. Therefore, while reduction does not change the activity of the trans isomer, it increases both the activity and the speed of onset of action of the cis isomer. The responses after 150 min. (Table V) were analyzed statistically (Table VI). The mean square due to preparations (term 1) is significant at the 5 percent level ($P < 0.05$). It can be seen from inspection of Table V that the major contribution to this term is made by the reduced cis isomer since the mean of the responses is lower than that to the trans and the reduced trans isomer. The differences between regression (term 2) and deviation from parallelism (term 3) are not significant at the 5 percent level. Fig. 19 shows the dose response curves when responses are plotted against the log of the doses using the regression equation calculated from the data.

The relative potencies and their fiducial limits were calculated and expressed in terms of the trans isomer of vit. K_1: 1 μg of the reduced trans isomer is estimated to have the potency of 0.92 μg of trans, with 5 percent fiducial limits at 0.84 μg and 1 μg, while 1 μg of reduced cis isomer is estimated to have the potency of 0.793 μg of trans K_1, with 5 percent fiducial limits at 0.72 μg and 0.87 μg. As reported in the methods section the NMR spectra of the reduced cis isomer indicated the presence of impurities which could not be eliminated because of the limitations

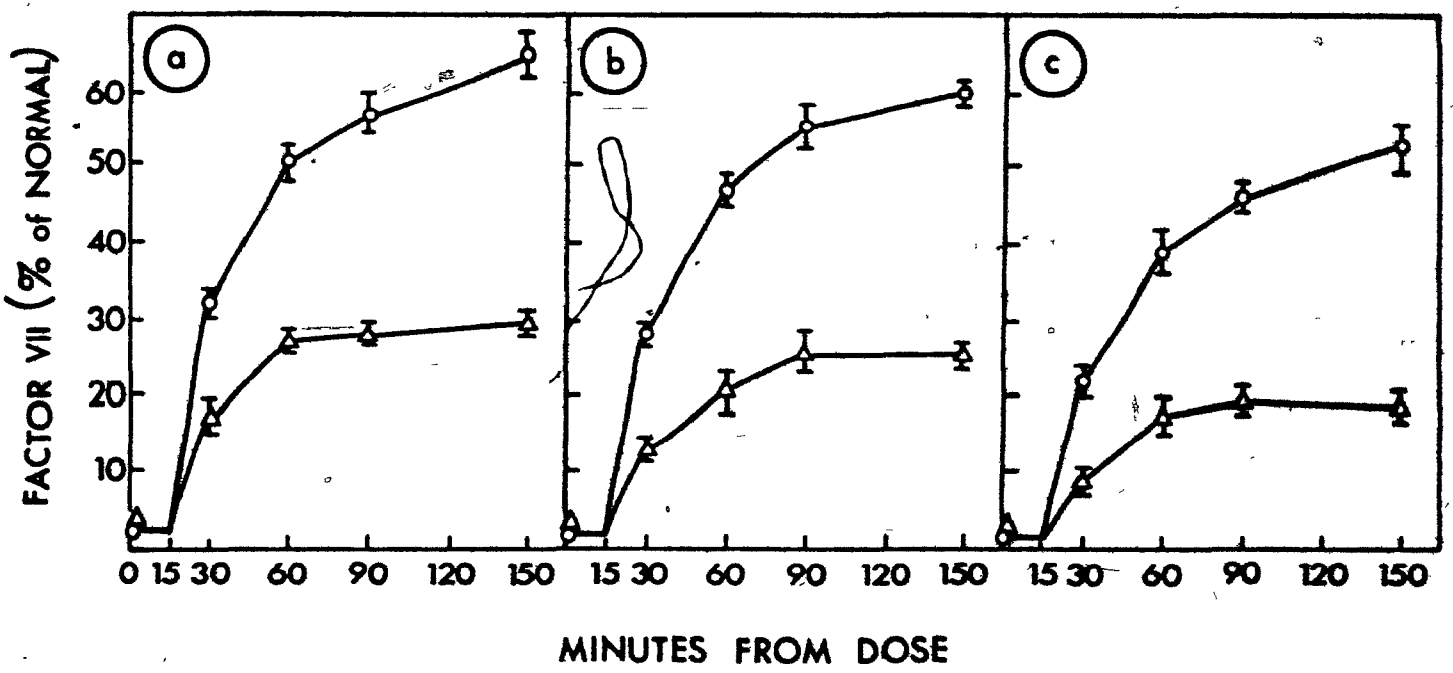


Fig. 18. Time course of response to graded doses of: (a) the trans isomer of vit. K_1, (b) 2',3'-dihydro vit. K_1 from the reduction of trans vit. K_1, (c) 2',3'-dihydro vit. K_1 from the reduction of cis vit. K_1, 20 µg/100 g, 40 µg/100 g. (All results are the mean of at least 5 different experiments in coumarin anticoagulant pretreated rats).

	Dose of the trans vit. K_1 isomer		Dose of 2',3'-dihydro vit. K_1 (from trans)		Dose of 2',3'-dihydro vit. K_1 (from cis)	
	40 µg	20 µg	40 µg	20 µg	40 µg	20 µg
	71.5	30	63	20	60	17.5
	57	30	62.3	23	46.5	21.8
	70	30.5	53	28.6	54	17.8
	71.5	27.8	61	29	50.2	23.5
	56	26.9	61	27	50	13.8
Total	326	145.2	300.3	127.6	260.7	94.4
Mean	65.2	29.04	60.06	25.52	52.14	18.88

Table V. Increase of the plasma level of factor VII in coumarin anticoagulant pretreated rats 150 min. after administration of 20 and 40 µg/100 g of the trans isomer, 2',3'-dihydro vit. K_1 from the reduction of trans vit. K_1 and 2',3'-dihydro vit. K_1 from the reduction of cis vit. K_1.

