

VITAMIN K

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I. INTRODUCTION

Owing to the methods contributed and perfected by biologists and chemists, the tempo of research in the vitamin field during the past twenty to thirty years has changed from a snail's pace to one so rapid that a worker in a particular field is often taxed to assimilate the numerous papers that appear each month. This is best illustrated by the recent developments with vitamin K. Since 1934, when Dam showed that an unknown fat-soluble dietary factor (vitamin K) is essential for the coagulation of blood in the chick, two different naturally occurring vitamins (K_1 from alfalfa and K_2 from putrefied fish meal) have been isolated, their structures determined, and one of them synthesized; numerous highly potent simple antihemorrhagic compounds have been prepared to meet requirements of physicians; and clinical investigation has made remarkable progress in the therapeutic applications of vitamin K. It is the purpose of this paper to review the story of vitamin K, which was so rapidly told in the pages of many journals during the past six years.

II. DISCOVERY OF VITAMIN K

The first observation of the symptoms which we now know are attributable to vitamin K deficiency was reported by Dam (52) in 1929. During the course of some experiments on cholesterol metabolism in the chick, he noted that chicks which had been kept on an ether-extracted diet became anemic and developed subcutaneous and intramuscular hemorrhages, and that in one chick the clotting time of the blood was prolonged. In 1930 (53), while still studying cholesterol metabolism, he observed the same hemorrhagic condition in chicks which had been kept on the ether-extracted diet supplemented with lemon juice (which contains the antiscorbutic vitamin). Since these observations were not mentioned in the summaries of these papers, it seems unlikely that Dam at that time fully appreciated the significance of this by-product of his investigation.

During a study of the fat-soluble vitamin requirements of the chick,

McFarlane (142, 143) and coworkers (1931) observed that chicks kept on a diet containing ether-extracted fish meal showed a high mortality during the third and fourth weeks, owing to hemorrhage following the insertion of wing bands. The blood of these chicks did not clot on standing overnight. There were no losses due to hemorrhage when casein or untreated fish meal was used in the diet.

Holst and Halbrook (111), in 1933, described the hemorrhagic condition and stated that it was cured by the addition of cabbage to the diet. They believed that the condition was due to a lack of vitamin C.

Continuing his work, Dam (54, 64) reported in 1934 that the hemorrhagic condition was not due to a lack of vitamins A, C, D, E, B, B₂, of fat, or of cholesterol, and in 1935 (51, 55) he suggested that the hemorrhages and prolonged clotting time were due to the lack of a new fat-soluble factor which he named vitamin K (from *Koagulations-Vitamin*). This terminology was accepted by other investigators but, following the isolation of two compounds having vitamin K activity, subscripts were added to the K for purposes of designation.

III. BIOASSAY OF ANTIHEMORRHAGIC COMPOUNDS

In every investigation directed toward the separation of an active principle (vitamins, hormones, alkaloids, etc.) from a natural source in which the concentration usually is less than 0.001 per cent, success depends upon a procedure of quantitative determination. Each step in which two fractions are obtained must be accompanied by bioassays of both fractions in order to ascertain the result of the step. Consequently, the development of a method of purification leading to the isolation of an active principle must be guided by careful and accurate bioassays. Usually this necessitates thousands of assays if the quest of the unknown is to be successful.

Frequently, as the purification proceeds and information on the chemical or physical properties of the active principle is obtained, chemical or physical methods of detection and measurement may replace the biological. For example, with vitamin K₁, Karrer (120) used the extinction coefficient at λ 248 m μ as a guide in the purification. However, such a replacement of the bioassay is usually impossible until extensive progress in the purification has been attained.

A. DEFICIENT DIETS

The discovery of a vitamin is almost invariably due to the recognition of a new syndrome which depends on the absence of an unknown substance from the diet. In the case of vitamin K, the diet used by Dam (53) had been extracted with ether. The syndrome, consisting of subcutaneous and intramuscular hemorrhages, anemia, and a prolonged clotting time, was

not relieved by the known fat-soluble vitamins (54). Almquist (21) also obtained the syndrome in experiments with chicks in which the following diet was used:

ALMQUIST'S BASAL DIET	
Fish meal, ether-extracted.....	17.5
Brewer's yeast (dry), ether-extracted.....	7.5
Polished rice, ground.....	73.
NaCl plus small amounts of CuSO ₄ and FeSO ₄	1.0
Cod-liver oil.....	1.0

Almquist's diet has been used extensively in this country. In the experience of most laboratories, newly hatched chicks placed on this diet show a definite prolongation of clotting time within 1 week but, owing to biological variability and the mild degree of deficiency, it is necessary to continue chicks on the diet for at least another week. The chicks are suitable for assay after marked prolongation of the clotting time develops, which usually requires 2 or 3 weeks.

According to Ansbacher (28), the deficiency in a severe form can be produced in a much shorter period on a diet in which the fish meal, rice flour, and yeast are replaced by casein, heat-treated cereals, and pure vitamin B supplements, respectively.

B. BASIS OF ASSAY

The basis of all procedures of bioassay for vitamin K is the prolonged clotting time produced by a deficient diet. In normal chicks the clotting time varies from 1 to 5 min.; in chicks which show a severe form of deficiency, the blood frequently fails to clot in 3 hr. (183). Although assays may be conducted on the preventive principle, and Dam (51) and Almquist (22) in their early work used this type of procedure, practically all assays are now based on the curative principle (10, 27, 59, 66, 94, 107, 139, 167, 184, 185, 186). After maintaining a large group of chicks from the same hatch under uniform conditions of diet, etc., for about 2 weeks, the clotting times of a part of the group are determined. If the values are in excess of 60 min., the remainder of the group are suitable for the assay. The substance to be tested is administered, and after an interval blood is drawn to ascertain the response.

Many variations of the general procedure have been used. Dam and Schønheyder (65) administered the vitamin on three successive days and drew blood on the fourth day. Thayer *et al.* (185) used a similar procedure, but after Ansbacher (27) showed that the vitamin exerted its effect in a few hours, they altered their procedure to a single administration and a response period of 18 hr.

The determinations of clotting time range from the simplest, in which a wing vein is punctured, the blood caught in a crucible, and examined at room temperature until clotting occurs (51, 185), to the most refined, which include the taking of blood with a syringe or cannula, the separation of the plasma, the addition of thromboplastin, and the determination of the clotting time in a thermostat (10, 167).

Essentially, the latter procedure is the determination of prothrombin, and since a diminished concentration of this substance (167) is responsible for the prolonged clotting, assays based on its determination would theoretically be superior. However, in the purification leading to the isolation of vitamin K, assays based on the simple clotting time guided workers to the pure compound (185).

C. STANDARDS OF REFERENCE

Owing to the variability in the degree of deficiency produced by the same diet in different lots of chicks, it soon became obvious that a basic standard of reference would be necessary if accurate assays were to be obtained. Although the actual applications of the standard varied, essentially its potency was used as a base for the comparison of the potencies of the products. Dam and Glavind (59) prepared a standard spinach powder, and both Almquist (18) and Thayer (184) used a standard alfalfa extract. After the development of the field of pure antihemorrhagic compounds, Thayer *et al.* (180) suggested the adoption of 2-methyl-1,4-naphthoquinone as a standard and Dam (61) the adoption of 2-methyl-1,4-naphthohydroquinone diacetate.¹

Although the standards were necessary in the purification of the active principles of crude extracts, it appears that the adoption of the crystalline compounds as standards is not entirely satisfactory. Many factors must be considered in the comparison of a substance with a standard. Among the factors which affect the potency are: (1) the method of administration, i.e., oral, intravenous, etc.; (2) differences in the rate of absorption of the standard and the unknown; (3) the medium in which the compounds are administered; (4) differences in the rate of metabolism and excretion of the standard and the unknown; and (5) the time interval allowed for response. Consequently, it is not surprising that various investigators find different ratios of the potencies of 2-methyl-1,4-naphthoquinone and vitamin K₁.

In addition to the assay of compounds for antihemorrhagic potency, it is necessary to ascertain their toxicity before therapeutic use. The most extensive report (146) indicates that no fear should be entertained regarding the toxicity of either vitamin K₁ or 2-methyl-1,4-naphthoquinone in the dosages therapeutically effective.

¹ The solubility in water probably is so low that this compound could not be used as a standard for assays in which the substances are administered intravenously.

D. PROTHROMBIN DEFICIENCY IN MAMMALS

As every biologist knows, the choice of the experimental animal is highly important. Had Dam (53), McFarlane (143), and Almquist (23) used mammals in their experiments, it is doubtful whether vitamin K would have been discovered. After discovery of the hemorrhagic syndrome in chicks, Dam and Glavind (60, 62) had difficulty in producing prolonged clotting times in mammals, but eventually they succeeded with both rabbits and rats. From a large number of rats, Greaves (99) finally obtained a few showing prolonged clotting time as a result of a dietary deficiency. Recently, Elliott, Isaacs, and Ivy (68) have shown that rats fed on a diet containing mineral oil (20 per cent) soon showed a marked lowering of prothrombin, and have suggested that rats prepared by this method could be used for the bioassay of antihemorrhagic compounds. Rats in which the common bile duct has been ligated may also be used for assay (93).