Nature of variation	df	sum of squares	mean square	F	P
(1) Preparations	2	688.46	344.23	14.9	<0.05
(2) Regression	1	9006.40			
(3) Deviation from Parallelism	2	10.56	5.28	0.23	>0.05
(4) Between doses	5	9705.43			
(5) Error	25	554.41	23.10		
TOTAL	29	10259.84			

Table VI. Analysis of variance of the data from Table V.

(Details of calculation are given in the appendix)

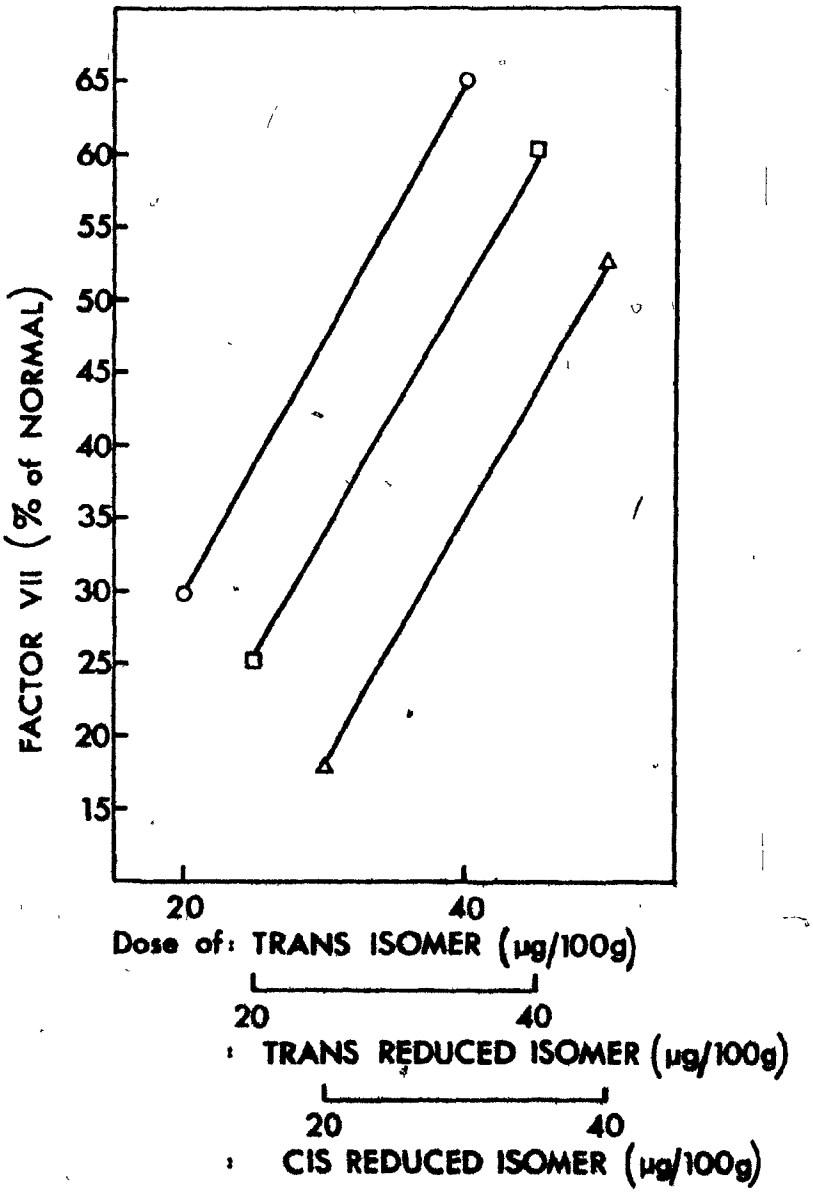


Fig. 19. Dose response curves for graded doses of the trans isomer of vit. K_1, 2',3'-dihydro vit. K_1 from the reduction of trans K_1, 2',3'-dihydro vit. K_1 from the reduction of cis vit. K_1. Curves are calculated from data of Table V, details of calculation are given in the appendix.

of the method of purification. There is also the possibility that the ratio of the optical isomers, due to the formation of a new asymmetric center at the 3'-position, may be different for the reduction of the two geometric isomers (131). Either one of these factors could be responsible for the lower activity of the reduction product of the cis isomer, but the first, the presence of impurities, is considered to be the more likely one.

In the previous experiments 1 μg of cis was estimated to have the activity of 0.11 μg of the trans isomer. / Reduction of the cis isomer, therefore, results in an approximately 8 fold increase of activity and also in a faster onset of action. Within statistical limits the reduced trans isomer has the same activity, while the reduced cis has only 79% of the activity of the trans isomer.

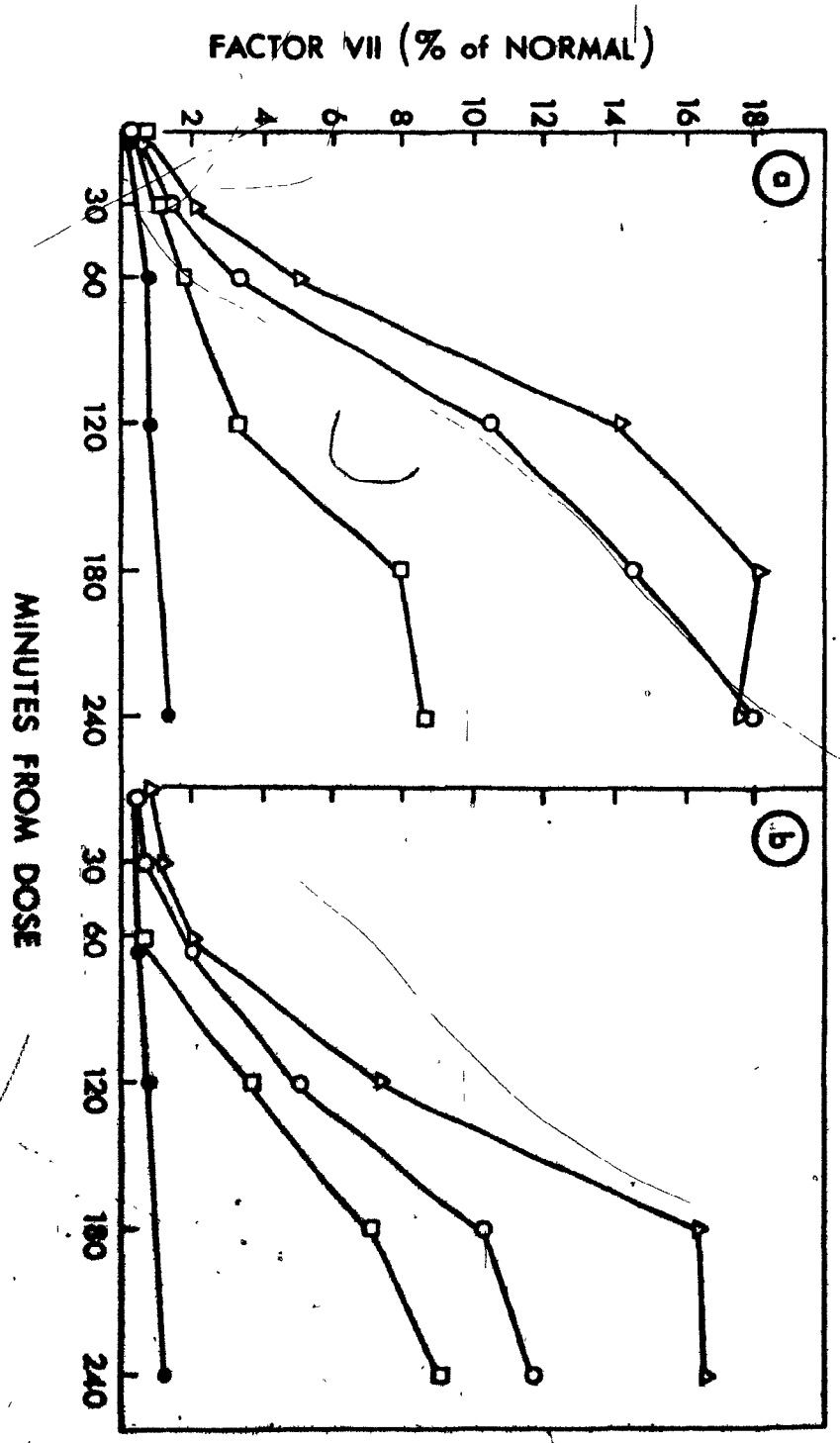
It was considered of interest to carry out preliminary experiments with the cis and the trans isomer with liver slices and microsomes from vit. K deficient animals. These systems have been used before in our laboratory and it has been shown that the formation and release of factor VII, after vit. K_1 (racemic) is added to such systems "in vitro", have physiological properties. For example, in these systems, as in intact animals, the response is inhibited by the chloro analogue of vit. K_1 and, in addition, the presence of factor VII and factor II can be demonstrated immunologically (106). Fig. 20 shows the results obtained with liver slices. The trans isomer gave an optimal response at a final concentration of 10^{-5} M. While 5×10^{-5} M gave the same response after 240 min., the rate of increase was slightly slower. The cis isomer gave the same optimal response as the trans isomer but at a concentration 10^{-3} M.

Fig. 20. Comparison of the activity of the trans and cis isomers of vit. K_1 in liver slices from vit. K deficient rats.

(a) trans isomer; • no vitamin, □ 10^{-6} M, ○ 10^{-5} M, △ 5×10^{-5} M.

(b) cis isomer; • no vitamin, □ 5×10^{-5} M, ○ 5×10^{-4} M, △ 10^{-3} M.

0.5 g of slices in 5 ml of bicarbonate buffer, pH 7.3. The incubation was carried out in a Dubnoff shaker at 37°C under an atmosphere of 95% O_2: 5% CO_2. Concentration of factor VII is expressed as percent of its concentration in normal rat plasma. Vit. K_1 isomers solubilized with Tween 80. Slices prepared by the method of Deutsch (132). At the times indicated a sample was removed from the medium for determination of factor VII.


FACTOR VII (% of NORMAL)
2
4
6
8
10
12
14
16
18
a
b
MINUTES FROM DOSE
30
60
120
180
240

This difference is of the same order of magnitude, about 50 to 100 times, as in vit. K deficient rats. It is also of interest to note that the difference between the onsets of action is the same as in the intact animals. The trans isomer already gave a slight, but measurable response after 30 min., while with the cis isomer there was no response after 30 min. and only after 60 min. was there a response.