IV. OCCURRENCE AND DISTRIBUTION OF ANTIHEMORRHAGIC SUBSTANCES

Following the early development of bioassay procedures by Dam (51), Schönheyder (167, 168), and Almquist (3, 20), many possible sources of the vitamin were surveyed. Dam and his collaborators (49, 51, 56, 58, 65) showed that the antihemorrhagic factor is widely distributed in green leaves and vegetables. Chestnut leaves are the most potent source, but alfalfa, cabbage, spinach, grass, cauliflower, and nettle also are rich sources of the factor. Seeds (sunflower, hemp, soybean, pea, oats, wheat, and yellow corn), roots (carrots and potatoes), fruits (strawberry, hips, and ripe tomatoes), and the lower plants (moss, lichen, fungus, seaweed, and mushroom) contain only a limited amount of the vitamin. Cod-liver oil and wheat-germ oil, which are good sources of vitamins A and E, respectively, contain no vitamin K. Furthermore, Dam (55) reported that the vitamin could not be detected in beef muscle, lung, kidney, adrenals, calf brain, and thymus, but that a diet containing 20 per cent of hog liver will prevent the disease. In his early work, hog-liver fat was used as the source of the antihemorrhagic substance. Almquist and Stokstad (20, 23) reported in 1935 that the addition of 0.5 per cent of dry alfalfa to the deficient diet (see page 480) prevented the appearance of hemorrhagic symptoms, and from that time alfalfa has been one of the main sources of the vitamin.

In contrast to the distribution of carotene in carrots, vitamin K occurs in the leafy portion, but not in the roots (9). This suggested a relationship between photosynthesis and the abundance of vitamin K. Further evidence in favor of this view was furnished by Dam (58), who showed that

a much larger amount of vitamin K is found in peas grown in light than in peas grown in the dark.

In the egg the vitamin is localized in the yolk (21, 51). The reserve in the newly hatched chick is influenced by the level of vitamin K in the diet of the hen (21). The liver of the chick contains very little vitamin (21, 65), whereas, as previously noted, 20 per cent of hog liver in the diet will prevent the disease (55).

Discovery of a second antihemorrhagic factor resulted from an observation of Almquist and Stokstad (20, 23) that the protein foods used in the diets often contained a protective factor and that rice bran, fish meal, and other foods which had been stored in a moist condition developed vitamin K activity. That this production was due to the action of microorganisms was definitely concluded from Osterberg's (149) work, which showed that bacterial putrefaction of fish meal gives a highly potent antihemorrhagic product and from the report (Almquist, Pentler, and Mecchi (19)) that a large number of bacteria, including *M. tuberculosis*, *B. coli*, *B. cereus*, and *B. subtilis*, synthesize a fat-soluble antihemorrhagic factor which is not released or excreted into the culture medium. In general, microorganisms of the mold, yeast, or fungus types are inactive (9). Limburger cheese is active, whereas acidophilus milk and buttermilk contain no detectable antihemorrhagic potency.

Almquist and Stokstad (21) showed that a large amount of an ether-extractable antihemorrhagic factor is present in the fecal matter of chicks receiving a vitamin K deficient diet. Apparently this substance is synthesized by bacteria in the lower portions of the intestinal tract. McKee *et al.* (145) found appreciable quantities of vitamin K in horse, cow, sheep, hog, and human feces.

V. ISOLATION OF VITAMIN K

A. VITAMIN K₁

1. *Properties of impure preparations*

The early work on the purification of vitamin K was conducted chiefly by Almquist and Dam. In 1935, Dam (51, 55), working with hog-liver fat, reported that the active factor was extractable with ordinary fat solvents, was partially destroyed by cold saponification, and occurred in the non-saponifiable non-steroid fraction. Heating at 100°C. for 12 hr. did not destroy the activity. In the same year, Almquist (5, 20) showed that the factor in alfalfa occurred in the ether-soluble non-saponifiable fraction and furnished evidence which indicated that the vitamin was not an acid, ester, or base. He likewise found the vitamin to be thermostable. This observation led him (4) to introduce molecular distillation as a means

of purification. Almquist (3, 5, 8) found that the vitamin was labile toward alkali, and this observation has been confirmed repeatedly by other investigators (9, 65). In the same paper (3) Almquist reported the preparation of crude concentrates. He found that less extraneous material was extracted with the active factor from dehydrated alfalfa by hexane than by other solvents. Since this report, hexane and petroleum ether have been extensively used for preparing crude extracts.

Chlorophyll and xanthophyll were removed from hexane extracts by adsorption on activated magnesium oxide (Micron brand) and activated carbon, respectively. Other impurities were removed by partition between 90 per cent methanol and petroleum ether, the vitamin remaining in the methanol layer. The products thus obtained were subjected to molecular distillation (4, 5), the active material distilling at 120° to 140°C. at 10^{-6} mm. In 1937, Almquist (8) reported that concentrates of this type gave an active crystalline preparation when absolute methanol solutions were chilled with solid carbon dioxide. However, in the light of the present knowledge of the vitamin, it seems likely that the activity must have been due to adsorption on the surface of the crystals. Later, Almquist modified his concentration procedure somewhat, using Lloyd's reagent (7) and phosphotungstic acid (126) instead of magnesium oxide for the removal of plant pigments.

Dam (50, 63, 65) used acetone for extraction of the vitamin. He obtained partially purified products by precipitating impurities by chilling ethanol and acetone solutions and by partition between 90 per cent methanol and petroleum ether. However, differing from Almquist, he obtained the vitamin in the petroleum ether layer (65). Adsorptions on calcium carbonate and sucrose were effective in removing some of the impurities, and distillation at 115–140°C. at 10^{-3} mm. increased the potency of the product. In a study of adsorbents, he reported that the vitamin was destroyed by aluminum oxide, magnesium oxide, silicic acid, and calcium sulfate and was only weakly adsorbed by calcium carbonate and sugar. Still another adsorbent—florex—was used by Riegel (163) to effect a partial purification of vitamin K_1 .

Almquist (11) reported that the vitamin could be separated from concentrates as a choleic acid. However, other investigators, using more highly purified products (37, 163), have been unable to prepare a choleic acid of the vitamin.

(a) Characterization

Although it seems likely that none of the preparations described prior to 1939 contained more than a few per cent of the vitamin and that chemical data on such products are often of little value and sometimes actually mis-

leading, a number of significant observations were made. Almquist reported the absence of sulfur, phosphorus, and nitrogen in his most active preparations (7). The potency was destroyed by reagents which react with double bonds, e.g., bromine, chromic acid, nitric acid, perbenzoic acid, ferric chloride, sulfuric acid, and hydrogen iodide (5, 6, 127). Reagents which attack hydroxyl groups,—e.g., phenylisocyanate, 3,5-dinitrobenzoyl chloride, benzoyl chloride, acetic anhydride and cyanic acid,—failed to alter the activity (5, 6, 63, 127). The failure of ketonic reagents,—such as 2,4-dinitrophenylhydrazine, Girard's reagent T, and hydroxylamine—to react, indicated an absence of ketonic groups (6, 63, 127). Another important point contributed by Almquist was the discovery that the vitamin is destroyed by ultraviolet light (6). This finding and the extensive and unexplained destruction of vitamin during the course of purification led MacCorquodale *et al.* (135) to study the effect of illumination from ordinary Mazda light bulbs. They found that the purified vitamin, dissolved in benzene or ethanol, is completely destroyed after a few hours' exposure.

Thayer, MacCorquodale, Binkley, and Doisy (182) reported in 1938 that they had isolated a white crystalline compound, melting at 69°C., which after four recrystallizations possessed vitamin K activity. Subsequent work failed to confirm this report, and the error was acknowledged (37, 135). The apparent response to this compound was probably due to the bleeding of the chicks prior to the assay to determine whether they had been depleted of vitamin K (see also reference 46).

2. *The product of Dam and Karrer*

In March, 1939, Dam, Karrer, *et al.* (57) reported the isolation of vitamin K₁ in a pure or approximately pure form. Later (119) they stated that this product was pure vitamin K₁.² The data presented in the first paper for the chromatographically homogeneous oil are the percentages of carbon and hydrogen, the potency by bioassay, the ultraviolet absorption curve, the extinction coefficient at 248 m μ , and the effect of catalytic reduction in hexane on the ultraviolet absorption. Of these data the only accurate criterion of purity is the extinction coefficient. A comparison of the value $E_{1\text{cm.}}^{1\%} = 280$ at λ 248 m μ with a large number of values obtained by other investigators (to be discussed in a later section) indicates that their product was not entirely pure.

The product of Dam and Karrer (120), the preparation of which was not described until late in 1939, was obtained from petroleum ether extracts of alfalfa by the following method: removal of the chlorophyll by adsorp-

² Dam and Karrer have given vitamin K₁ the name α -Phyllochinon (α -phylloquinone), but thus far this name has not received wide acceptance.

tion on zinc carbonate; precipitation of impurities from petroleum ether; molecular distillation; crystallization of impurities from acetone; and, finally, chromatographic adsorption on magnesium sulfate and zinc carbonate. After seven or eight adsorptions on zinc carbonate, further adsorption failed to increase the potency of the product. A 15 to 20 per cent yield of the vitamin present in the active distillate was obtained by this process.

3. *The product of the St. Louis University group*

(a) Method of isolation

From the properties of the vitamin as determined by Dam and Almquist on the crude concentrates, it was evident that pure preparations of the vitamin could not be obtained by chemical means, e.g., removing impurities by saponification, separation of the activity into a ketonic fraction or alcoholic fraction, etc. The results of partition between various solvents and of distillation had likewise proved disappointing. As a result, investigators had turned to the possibility of chromatographic adsorption (37, 63, 74, 120, 163) as a means of purification. The method developed by Binkley *et al.* (37) led to the first isolation of vitamins K_1 and K_2 in a form of established purity. Although a large number of adsorbents (alumina, magnesium oxide, infusorial earth, Fuller's earth, supercel, magnesium oxide plus supercel, sucrose, decalso, permutit, norite, darco, nuchar, calcium sulfate, and calcium carbonate) were studied under varying conditions, the most satisfactory were permutit and decalso—two artificial zeolites used for water-softening purposes—and darco. With a modified chromatographic adsorption method, it was found that decalso and permutit are very satisfactory for the concentration of crude preparations, while darco is advantageous with preparations containing more than 10 per cent of the vitamin. The vitamin is stable toward these adsorbents; it is easily eluted; the adsorptive properties are such that a twenty- to forty-fold concentration can be obtained by a single adsorption; and the process is practically quantitative from start to finish. Decalso and permutit are suitable for handling large quantities (extract of 1000 pounds of alfalfa leaf meal) of crude extracts in one operation.

Vertical glass cylinders fitted with perforated porcelain bottoms which were covered with cotton were used in developing the process of chromatographic adsorption. For quantity production large copper percolators were used as containers for the adsorbents. The vitamin was adsorbed by allowing a petroleum ether extract of artificially dried alfalfa leaf meal to flow through the adsorbent. It was eluted by washing successively with 1:10, 1:7, and 1:5 mixtures of benzene and petroleum ether. By

using proper solvents for selective elution of the vitamin, by constantly observing the movement of the colored layers in the column, and by careful fractionation of the solvents which percolated through, a high degree of purification was attained. Three repetitions of this adsorptive process gave a reddish oil containing from 20 to 50 per cent of the vitamin. Additional adsorptions on permutit did not give any detectable purification. Further purification by adsorption on darco, followed by fractional elution, gave a lemon-yellow oil (potency 1000 units per milligram) which crystallized in yellow rosettes from acetone or ethanol at -70°C . These crystals melted at approximately -20°C . (184, 185).

Fernholz *et al.* (74), using a heat-activated calcium sulfate as an adsorbent, prepared vitamin K concentrates which had a potency comparable to that of vitamin K_1 (36).

(b) Preparation of a crystalline derivative

In order to establish definite proof of the isolation (144) of the vitamin, a crystalline derivative (136),—the diacetate of dihydrovitamin K_1 (m.p. $62\text{--}63^{\circ}\text{C}$.),—was prepared by reductive acetylation. This compound, on hydrolysis by means of the Grignard reagent and subsequent oxidation with air, gave a lemon-yellow oil identical in every respect with the original vitamin.

B. VITAMIN K_2 ; ISOLATION OF CRYSTALLINE PRODUCT

By application of the adsorption procedure developed for the isolation of vitamin K_1 (37), McKee *et al.* (145) isolated a different antihemorrhagic factor (36, 144) from the petroleum ether extracts of putrefied fish meal. Three adsorptions on decalso or permutit yielded a reddish yellow oil which crystallized on standing at -5°C . After several recrystallizations from an acetone-ethyl alcohol mixture or from a mixture of methyl alcohol and chloroform (1:1), a pure yellow crystalline compound melting at $53.5\text{--}54.5^{\circ}\text{C}$. was obtained. This compound had a potency of approximately 660 units per milligram (185). Evidence that the crystalline compound was actually a vitamin was based on (a) recovery of the crystals with unchanged melting point and potency after partial destruction by passage through a column of alumina, after partial oxidation with permanganate and after partial destruction during distillation, (b) the similarity of the ultraviolet absorption spectra, of the lability toward light, and of the chemical properties of the compounds isolated from alfalfa and from putrefied fish meal, (c) twenty recrystallizations from a variety of solvents without loss of potency, and (d) the preparation of several different batches having the same melting point and potency.

Analyses and molecular weight determinations indicated a formula of about $C_{40}H_{54}O_2$ (144, 145). However, degradation studies (38) later showed that the correct formula is $C_{41}H_{56}O_2$.

VI. CONSTITUTION OF VITAMIN K

A. VITAMIN K_1

On the basis of analyses for carbon and hydrogen and of molecular weight determinations, McKee *et al.* (144) proposed an empirical formula of $C_{32}H_{46}O_2$ for vitamin K_1 . Since it is not possible to determine accurately from these data the number of carbon atoms of compounds of such high molecular weight, it is not surprising that shortly thereafter (35, 137) degradative studies demonstrated that the correct formula is $C_{31}H_{46}O_2$.

1. Evidence of quinonoid structure

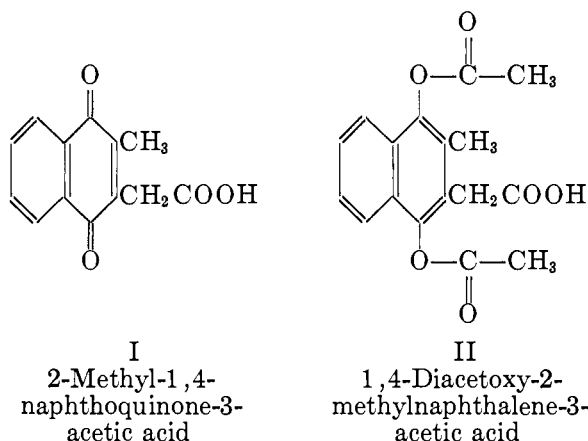
Catalytic hydrogenation (144) induced an uptake of eight atoms of hydrogen, with the formation of a colorless reduction product which upon exposure to air was converted to a yellow compound. Upon catalytic hydrogenation this yellow product absorbed two atoms of hydrogen, with the production of a colorless compound. This behavior, the absorption spectrum, the lability toward light and alkali, and the presence of two atoms of oxygen per mole led McKee *et al.* (144) to propose a quinonoid structure for vitamin K_1 . This conclusion was confirmed by the preparation of the crystalline hydroquinone diacetate, m.p. 62–63°C. Treatment of this diacetyldihydrovitamin K_1 with methylmagnesium iodide produced the hydroquinone which, upon being shaken with air, was rapidly oxidized to the vitamin.

The pure yellow color of the vitamin indicated that the substance probably belonged to the *p*-quinone series. This conclusion was supported by the discovery that, of a considerable variety of quinones, only α -naphthoquinones possessed vitamin K activity (181). Moreover, the ultra-violet absorption (71) curve indicated a close relationship to α -naphthoquinones. Since the vitamin absorbed two atoms of hydrogen in addition to the six atoms necessary for the formation of the tetrahydro derivative of the hydroquinone, it was apparent that an ethylenic linkage is present. Since the vitamin did not respond to Craven's color test, it was concluded that the vitamin is a 1,4-naphthoquinone with or without substituents in the benzenoid ring, and with hydrocarbon radicals in the 2- and 3-positions.

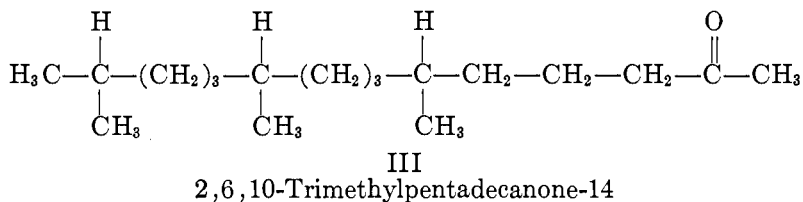
2. Oxidative degradation

Oxidation of vitamin K_1 with chromic acid resulted in the formation of a mixture of substances from which two acids were isolated. The identification of phthalic acid demonstrated that the benzenoid ring in the vitamin

is unsubstituted. The other acid, a quinone acid, was obtained as pale yellow crystals melting with decomposition at 210°C. On the basis of one analysis, it was suggested that the acid was 2-ethyl-1,4-naphthoquinone-3-acetic acid (136). However, when synthetic acids were prepared for comparison, it was found that 2-methyl-1,4-naphthoquinone-3-acetic acid had the same decomposition temperature, 210°C., whereas the 2-ethyl homolog melted with decomposition at 185°C. The methyl esters of the 2-methyl-1,4-naphthoquinone-3-acetic acid and the acid obtained from the vitamin had the same melting point (127.5–128.5°C.) and the mixture showed no depression. The quinone acid was, therefore, 2-methyl-1,4-naphthoquinone-3-acetic acid (I) (35, 137).