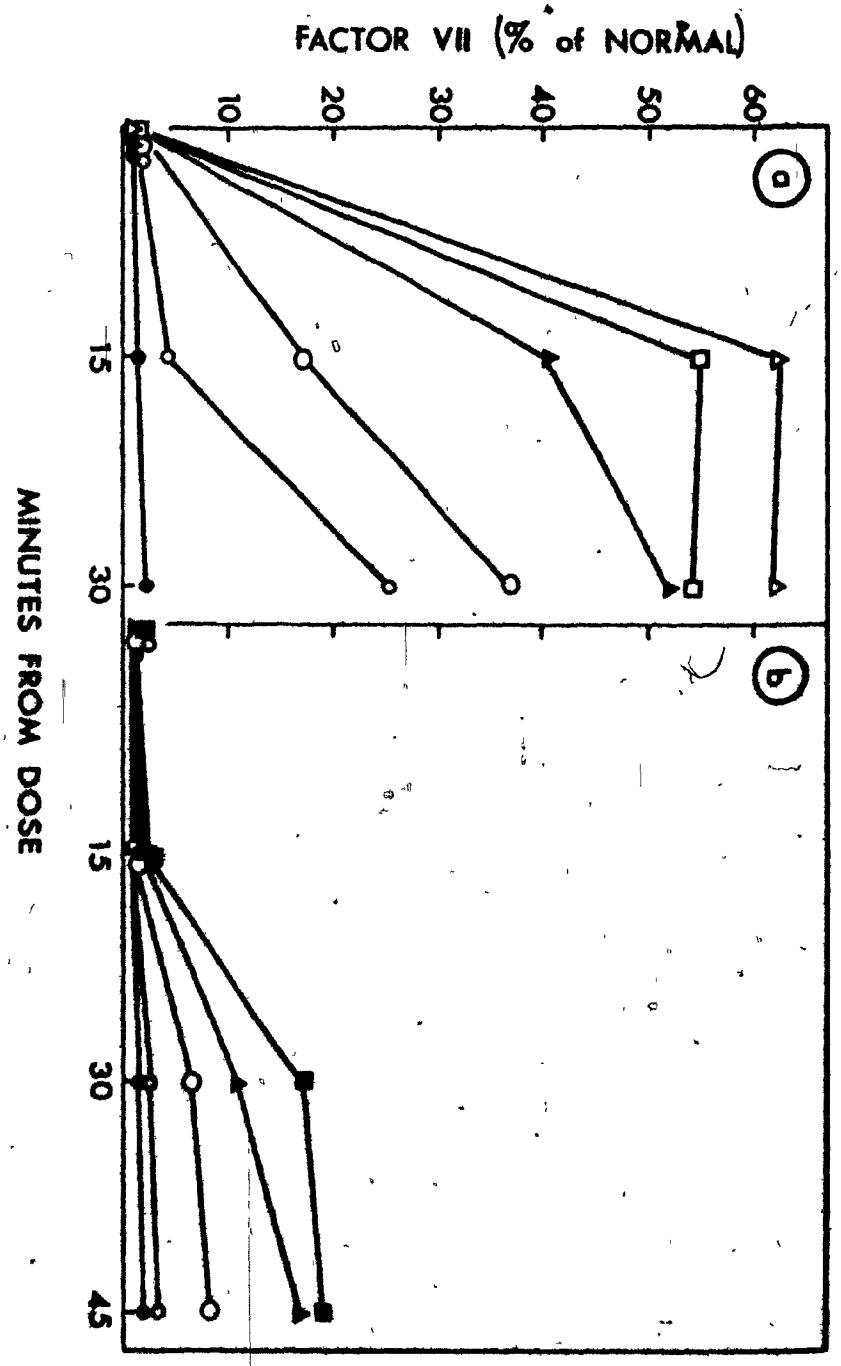
A similar comparison was carried out with liver microsomes from vit. K deficient animals (Fig. 21). This preparation will release factor VII after addition of the hydroquinone of vit. K_1 without the requirement for any other cofactors (106), provided vit. K_1 is solubilized with the nonionic detergent Lubrol. In this system the trans isomer gives an optimal response at a concentration between 5×10^{-5} and 10^{-4} M. The response to the cis isomer, at a final concentration of 10^{-3} M, is considerably less than that to the trans isomer, however, it is not possible to test in this system concentrations higher than 10^{-3} M. There is also a difference in the onset of action after 15 min. The trans isomer has already produced an effect, while there is no effect with the cis isomer. The difference between the activity and the onset of action of the two isomers are, therefore, similar in the "in vitro" system and in intact animals.

Fig. 21. Comparison of the activity of the trans and cis isomers of vit. K_1 in liver microsomes (100 x 10^3 g pellet) from vit. K deficient rats. Microsomes suspended in one-sixth of the volume of the postmicrosomal supernatant. 0.5 ml of suspension in 5 ml incubation media. Incubation media in mmoles/L: NaCl 110.0, KCL 2.0, $MgSO_4.7H_2O$ 0.9, $MgCl_2.6H_2O$ 1.0, Na_2HPO_4 2.0, KH_2PO_4 6.0, $KHCO_3$ 27.0, $CaCl_2$ 0.8, (133). Incubation carried out in a Dubnoff shaker at 37°C under an atmosphere of 95% O_2: 5% CO_2.

(a) trans isomer of vit. K_1; • no vitamin, ○ 10^{-5} M, ○ 2.5×10^{-5} M, ▲ 5×10^{-5} M, □ 7.5×10^{-5} M, △ 10^{-4} M.