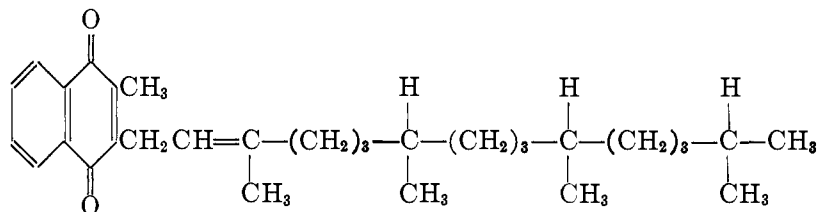


When diacetyldihydrovitamin K₁ was oxidized with chromic acid, a fairly good yield of two products was formed by cleavage at the double bond. One product, an acid having the composition C₁₇H₁₆O₆ and melting at 209–210°C., was identified as 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid (II) by comparison of the methyl ester with a synthetic specimen. The second product was a liquid ketone. It was found that this ketone could be obtained more simply and in excellent yield by ozonolysis of the diacetate of dihydrovitamin K₁. The ketone, isolated as the semicarbazone, proved to be identical with the semicarbazone of 2,6,10-trimethylpentadecanone-14 (III).



3. Structure of vitamin K₁

The identification of these degradation products of vitamin K₁ and its diacetyldihydro derivative clearly indicated that the constitution of the vitamin is that of 2-methyl-3-phytyl-1,4-naphthoquinone (IV).



IV
Vitamin K₁
or
2-methyl-3-phytyl-1,4-naphthoquinone

B. VITAMIN K₂

1. Evidence of quinonoid structure

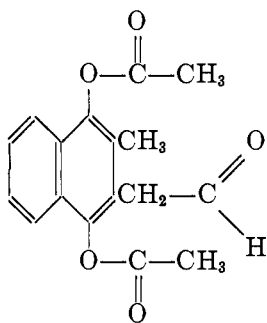
The chemical behavior of vitamin K₂ was quite similar to that of vitamin K₁. Upon catalytic hydrogenation it absorbed 9 moles of hydrogen to produce a colorless compound which on exposure to air was oxidized to a yellow compound. This yellow compound absorbed 1 mole of hydrogen to give a colorless solution which on oxidation with air returned to the original yellow color. The yellow color, the instability to light and alkali, and the behavior in hydrogenation experiments were suggestive of the 1,4-quinones. Reductive acetylation, a reaction characteristic of quinones, gave a white crystalline diacetate of dihydrovitamin K₂ (m.p. 59.5–60°C.). Catalytic hydrogenation of this diacetate caused an uptake of 8 moles of hydrogen. The addition of 6 moles of bromine indicated the presence of six double bonds in the side chains of the molecule.

The ultraviolet absorption curves (71) for vitamins K₁ and K₂ and for 2,3-dimethyl-1,4-naphthoquinone showed a striking similarity (figure 1); likewise, the curves for the diacetates of the corresponding hydroquinones showed close agreement (figure 2). This evidence not only supported the conclusion that vitamin K₂ is a 1,4-quinone but, together with the hydrogenation data, indicated that it is a 1,4-naphthoquinone. Since, under the conditions of reduction, 3 moles of hydrogen were needed to form a tetrahydronaphthoquinone, the other 6 moles of hydrogen must have been used in the saturation of six double bonds in the side chains. This interpretation harmonized with the addition of 6 moles of bromine

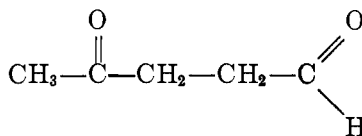
by the diacetate of dihydrovitamin K_2 . Since the vitamin did not respond to Craven's color test and did not react with maleic anhydride, it was concluded that vitamin K_2 is a 2,3-disubstituted 1,4-naphthoquinone with six double bonds, arranged without conjugation, in the side chains.

2. Oxidative degradation

Treatment of the diacetate of dihydrovitamin K_2 in glacial acetic acid with ozone, followed by decomposition of the ozonide with zinc in ether,



V
1,4-Diacetoxy-2-methylnaphthalene-
3-acetaldehyde

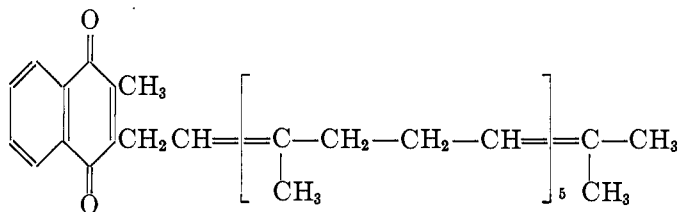


VI
Levulinaldehyde

gave a good yield of 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde (m.p. $115^{\circ}\text{C}.$) (V), which was characterized as the semicarbazone (m.p. $206^{\circ}\text{C}.$). Mixed melting points showed that this aldehyde was identical with 1,4-diacetoxy-2-methylnaphthalene-3-acetaldehyde, obtained from the diacetate of dihydrovitamin K_1 under the same experimental conditions. The isolation of this aldehyde demonstrated conclusively that vitamin K_2 is a 2-methyl-1,4-naphthoquinone. From the water-soluble products of the ozonization reaction, levulinaldehyde (VI) was isolated as the bis-2,4-dinitrophenylhydrazone. On the assumption that 5 moles of levulinaldehyde would originate from 1 mole of vitamin K_2 , a yield of 93 per cent was obtained. The third compound isolated from the ozonization reaction was acetone, which was identified as the 2,4-dinitrophenylhydrazone.

3. Structure of vitamin K_2

Since the fragments isolated from the oxidative degradation of vitamin K_2 give a total of forty-one carbon atoms, $\text{C}_{41}\text{H}_{56}\text{O}_2$ has been proposed as the correct empirical formula. The most probable arrangement of the units is expressed by structural formula VII.



VII
Vitamin K₂

VII. SYNTHESIS OF VITAMIN K₁

A. FIESER'S HYPOTHESES REGARDING THE STRUCTURE OF VITAMIN K₁

While the group of investigators at St. Louis University was approaching the problem of the structure of vitamin K₁ by degradation, followed by synthesis of the indicated structure (35, 136, 137), Fieser *et al.* (82, 83, 85) were approaching the problem from a synthetic point of view. "On the basis of Doisy's reports in the May and June Journal concerning the properties of pure vitamins K₁ and K₂, and from the observations of the Almquist, Dam-Karrer, and other groups, we advanced the hypothesis early in June that the substances are 2,3-dialkyl-1,4-naphthoquinones . . ." (84). In the July, 1939, number of the *Journal of the American Chemical Society*, Fieser, Riegel, and their collaborators (82, 86) suggested as a specific hypothesis that vitamin K₁ might be 2,6(?)-dimethyl-3-phytyl-1,4-naphthoquinone (or the 2-monomethyl compound) and that vitamin K₂ might be 2,3-difarnesyl-1,4-naphthoquinone. Fieser proceeded to test this hypothesis by an experimental attack from the synthetic approach.

B. SYNTHESSES OF VITAMIN K₁

In the September, 1939, issue of the *Journal of the American Chemical Society* the synthesis of vitamin K₁ was described by three different groups of workers. Binkley *et al.* (35) synthesized it by condensing phytyl bromide with the monosodium salt of 2-methyl-1,4-naphthohydroquinone, using benzene as a solvent. The product was purified by chromatographic adsorption and distillation and identified as the diacetate of dihydrovitamin K₁. This synthetic product (138) gave the same degradative products and possessed the same potency as the natural vitamin. This same group of investigators reported that phytol condensed with 2-methyl-1,4-naphthohydroquinone in the presence of zinc chloride as a condensing agent (137).

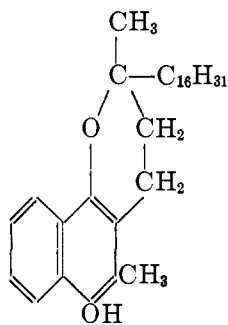
Fieser (76, 77, 78, 79, 81) found that 2-methyl-1,4-naphthohydroquinone condenses with phytol in dioxane in the presence of oxalic acid at 75°C.

The unreacted 2-methyl-1,4-naphthohydroquinone was removed with dilute alkali, and dihydrovitamin K₁ was separated on the basis of its insolubility in petroleum ether. Oxidation with silver oxide gave vitamin K₁.³ The synthetic compound was identified by its absorption spectrum, by its biological activity, and by its conversion to the diacetate of dihydrovitamin K₁. Trichloroacetic acid, phosphoric acid, acetic acid, and heat (89) were also used successfully as condensing agents.

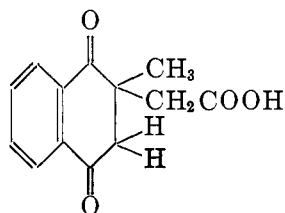
At the same time, Fieser (77) showed that natural vitamin K₁ could be extracted from partially purified concentrates by converting it to the hydroquinone, which can be extracted from petroleum ether with Claisen's alkali containing sodium hydrosulfite. After dihydrovitamin K₁ is precipitated by dilution, it is extracted with ether and purified by digestion with petroleum ether. Comparison of the diacetates of the natural and synthetic products left no doubt concerning the success of the synthetic work.

The third synthesis was reported by Almquist and Klose (15, 17), who condensed 2-methyl-1,4-naphthoquinone and phytyl bromide in petroleum ether, using zinc and acetic acid to effect the condensation. The product was purified by molecular distillation.

The synthetic procedures outlined above gave at best only moderate yields of the desired 2-methyl-3-phytyl-1,4-naphthohydroquinone and a considerable amount of a liquid by-product. In view of the formation of tocopherols by similar condensations (174), this substance was regarded as a naphthotocopherol (VIII) (86). In an extended study of the by-



VIII



IX

2-Methyl-2,3-dihydro-
1,4-naphthoquinone-
2-acetic acid

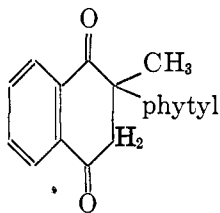
³ Frank, Hurwitz, and Seligman (96) used Fieser's synthetic vitamin K₁ in the successful treatment of patients. This was the first therapeutic use of synthetic vitamin K₁.

product, Tishler *et al.* (188) showed this hypothesis to be untenable. Naphthocopherol was prepared by refluxing vitamin K₁ in acetic acid with stannous chloride. A study of its chemical properties and absorption spectrum showed it to be quite different from the oily by-product. Furthermore, differences in the absorption spectra of 2-methyl-1,4-naphthoquinone monoethyl ether and of the oily by-product indicated that the latter is not a phytol ether.

Chromic acid oxidation of the by-product gave 2-methyl-2,3-dihydro-1,4-naphthoquinone-2-acetic acid (IX) and 2,6,10-trimethylpentadecanone-14; reduction with aluminum isopropoxide yielded a secondary diol. Formation of a crystalline hydrazone likewise indicated the presence of two carbonyl groups. This evidence showed that the compound is 2-methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone (X). The substance possesses antihemorrhagic activity and can be converted in small part into vitamin K₁ by pyrolysis.

C. SYNTHESIS OF VITAMIN K₂

To date, a synthesis of vitamin K₂ has not been reported. However, degradation of the vitamin has shown conclusively that it is 2-methyl-3-(3',7',11',15',19',23'-hexamethyl-2',6',10',14',18',22'-tetracosahexenyl)-1,4-naphthoquinone (38).



X

2-Methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone

VIII. PHYSICAL AND CHEMICAL PROPERTIES OF THE NATURAL VITAMINS

Vitamin K₁ is a lemon-yellow oil at room temperature. At -70°C . it separates from acetone or ethyl alcohol in light yellow rosettes which melt at about -20°C . into an oil, plus solvent. As the temperature rises, the oil gradually passes into solution. The vitamin is soluble in the ordinary fat solvents,—ethyl alcohol, acetone, hexane, benzene, chloroform, and dioxane. It is insoluble in water and only sparingly soluble in methyl alcohol.

Vitamin K₂ is a lemon-yellow crystalline compound melting at $53.5\text{--}54.5^{\circ}\text{C}$. It may be crystallized from ethyl alcohol, acetone, or a mixture

(1:1) of methyl alcohol and chloroform. In general, it is slightly less soluble than vitamin K₁.

The diacetates of dihydrovitamin K₁ and dihydrovitamin K₂ melt at 62–63°C. and 59.5–60°C., respectively. The dibenzoate of dihydrovitamin K₁ melts at 85–86°C. (78). These derivatives may be crystallized from ethyl or methyl alcohol.

Neither vitamin rotates polarized light at a concentration of 1 per cent in ethyl alcohol.

A. ULTRAVIOLET ABSORPTION

Almquist (6), working with crude vitamin K preparations, found strong absorption in the ultraviolet, while Dam and Lewis (63) obtained no char-

TABLE 1

Ultraviolet absorption of vitamin K₁, vitamin K₂, and some 1,4-naphthoquinones

COMPOUND	λ	LOG E_{molar}
	$m\mu$	
Vitamin K ₁	248	4.26* (4.29)†
Vitamin K ₂	248	(4.27)†
1,4-Naphthoquinones:		
2,3-dimethyl.....	249	4.24* (4.29)†
2,3-diallyl.....	249	4.24*
2-methyl-3-(β, γ, γ -trimethylallyl).....	249	4.27*

* Values reported by Tishler *et al.* (188), using alcohol as the solvent.

† Values reported by Ewing (unpublished data), using hexane as the solvent.

acteristic absorption with similar preparations. Dam, Karrer, *et al.* (57) and Karrer *et al.* (119, 120) reported maxima at 248, 261, 270, and 328 $m\mu$ for a preparation which they considered to be the pure or nearly pure vitamin. McKee *et al.* (144), in their first report on the isolation of vitamin K, found that vitamin K₁ shows maxima at 243, 248, 261, 270 and 323 $m\mu$ and vitamin K₂ shows maxima at 249, 261, 269, and 320 $m\mu$. Whereas Dam, Karrer, *et al.* (57) and Karrer *et al.* (119, 120) found an extinction coefficient of $E_{1\text{cm}}^{1\%} = 280$ at 248 $m\mu$, McKee *et al.* reported the value 385. Since the intensity of absorption, as measured by the extinction coefficient, is an accurate index of the relative purity of different samples, the American workers concluded that the Dam-Karrer product was 70 per cent pure.⁴

⁴ In order to obtain more information on the cause of the discrepancy, specimens of the same preparations of natural and synthetic vitamin K₁ were supplied to both Karrer and Ewing for the determination of the extinction coefficient. The values reported for $E_{1\text{cm}}^{1\%}$ at λ 248 $m\mu$ by Ewing were 421 for the natural vitamin K₁ and 417 for the synthetic; those reported by Karrer were 318 and 324, respectively (un-

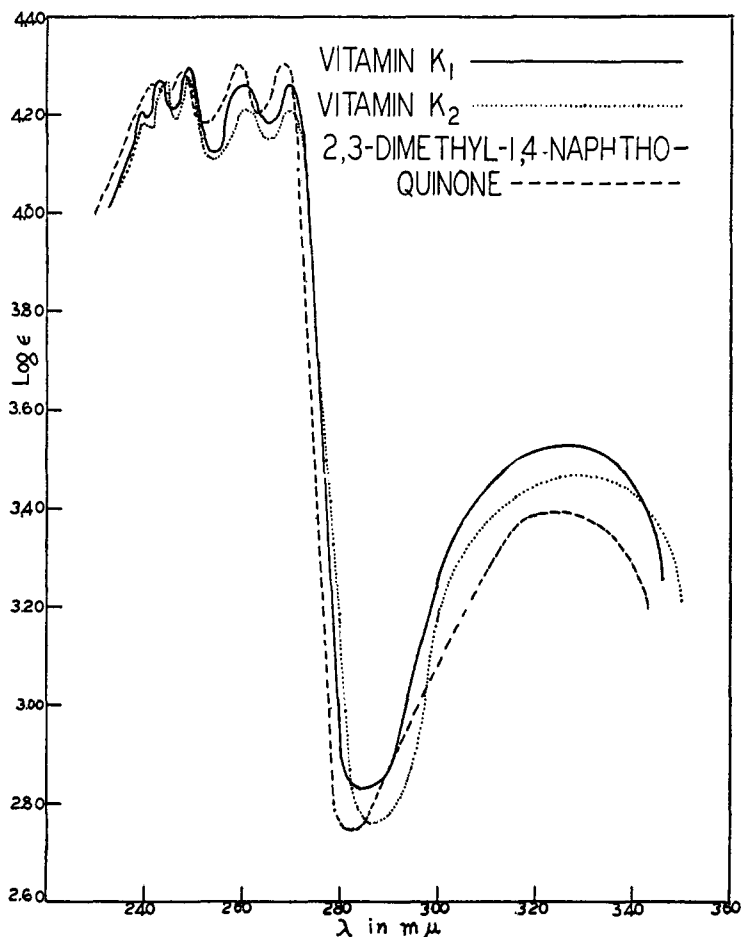


FIG. 1. Ultraviolet absorption curves of vitamin K_1 , vitamin K_2 , and 2,3-dimethyl-1,4-naphthoquinone.

Later Binkley *et al.* (36) reported a value of 540 for the extinction coefficient of a single vitamin preparation. Since all subsequent determinations agree approximately with the value given in the first paper, the figure

published data). Ewing subsequently made additional determinations on specimens of the same preparations and obtained 432 and 439 for the natural and the synthetic product, respectively. In view of the agreement between the values for the extinction coefficient of vitamin K_2 reported by Karrer (119, 120) and by Ewing (71), the cause for the difference in the values for vitamin K_1 is not apparent.