(b) cis isomer of vit. K_1; • no vitamin, ○ 10^{-4} M, ○ 2.5×10^{-4} M, ▲ 5×10^{-4} M, ■ 10^{-3} M.

Vit. K_1 isomers solubilized in Lubrol and reduced to hydroquinone by addition of equimolar amount of sodium hydrosulfite ($Na_2S_2O_4$). At the times indicated a sample was removed from the medium for determination of factor VII.


FACTOR VII (% of NORMAL)
10
20
30
40
50
60
a
15
30
b
15
30
45
MINUTES FROM DOSE

DISCUSSION

The present results demonstrate that the trans isomer of vit. K_1 has greater biological activity than the cis isomer and allow a quantitative estimate of this difference in vit. K deficient and coumarin anticoagulant pretreated animals. In the vit. K deficient rats the trans isomer is approximately 67 times more active than the cis isomer, while in the coumarin anticoagulant pretreated animals, the trans isomer is approximately 9 times more active. In addition, the trans isomer has a faster onset of action, particularly in coumarin anticoagulant pretreated animals (about 30 min. compared to 60 min.).

These results are at variance with the report of Matschiner and Knauer (117) that the cis isomer lacks biological activity. In their experiments the activities of partially purified preparations of the two isomers, containing 90% trans: 10% cis and 90% cis: 10% trans were compared. After injection of a single dose of 2 µg per animal to vit. K deficient rats, 2 µg of the cis isomer showed less activity than 0.2 µg of a sample containing predominantly the trans isomer (95% trans: 5% cis). Since the sample of the cis isomer (2 µg) contained approximately this amount of the trans isomer (10% or 0.2 µg), Matschiner concluded that the cis isomer lacked activity. In our experiments the cis isomer had a purity of greater than 99% and 1.6 µg per 100 g of body weight was found to be the lowest dose that gave a response in vit. K deficient animals. Since in Matschiner's report the doses (2 µg) are expressed per rat, assuming a weight per animal of 200 to 300 g, the dose per 100 g would have been only from 0.6 to 1 µg per 100 g and would, therefore, have been insufficient to produce a response.

Is it possible that the activity of the cis isomer observed in our experiments is, likewise, the result of contamination by the trans isomer because of isomerization during the preparation and handling of the solutions before injection? Since the trans isomer is 67 times more active in vit. K deficient animals, transformation of as little as 1.5% of the dose of the cis isomer would have been sufficient to produce the responses observed. In coumarin anticoagulant pretreated animals, however, the trans isomer is only 9 times more active and the transformation of approximately 10% of the cis would have had to occur to account for the activity. Furthermore, if isomerization had occurred before administration one would expect to see a faster onset of action as occurs with the trans isomer. Moreover, reproducible results were obtained with different preparations of the cis isomer at different times, making it unlikely that "uncontrolled" isomerization is responsible for the activity obtained.

The present results have been obtained in intact animals. In contrast to studies with "in vitro" systems, their physiological significance cannot be open to doubt. On the other hand, the final effect measured, the increase of the plasma level of factor VII, is the resultant of an unknown number of secondary interactions such as transport, uptake, distribution and rate of metabolization, in addition to the primary interaction of the vitamin with its specific site of action. Differences between the two isomers at any of these levels could account for the different biological responses. The finding that in slices and in a cell-free system the difference is of the same order of magnitude as in intact animals excludes the possibility that the secondary interactions

are responsible for the difference. From this, it can be concluded that the difference of the activity of the two isomers can be traced to the specific site of action.

What is the functional significance of these findings? The mechanism of action of vit. K at the molecular level, including its possible role in the carboxylation of clotting factors is still unknown. Vit. K_1 is one of a group of lipophilic para-quinones including vit. K_2 (2-methyl-3-difarnesyl-1,4-naphthoquinone) which is found in the membranes of certain bacteria, coenzyme Q (2-methyl-5,6-dimethoxy-3-polyisoprenoid-1, 4-benzoquinone) which is present in mitochondrial and bacterial membranes, and plastoquinone (5,6-dimethyl-1,4-benzoquinone) which is present in chloroplasts. In general, these quinones function as part of electron transport systems because they can be reversibly reduced and oxidized, but in many cases their exact function is still uncertain. The most extensively studied member of this group is ubiquinone or coenzyme Q, whose aromatic moiety has a 2-methyl-5,6-dimethoxy-para-benzoquinone structure. This quinone is a component of the mitochondrial terminal electron transport chain and functions between the flavin dehydrogenases and the cytochrome system. When originally isolated, it was believed that it might act as a coupling factor between terminal electron transport and oxidative-phosphorylation. Failure to establish such a role for ubiquinone or to demonstrate the presence of coupling factors in general led Mitchell to propose in 1961 the chemi-osmotic theory of oxidative-phosphorylation, according to which the association of terminal electron transport with proton dislocation across the mitochondrial membrane is responsible for oxidative-phosphorylation (134). Klingenberg has made the additional suggestion that ubiquinone, because it can be reduced and