Although the value of the extinction coefficient of our preparations differed from the value given by Karrer, Almquist's (16, 18) assays of specimens of vitamin K_1 supplied by the two research groups showed close agreement.

of 540 should not be considered as characteristic of pure vitamin K_1 . After determining the extinction coefficients of a large number of samples of both natural and synthetic vitamin K_1 , Dr. Ewing (unpublished data)

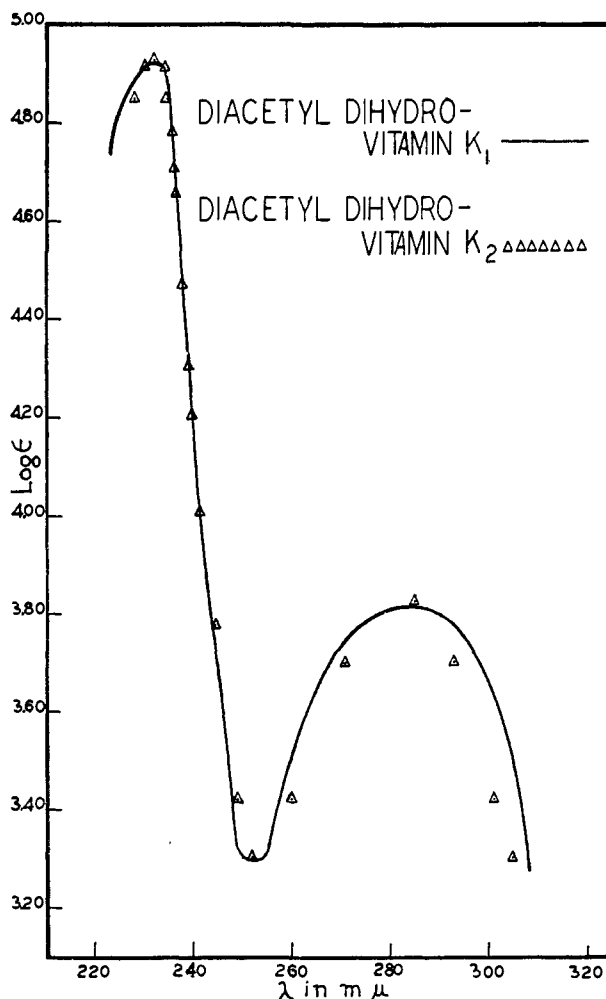


FIG. 2. Ultraviolet absorption curves of diacetate of dihydrovitamin K_1 and diacetate of dihydrovitamin K_2 .

concluded that with hexane as solvent $E_{1\text{cm.}}^{1\%} = 430 \pm 10$ ($\log E_m = 4.29$). This agrees with the most recent values reported by Tishler *et al.* (188), who state that the most reliable value for vitamin K_1 is $\log E_m = 4.26$ (alcohol). Since Ewing *et al.* (71) found that, by using hexane as a

solvent, slightly higher maxima were obtained and the fine structure was better defined than in ethyl alcohol, the values reported by the two groups are well within the experimental error (see table 1). Through the courtesy of Dr. Ewing, curves for vitamin K_1 , vitamin K_2 , and 2,3-dimethyl-1,4-naphthoquinone are reproduced (figure 1). It should be noted that a maximum which had previously escaped detection has been found at 240 $m\mu$.

Table 1 gives the molar extinction coefficients found for the most intense absorption band of vitamins K_1 and K_2 and of several simple 2,3-dialkyl-1,4-naphthoquinones. It is significant that the values for all of these compounds show such good agreement, especially when the difference due to the use of different solvents is taken into account. This observation is excellent evidence that $\log E_m = 4.27$ to 4.29 (hexane) for vitamins K_1 and K_2 is the correct value.

The ultraviolet absorption curves for the diacetates of dihydrovitamins K_1 and K_2 are reproduced in figure 2. The values for $\log E_m$ at 232.5 $m\mu$ for the two compounds show good agreement (4.93 for the diacetate of dihydrovitamin K_1 , and 4.93 for the diacetate of dihydrovitamin K_2). Fieser (78) reports a slightly higher value ($\log E_m = 4.98$) for the diacetate of vitamin K_1 , whereas Karrer *et al.* (120) find that $\log E_m = 4.93$.

B. OXIDATION-REDUCTION POTENTIAL

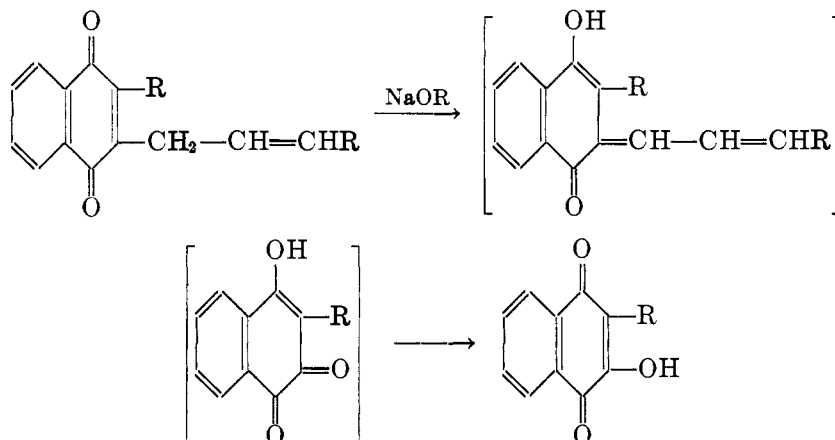
Karrer *et al.* (119) reported an oxidation-reduction potential $E_m = +0.005$ volt for the vitamin from alfalfa. Riegel (162, 164) found the potential E_0 for pure vitamin K_1 to be 363 millivolts at 20°C. According to Riegel, Karrer did not use the customary method for determining the oxidation-reduction potentials of quinones; consequently, a satisfactory comparison of the two values cannot be made. From a consideration of the pH of the solvent used, Riegel calculated Karrer's value of the oxidation-reduction potential E_0 to be about 400 millivolts.

C. COLOR REACTIONS

Dam *et al.* (57) observed that sodium ethylate reacts with vitamin K_1 to give a transient blue color which fades to reddish brown. Almquist (12), working with partially purified preparations, found that the amount of color developed agreed well with bioassays, whereas Fernholz (74) found that some potent preparations of the vitamin failed to give the reaction. All investigators now agree that the reaction is characteristic of 2-methyl-3-phytyl-1,4-naphthoquinone.

Fieser *et al.* (85) obtained the characteristic color with 1,4-naphthoquinones containing at least one allyl group in the quinonoid ring and isolated 2-hydroxy-3-allyl-1,4-naphthoquinone as the end-product of the

reaction of sodium ethylate with 2,3-diallyl-1,4-naphthoquinone. Later it was established that synthetic vitamin K₁ gave phthiocol as one product of the color reaction (77, 78). The following mechanism was proposed for this reaction (85):



Essentially the same interpretation of the first phase of this color reaction was advanced by Karrer (117).

The formation of phthiocol from vitamin K₁ by alkali led to the suggestion that the phthiocol isolated from the human tubercle bacilli may have arisen from the alkaline cleavage of a K-type vitamin.

Another color reaction which proved to be useful in determining the structure of vitamins K₁ and K₂ was Craven's color test (38, 48, 137). 1,4-Naphthoquinones substituted in the 2-position give a deep blue color with ammoniacal alcoholic ethyl cyanoacetate, while 2,3-disubstituted derivatives give no color.

IX. SIMPLE 1,4-NAPHTHOQUINONES AND RELATED COMPOUNDS

As soon as McKee *et al.* (144) announced that vitamins K₁ and K₂ are quinones and gave data which could be interpreted only in terms of the α -naphthoquinones, a number of investigators became interested in the potencies of the naphthoquinones. The first report was by Almquist (13), who apparently correlated the production of vitamin K potency by bacteria with Anderson's phthiocol from tubercle bacilli. Although this compound possesses potency, its activity is not comparable with that of the vitamin from alfalfa. A very important observation was made by Ansbacher (29, 31, 72), who found that 2-methyl-1,4-naphthoquinone is more active than vitamin K₁. Although other groups of investigators (14, 15, 181) failed in their early work to observe the great activity of this

compound, they now agree that it is at least twice as active as vitamin K₁ (16, 18, 67, 69, 161, 180, 183, 190).

A. QUINONES WITHOUT POTENCY

The following quinones, which do not belong to the 1,4-naphthoquinone series, have been prepared and tested for vitamin K activity: benzoquinone (15, 30, 181), toluquinone (30, 181), *p*-xyloquinone (141, 181), phlorone (30), diallyl-1,4-benzoquinone (82), 2-methoxy-3-methyl-1,4-benzoquinone (141), 2,3-dimethyl-1,4-benzoquinone (141), trimethylbenzoquinone (30, 141), triethylbenzoquinone (61), thymoquinone (61, 181), diamylhydroquinone (181), duroquinone (30, 61, 141), 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone (90), trimethyl- γ -oxybutylbenzoquinone (61), diallyl-1,4-benzohydroquinone diacetate (82), α -tocopherylquinone (61, 133), anthraquinone (15, 61, 141), 1,2-dihydroxyanthraquinone (15), anthraquinone- β -sulfonic acid (181), 1,1,3-trimethyl-1,4-dihydroanthraquinone (92), 2,7-dinitrophenanthraquinone (141), 2-hydroxy-3-methylanthraquinone (141), quinalizarin (141), rufigallol (141), purpurin (141), 1,1-dimethyl-3-*tert*-butyl-1,4-dihydroanthraquinone (92), 2-(δ -methyl- γ -pentenyl)-1,4-dihydroanthraquinone (92), dihydroanthraquinone diacetate (181), and phenanthraquinone (61, 141, 181). Of these quinones only a few have been reported to possess antihemorrhagic activity. Ansbacher (30) found phlorone to be active at 1 mg. Kuhn *et al.* (133) reported that α -tocopherylquinone is active at 10 mg., but Dam *et al.* (61) found it to be inactive at a level of 0.0044 mg. per gram of body weight. Triethylbenzoquinone shows slight activity (61). Martin and Lischer (141) found that purpurin is active at 0.1 mg. and that rufigallol, anthragallol, and duroquinone are active at 10 mg.