oxidized reversibly by virtue of its para-quinone structure, could function as a proton carrier. Such a function would require also a certain mobility within the membrane structure, so that protons could be accepted and donated at opposite interfaces of the membrane (135). It seems not unreasonable to assume as a working hypothesis that, because of its similar structure, vit. K_1 could function at the molecular level in a similar manner and to try to explain the difference between the biological activity of the trans and cis isomers within the context of this hypothesis. Such a function would require, in addition to a certain mobility and flexibility, a precise orientation within the three dimensional membrane structure of the part of the vit. K_1 molecule that carries the functional group, the para-naphthoquinone; this part of the molecule would have to make contact or combine with the conjugate systems that donate or accept protons on the opposite interfaces of the membrane. The finding that the particular microsomal system that responds to vit. K_1 is derived from an intracellular membrane structure lends additional support to this hypothesis. It has been pointed out already in the discussion of structural requirements for vit. K-like activity, that the size of the aromatic nucleus, in general, and that of the substituent at the 2-position, in particular, appear to be critical for combination (affinity), and it can therefore be assumed that this part of the vit. K molecule, which also carries the functional group, the para-quinone structure, combines with the active sites of the conjugate systems. If, for example, the 2-methyl-1,4-naphthoquinone moieties of the cis and trans isomers are oriented identically, it can be seen from molecular models that the orientation of the 3-lipophilic side chain will be

different, because of the absence of free rotation about the 2',3-double bond (Fig. 22 (a) and (b)). The same considerations would apply if the naphthoquinone moiety of either isomer were to combine identically with the active sites of an enzyme.

This difference in orientation of the 3-side chain might be sufficient to abolish or reduce the ability of the cis isomer to function as a proton carrier. In the first case, the cis isomer would, therefore, only become active after it has been transformed "in vivo" to the trans isomer. In vit. K deficient animals, where only a transformation of 1 to 2% would be required, the cis isomer shows a slightly slower onset of action (Fig. 12). In coumarin pretreated animals, where a transformation of approximately 10% is required to account for the activity observed, the onset of action of the cis isomer is considerably slower, 60 min. compared to 30 min. (Fig. 15). On the other hand, the different orientation of the side chain, by restricting the mobility or changing the orientation, may simply reduce the efficiency of the cis isomer as a proton carrier. The experimental evidence at hand is insufficient to distinguish between these two possibilities, although the difference in the onset of action is more easily explained by the first.

Reduction of the 2',3-double bond of the trans isomer leaves the activity unchanged, but increases the activity and the speed of onset of the cis isomer to that of the trans isomer. Inspection of molecular models shows that the spatial orientation of the 2',3-dihydro derivative is similar to that of the trans isomer (Fig. 22(a) and (b)). Furthermore, after reduction there is free rotation about the bond at the 2',3-position and, therefore, increased flexibility.

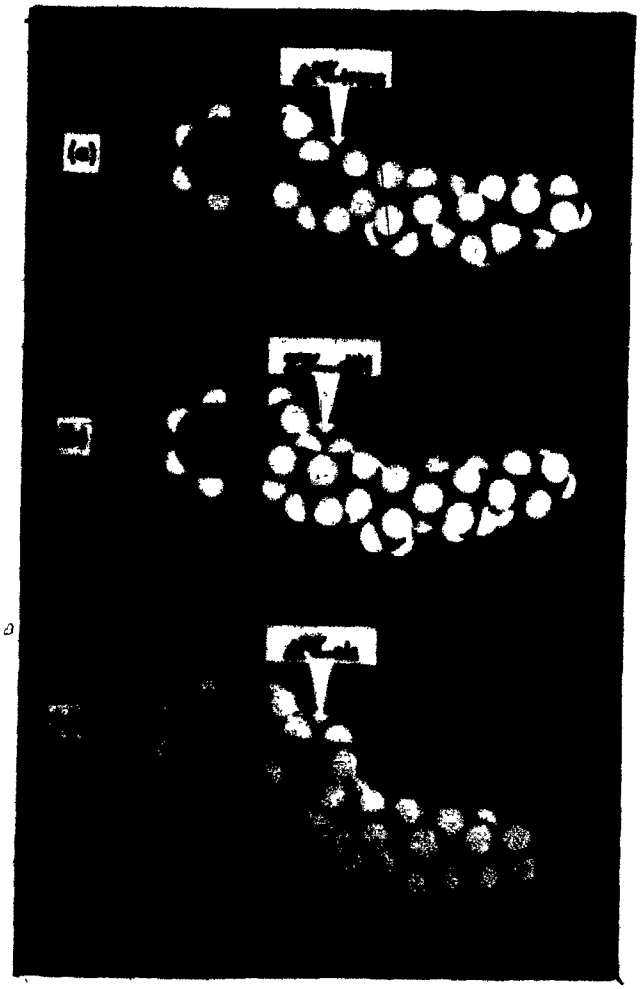


Fig. 22. CPK molecular models of: (a) the trans isomer of vit. K_1, (b) 2',3'-dihydro vit. K_1, (c) the cis isomer of vit. K_1.

The 2',3'-dihydro derivative of vit. K_1 was first synthesized by Karrer in 1940 (118) who reported that it had 1/24 of the activity of vit. K_1. Fieser et al (111) prepared the dihydro derivative by a different method in 1941 and reported 1/8 of the activity of vit. K_1. Since these results were reported, more efficient methods for the purification, such as T L C, and for determination of the purity, such as N M R, have become available. We also found that catalytic reduction of vit. K_1 gave, in addition to the reduction of the 2',3'-double bond, some reduction of the naphthoquinone nucleus, and encountered difficulties, particularly with the reduction product of the cis isomer, in eliminating these byproducts. These byproducts differ only by 2 or 4 hydrogen atoms, so that the contamination would not have been detected by the techniques of carbon hydrogen analysis and ultraviolet spectra used by Karrer and by Fieser as criteria for purity. They do, however, show up in the N M R spectra, particularly for the byproducts of the cis isomer.

The retention of activity after reduction of the double bond makes it unlikely that trans-cis isomerization plays a role in the mechanism of action of vit. K_1. This possibility was suggested by Brodie (136) in connection with vit. K_1 dependent oxidative-phosphorylation in vit. K depleted preparations from M. Phlei. Brodie reported that only the trans isomer of vit. K_1 was capable of restoring activity and that this was associated with its isomerization to the cis isomer. This could not be confirmed by Di Mari et al (137) who traced the isomerization to non-specific photocatalysis because of poor experimental controls.

If vit. K_1 were to act as a proton carrier within or across a

lipophilic membrane structure, how could such a function be related or directly coupled to the carboxylation of a saturated aliphatic acid like the glutamyl residues of a polypeptide? Structural analysis leaves little doubt that the vit. K-dependent clotting factors contain γ-carboxyglutamic acid residues and that the inactive variants found in the plasma of vit. K deficient and coumarin anticoagulant pretreated animals lack the ability to bind Ca ion because of their absence (26,92,93). Moreover, vit. K_1-dependent carboxylation in cell-free systems has been measured by the incorporation of C^{14} labelled in CO_2 in at least seven laboratories (95,100,101,102,103,104,105). It is of course possible that in partially purified cell-free systems the formation or activation of the clotting factors involves an interrelated sequence of reactions, including carboxylation, in addition to a separate vit. K-dependent step. This argument cannot, however, apply to the experiments in which it has been claimed that simple glutamyl containing pentapeptides are carboxylated in the presence of vit. K_1 by a microsomal preparation (105,138). Assuming the validity of these experiments, one must, therefore, assume that the carboxylation-dependent on vit. K takes place by a mechanism entirely different from any of the presently known biological carboxylations. While the hydroquinone of vit. K_1 could supply the reducing equivalents and energy for a reductive type of carboxylation, there still remains the problem of how an aliphatic acid could be carboxylated by such a mechanism without energy-dependent activation. In addition it should be noted that the response to vit. K_1 in the cell-free system must also involves the release of the clotting factor protein from the particulate microsomal preparation. By what mechanism and how closely this release is coupled

or integrated with the carboxylation is a problem that does not seem to have until now received any attention.

A systematic study of the structural requirements for vit. K-like activity in intact animals may provide additional information and an indication of the as yet unknown mechanism of action of the vitamin. Since there should be a correspondance between the activity in intact animals and in "in vitro" system, such studies should also be useful to evaluate the significance of results obtained with "in vitro" systems.

SUMMARY

(1) Vit. K_1 has been separated into its 2',3'- trans and cis isomers by TLC to a purity of better than 99% as indicated by TLC and NMR.

(2) The activity of each of the two isomers was estimated in vit. K deficient and coumarin anticoagulant pretreated rats after intravenous administration. The results were evaluated by an analysis of variance as a 6 point parallel line assay.

(a) In vit. K deficient animals the cis isomer had 1.5% of the potency of the trans isomer with 5% fiducial limits at 1.1% and 1.9%.

(b) In coumarin anticoagulant pretreated rats the cis isomer had 11% of the potency of the trans isomer with 5% fiducial limits at 9% and 13%.

(c) In addition, the cis isomer had a slower onset of action, particularly in coumarin pretreated rats (60 min compared to 30 min.).

(3) The trans and cis isomers were reduced catalytically to 2',3'-dihydro vit. K_1. Hydrogenation did not change the activity of the trans isomer, but increased the activity of the cis isomer to that of the more active trans isomer.

(4) Approximately the same difference was found between the activity of the two isomers on the release of factor VII from liver slices and microsomes from vit. K deficient rats when added to these systems "in vitro".

(5) The results can be explained by the hypothesis that the mechanism of action of vit. K involves proton transport across a lipophilic membrane structure, and that the difference between the activity of the trans and cis isomers arises from the different spatial orientation of the phytyl side chain because of the presence of the 2',3'-double bond and the effect this will have on the orientation and flexibility of the naphthoquinone

moiety of the vit. K_1 molecule. The finding that when the double bond is reduced, activity of the trans isomer is retained and activity of the cis isomer is increased to that of the trans isomer is in agreement with this explanation, and also demonstrates that the double bond is not an obligatory requirement for activity.

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APPENDIX (Summary of Statistical Calculations)

Component	Polynomial coefficients t_1	t_2	t_3	C_1	C_2	C_3	Division
(1) Preparations	-1	-1	-1	1	1	1	30
(2) Regression	-1	0	1	-1	0	1	20
(3) Deviation from Parallelism	1	0	-1	-1	0	1	20
(4) Curvature	1	-2	1	1	-2	1	60
(5) Diff. from curvature	-1	2	-1	1	-2	1	60

Table VII. Polynomial coefficients for the calculation of the component of analysis of variance for data from Table I and Table III.

Analysis of variance and estimation of relative potency of the cis and trans isomersof vit. K_1 in vit. K deficient rats: Data from Table I.

(1) Preparations = $[-78.4-144.4-214.9+38.9+77.2+193.2]^2 \div 30$

= 549.552

(2) Regression = $[-78.4+214.9-38.9+193.2]^2 \div 20$

= 4228.23

(3) Deviation from Parallelism

= $[(214.9-78.4)-(193.2-38.9)]^2 \div 20$

= 15.488

(4) Curvature = $[(78.4+214.9)-2(144.4)+(38.9+193.2)-2(77.2)]^2 \div 60$

= 112.61

(5) Difference of Curvature

$$= [(78.4+214.9)-2(144.4)-(38.9+193.2)-2(77.2)]^2 \div 60$$

$$= 89.3$$

$\bar{y}_t$ = intercept of trans curve

$\bar{y}_c$ = intercept of cis curve.