A large number of naphthoquinones containing hydroxyl groups have been studied. These include phthiocol (13, 18, 29, 72, 79, 181), 3,4-dihydroxy-1,2-naphthoquinone (134), 2-allyl-3-hydroxy-1,4-naphthoquinone (134), 2-*n*-butyl-3-hydroxy-1,4-naphthoquinone (134), α -lapachone (80, 134), lapachol (14, 80, 82, 85, 133), lomatiol (14, 80, 82), hydrolapachol (15, 80, 82), phthiocol ethyl ether (15), phthiocol octadecyl ether (15), phthiocol phytyl ether (15), dihydrophthiocol triacetate (15), phthiocol monoacetate (15, 18), hydroxyhydrolapachol (80, 82), lomatiol methyl ether (82), lapachol methyl ether (82), juglone (133), 3,5,6,7,8-pentaoxy-2-allyl-1,4-naphthoquinone (133), 2- α -heptenyl-3-hydroxy-1,4-naphthoquinone (83), 2-*n*-heptyl-3-hydroxy-1,4-naphthoquinone (83), and β , β -dimethyldihydrofurano-1,4-naphthoquinone (134). As compared with vitamin K₁ or 2-methyl-1,4-naphthoquinone, these compounds are relatively inactive. Phthiocol, which has been reported to be from $\frac{1}{2}$ to $\frac{1}{10}$ as active as the vitamin, has special significance, since it

was the first simple 1,4-naphthoquinone reported to have vitamin K activity.

TABLE 2
2-Substituted 1,4-naphthoquinones and derivatives

COMPOUND AND REFERENCE	APPROXIMATE WEIGHT OF ONE UNIT
	γ
Naphthalene (15).....	—
1,2-Naphthoquinone (73, 181).....	—
1,4-Naphthoquinone (15, 61, 73, 133, 181).....	1000
2-Methyl-1,4-naphthoquinone (XIV) (18, 29, 61, 67, 72, 134, 170, 180, 181, 183, 190).....	0.5
2-Ethyl-1,4-naphthoquinone (73, 170, 181).....	100
2- <i>n</i> -Propyl-1,4-naphthoquinone (73, 85).....	1000
2-Allyl-1,4-naphthoquinone (73, 82, 83, 181).....	>1000
2- <i>n</i> -Hexadecyl-1,4-naphthoquinone (73).....	>1000
2- <i>n</i> -Octadecyl-1,4-naphthoquinone (73).....	>1000
2-Geranyl-1,4-naphthoquinone (89, 90).....	—
2-Farnesyl-1,4-naphthoquinone (89, 90).....	—
2-Phytyl-1,4-naphthoquinone (61, 89, 90, 121).....	50
1,4-Naphthohydroquinone diacetate (181).....	2000
2-Oxymethyl-1,4-naphthoquinone acetate (61).....	Weak
2-Methyl-1,4-naphthohydroquinone diacetate (29, 61, 72, 181).....	1
2-Phytyl-1,4-naphthoquinone oxide (91).....	—
2-Farnesyl-1,4-naphthoquinone oxide (91).....	500

Owing to the differences in assay procedures used in various laboratories and our utilization of data published by other investigators, the values given in these tables for the potencies of these compounds are only approximations. However, there is little doubt that the order of magnitude of the potencies is correct.

Many different units are in use, but in these tables the values given are in terms of our unit. This unit is the specific antihemorrhagic activity of 0.8 mg. of a standard alfalfa extract. On the basis of this unit and by the procedure of assay used in this laboratory, the potency of 2-methyl-1,4-naphthoquinone is 2000 units per milligram and that of vitamin K₁ is 1000 units per milligram.

As an example, the following method was used in assigning potencies to compounds which have not been assayed in this laboratory: Dam gives the activity of vitamin K₁ as 12,000,000 and that of 2-methyl-1,4-naphthoquinone monoxime as 5,000,000 of his units. The weight of one of our units of the oxime is therefore 2.4 γ ; the approximate figure 2 γ is given in the table. In these tables, — in the column giving the weight of the unit indicates that the compound was inactive for the amount used in the assay.

The following naphthoquinones which are substituted in the benzenoid ring failed to show appreciable vitamin K activity: 2,6-dimethyl-1,4-naphthoquinone (73, 83), 2,7-dimethyl-1,4-naphthoquinone (83), 2,3,5-trimethyl-1,4-naphthoquinone (61), 2,3,6-trimethyl-1,4-naphthoquinone

(61), 3,5,7-trimethyl-1,4-naphthoquinone (61), 3,6,7-trimethyl-1,4-naphthoquinone (61), 3,5,7-trimethyl-1,2-naphthoquinone (61), and 2,6-dimethyl-3-phytyl-1,4-naphthoquinone (78).

Table 2, which includes the 2-substituted 1,4-naphthoquinones and derivatives, shows that, if the alkyl group is increased beyond the methyl group, the potency is lost and is not regained even when the alkyl group contains sixteen to twenty carbon atoms.

TABLE 3
2,3-Disubstituted 1,4-naphthoquinones and derivatives

COMPOUND AND REFERENCE	APPROXIMATE WEIGHT OF ONE UNIT
	γ
2-Methyl-1,4-naphthoquinone (XIV) (18, 29, 61, 67, 72, 134, 170, 180, 181, 183, 190).....	0.5
2,3-Dimethyl-1,4-naphthoquinone (82, 85, 133).....	50
2-Methyl-3-bromo-1,4-naphthoquinone (181).....	10,000
2-Methyl-3-amino-1,4-naphthoquinone (18).....	75
2-Methyl-3-benzyl-1,4-naphthoquinone (78, 87).....	>100
2-Methyl-3-trimethylallyl-1,4-naphthoquinone (87).....	>100
2-Methyl-3-cinnamyl-1,4-naphthoquinone (78, 87).....	100
2-Methyl-3-geranyl-1,4-naphthoquinone (78).....	25
2-Methyl-3-farnesyl-1,4-naphthoquinone (89).....	10
2-Methyl-3-phytyl-1,4-naphthoquinone (18, 33, 61, 69, 72, 76, 137, 144, 183, 185).....	1
2-Methyl-3-palmityl-1,4-naphthoquinone (18).....	4
2-Methyl-3- <i>n</i> -octadecyl-1,4-naphthoquinone (73).....	1,000
2-Ethyl-3-phytyl-1,4-naphthoquinone (78).....	>160
2,3-Diallyl-1,4-naphthoquinone (73, 82).....	>1,000
2-Methyl-1,4-naphthoquinone dimer (18).....	20
2,3-Dibromo-2-methyl-1,4-dioxotetrahydronaphthalene (181).....	10,000
2,3-Dimethyl-1,4-naphthoquinone oxide (91).....	25
2-Methyl-3-cinnamyl-1,4-naphthoquinone oxide (91).....	Weak
Disodium 2,3-dimethyl-1,4-naphthohydroquinone disulfate (88)....	500

B. POTENT 1,4-NAPHTHOQUINONES

When 2-methyl-1,4-naphthoquinone (table 3) is substituted in the 3-position by groups other than an alkyl group, e.g., bromine, amino, etc., the most potent derivative is only $\frac{1}{100}$ as active as the parent compound. 2,3-Dimethyl-1,4-naphthoquinone is about $\frac{1}{50}$ as active as the compound not substituted in the 3-position. From his study of a series of 2,3-dialkyl-1,4-naphthoquinones, Fieser (78) reported a certain specificity in structure associated with antihemorrhagic activity. With the exception of 2,3-dimethyl-1,4-naphthoquinone, activity began to appear as the alkyl

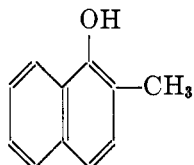
groups were increased in size beyond six to eight carbon atoms, became appreciable in the 2-methyl-3-cinnamyl (ten carbons) and 2-methyl-3-geranyl (eleven carbons) compounds, and reached a peak in vitamin K₁ (twenty-one carbons) and vitamin K₂ (thirty-one carbons). Fernholz *et al.* (73) pointed out that the inactivity of 2-*n*-octadecyl, 2-methyl-3-*n*-octadecyl, and similar long-chain substituted 1,4-naphthoquinones does not fit into this picture. However, there does appear to be an increase in activity with increase in the length of the chain in the 3-position, so long as this chain is β, γ -unsaturated and is made up of isoprenoid units. As pointed out in the discussion of table 2 and as illustrated by the contrasting inactivity of 2-ethyl-3-phytyl-1,4-naphthoquinone, the methyl group in the 2-position is highly specific. Since on a weight basis 2-methyl-1,4-naphthoquinone is twice as active as vitamin K₁, whereas, on a molar basis, the two are of about equal activity, Fieser postulated that 2-methyl-1,4-naphthoquinone merely served as a component for the synthesis of vitamin K₁ in the organism. Almquist and Klose (18) pointed out that the ratio of the activities of vitamins K₁ and K₂ is directly proportional to their respective contents of 2-methyl-1,4-naphthoquinone, and that the potencies of these vitamins are equivalent to about 80 per cent of the 2-methyl-1,4-naphthoquinone contained in them. These investigators propose that these relationships can be best explained by assuming that the long side chains of vitamins K₁ and K₂ are split off to the same extent.