$\bar{y}_t = (78.4+214.9) \div 10 = 29.33$

$\bar{y}_c = (38.9+193.2) \div 10 = 23.21$

b = slope for both curves

$b = (214.9-78.4+193.2-38.9) \div 20$

$b = 14.54$

Relative potency = R

$$\log R = \log \frac{0.125}{6.4} - 0.42 \times 0.301$$

$$= -1.835$$

$R = \text{antilog } \bar{2}.165$

$R = 0.015$

Fiducial limits of R

$t_{0.05,\ 24\ d.f.} = 2.06$

g criterium

$$g = \frac{50 \times (2.06)^2}{(14.54)^2 \times 20} = 0.05 \quad <0.1$$

M = difference between equally effective doses

V(M) = variance of M

$$V(M) = 50 \div (14.54)^2 \times [1/15 + 1/15 + (-0.42)^2 \div 20]$$

$$= (0.18)^2$$

Fiducial limit (below) = antilog $\bar{2}.06$

= 0.011

Fiducial limit (above) = antilog $\bar{2}.2767$

= 0.019

Analysis of variance and estimation of potency of the cis and trans isomers of vit. K_1 in coumarin anticoagulant pretreated rats: data from Table III. Polynomial coefficients were used from Table VII.

(1) Preparations = $[-48.5-139.3-253.5+28+106.7+235.5]^2 \div 30$

= 168.51

(2) Regression = $[-48.5+253.5-28+235.5]^2 \div 20$

= 8507.81

(3) Deviation from Parallelism

= $[(253.5-48.5)-(235.5-28)]^2 \div 20$

= 0.31

(4) Curvature = $[(48.5+253.5)-2(139.2)+(28+235.5)-2(106.7)]^2 \div 60$

= 90.04

(5) Difference of Curvature

= $[(48.5+253.5)-2(139.2)-(28+235.5)-2(106.7)]^2 \div 60$

= 11.88

Calculation of intercepts

$\bar{y}_t = (48.5+253.5) \div 10 = 30.2$

$\bar{y}_c = (28+235.5) \div 10 = 26.35$

$b = (253.2-48.5+235.5-32) \div 20$

$b = 20.43$

Calculation of R

$$\log R = \log \frac{10}{80} - 0.17 \times 0.301$$

$$= -0.953$$

$$R = \text{antilog } \bar{1}.047$$

$$R = 0.11$$

Fiducial limits of R

$$g = \frac{50 \times (2.06)^2}{(20.43)^2 \times 20} = 0.03 \quad < 0.1$$

$$V(M) = 50 \div 20.43^2 \times [1/15 + 1/15 + (-0.17)^2 \div 20]$$

$$= (0.127)^2$$

Fiducial limit (below) = antilog $\bar{2}.96814$

= 0.09293

Fiducial limit (above) = antilog $\bar{1}.12586$

= 0.13362

Analysis of variance and estimation of the relative potency of 2',3'-dihydro vit. K_1 obtained by reduction of the cis and trans isomers of vit. K_1 by comparison with the trans isomer of vit. K_1.

Table for calculations

	Preparations	low	High	Total	High-low
(1)	Trans	145.2	326	471.2	180.8
(2)	Trans reduced	127.6	300.3	427.9	172.7
(3)	Cis reduced	94.4	260.7	355.1	166.3
	TOTAL	367.2	887	1254.2	518.8

(1) Preparations $= (471.2^2 + 427.9^2 + 355.1^2) \div 10 - 1254.2^2 \div 30$

$= 688.46$

(2) Regression $= 519.8^2 \div 30$

$= 9006.4$

(3) Parallelism $= (180.8^2 + 172.7^2 + 166.3^2) \div 10 - 9006.4$

$= 10.56$

Calculation of intercepts

$\bar{y}_t = 326 + 145.2 \div 10 = 47.12$

$\bar{y}_{t\ \text{reduced}} = (127.6 + 300.3) \div 10 = 42.79$

$\bar{y}_{c\ \text{reduced}} = (94.4 + 260.7) \div 10 = 35.51$

$b = 518.8 \div 30$

$b = 17.33$

Relative potency trans- 2',3'-dihydro vit. K_1 (from trans vit. K_1 reduction)

$R = \text{antilog } \bar{1}.96239$

$R = 0.917$

Fiducial limits of R

$$g = \frac{23.10 \times (2.06)^2}{(17.33)^2 \times 30} = 0.0108 < 0.1$$

$V(M) = 23.10 \div 17.33^2 \times [1/10 + 1/10 + (-0.24986)^2] \div 30$

$= (0.12467)^2$

Fiducial limit (below) = antilog $\bar{1}.92373$

$= 0.84$

Fiducial limit (above) = antilog 1.99895

$= 1$

Relative potency trans- 2',3'-dihydro vit. K_1 (from cis vit. K_1 reduction)

R = antilog $\bar{1}.99916$

R = 0.793

Fiducial limits of R

$g = 0.0108 < 0.1$ (the same that above)

$V(M) = 23.10 \div 17.33^2 \times [1/10 + 1/10 + (-0.67)^2 \div 30]$

$= (0.12865)^2$

Fiducial limit (below) = antilog $\bar{1}.85927$

= 0.72

Fiducial limit (above) = antilog $\bar{1}.93905$

= 0.87

For further details see References 129 and 130.