C. OTHER POTENT COMPOUNDS

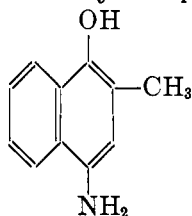
Tishler and Fieser and coworkers have prepared a number of methyl-naphthols (XI) and methyltetralones (XVI) (table 4). Of these, the compounds which on oxidation could give 2-methyl-1,4-naphthoquinone have potencies of the same order as vitamin K₁. β -Methylnaphthalene is active at 1 mg., but 1-amino-2-methylnaphthalene is considerably more active. In a study of the following series of compounds⁵—(1) β -methylnaphthalene, (2) 1-nitro-2-methylnaphthalene, (3) 1-amino-2-methylnaphthalene, (4) 2-methyl-1-naphthol (XI), (5) 4-amino-2-methyl-1-naphthol (XII), (6) 2-methyl-1,4-naphthohydroquinone (XIII), and (7) 2-methyl-1,4-naphthoquinone (XIV)—compounds 1, 2, and 3 showed low potencies, but compounds 5, 6, and 7 showed appreciably more activity. 2-Methyl-1-naphthol was about $\frac{1}{10}$ as active as 2-methyl-1,4-naphthoquinone.

Fieser made the significant observation that the oxides of 1,4-naphthoquinones possess the same order of potency as the compounds from which

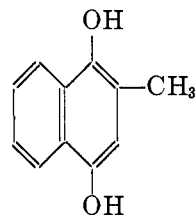
⁵ Richert, Binkley, Thayer, and Doisy (unpublished data and also references 80 and 187).

Antihemorrhagic compounds

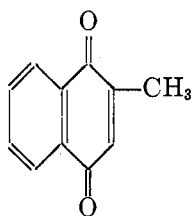
XI
2-Methyl-1-
naphthol



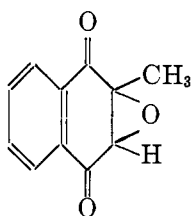
XII
4-Amino-2-methyl-1-
naphthol



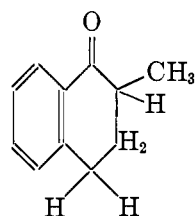
XIII
2-Methyl-1,4-
naphthohydroquinone



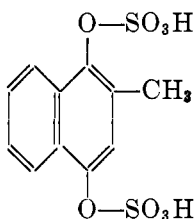
XIV
2-Methyl-1,4-
naphthoquinone



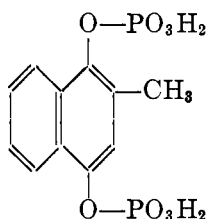
XV
2-Methyl-1,4-
naphthoquinone oxide



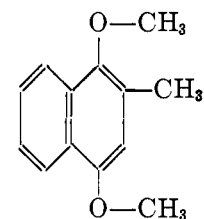
XVI
2-Methyl-1-
tetralone



XVII
2-Methyl-1,4-
naphthohydroquinone
disulfuric acid ester



XVIII
2-Methyl-1,4-
naphthohydroquinone
diphosphoric acid ester



XIX
1,4-Dimethoxy-2-
methylnaphthalene

they are derived (tables 4 and 5). These compounds are colorless, are easily reduced to quinones, and do not give the Dam-Karrer color test. On this basis, he postulated that vitamin K_0 (33) of Ansbacher might be the oxide of vitamin K_1 .

The hydroquinones are equal in activity to the corresponding quinones, whereas the diacetates are one-half as active. This statement holds for the hydroquinones and the diacetates of all 1,4-naphthoquinones that have been assayed (18, 31, 72, 181). However, Dam *et al.* (61) report considerably less activity for the diacetate of dihydrovitamin K_1 .

TABLE 4

Naphthols, tetralones, esters of 2-methyl-1,4-naphthoquinone, and related compounds

COMPOUND AND REFERENCE	APPROXIMATE WEIGHT OF ONE UNIT
	γ
2-Methyl-1-naphthol (XI) (187, 189).....	1
3-Methyl-1-naphthol (187, 189).....	1
1-Methyl-2-naphthol (187).....	—
3-Methyl-2-naphthol (187, 189).....	—
4-Methyl-1-naphthol (187, 189).....	—
2-Methyl-1-naphthylamine (187, 189).....	5
β -Methylnaphthalene (187).....	1000
3-Methyl-1-tetralone (187, 189).....	1
2-Methyl-1-tetralone (XVI) (187, 189).....	1
2-Methyl-5,6,7,8-tetrahydro-1,4-naphthoquinone (75).....	1000
2-Methyl-1,4-naphthoquinone oxide (XV) (79, 91).....	5
2-Methyl-1,4-dimethoxynaphthalene (XIX) (31).....	5
2-Methyl-1,4-naphthoquinone monoxime (61).....	2
2-Methyl-1,4-naphthohydroquinone diacetate (18, 31).....	1
2-Methyl-1,4-naphthohydroquinone dipropionate (31).....	1
2-Methyl-1,4-naphthohydroquinone dibenzoate (31).....	1
2-Methyl-1,4-naphthohydroquinone di- <i>n</i> -butyrate (31).....	1.25
2-Methyl-1,4-naphthohydroquinone diisobutyrate (31).....	5
2-Methyl-1,4-naphthohydroquinone di- <i>n</i> -valerate (31).....	1.25
2-Methyl-1,4-naphthohydroquinone diisovalerate (31).....	3
2-Methyl-1,4-naphthohydroquinone dimesitoate (187, 189).....	200
2-Methyl-1,4-naphthalenedioxydiacetic acid (32).....	2000

TABLE 5

Vitamin K₁ and derivatives

COMPOUND AND REFERENCE	APPROXIMATE WEIGHT OF ONE UNIT
	γ
Vitamin K ₁ (18, 33, 61, 69, 72, 76, 137, 144, 183, 185).....	1
Dihydrovitamin K ₁ diacetate (36, 137).....	2
β, γ -Dihydrovitamin K ₁ (90, 189).....	6
2-Methyl-3-phytyl-5,8-dihydro-1,4-naphthoquinone (90).....	5
$\beta, \gamma, 5, 6, 7, 8$ -Hexahydrovitamin K ₁ (75, 90, 189).....	>2
Vitamin K ₁ oxide (91).....	1
Dipotassium vitamin K ₁ hydroquinone disulfate (88).....	>500
Dihydrovitamin K ₁ diphosphoric acid (88).....	25
Naphthotocopherol (75, 90).....	300
	(>1000)
2-Methyl-3-isophytyl-1,4-naphthoquinone (33).....	15
2-Methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone (188).....	50

The high potency of 2-methyl-1,4-dimethoxynaphthalene (XIX) (table 4) is interesting. Owing to the difficulty of biological hydrolysis of methyl ethers of phenols,⁶ Ansbacher believed it possible that this compound acted as a whole. Likewise, he favored this explanation for the lower activity of the complex esters of 2-methyl-1,4-naphthohydroquinone rather than differences in the rate of hydrolysis or in the rate of absorption. As Tishler *et al.* (187) noted, another possibility to be considered is direct oxidation of the derivative to 2-methyl-1,4-naphthoquinone. However, these investigators consider the low activity of the dimesityl derivative of 2-methyl-1,4-naphthoquinone ($\frac{1}{100}$ as active as the dibenzoate) to be an indication that hydrolysis plays an important part. Since a biochemical study on the mode of action of these compounds has not been reported, any statement on this question is premature.

Table 5 is a compilation of the derivatives of vitamin K₁ and compounds which may be considered to be related in structure. Like the effect on the potency of 2-methyl-1,4-naphthoquinone, complete reduction of the benzenoid ring destroys the activity, while other reduction products show diminished activity. Fieser *et al.* (90) reported naphthotocopherol to be active at 300 γ , whereas Fernholz *et al.* (75) found it to be inactive at 1 mg., while the oxidation product was active at 300 γ . The disulfate and the diphosphate of dihydrovitamin K₁ show considerably less activity than vitamin K₁.

D. POTENT WATER-SOLUBLE COMPOUNDS

Since the 1,4-naphthoquinones and the natural vitamins are oil-soluble and must be used in conjunction with bile salts in oral therapy, and since a large proportion of patients in need of therapy cannot be treated orally because of nausea, intestinal obstruction, or other complications, it was important to find a compound of high activity which could be dissolved in an aqueous medium for intravenous use.⁷ Although 2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthohydroquinone have been used successfully, the solubility in saline is too low for their use in a convenient volume. The compounds listed in table 6 possess the same order of potency as 2-methyl-1,4-naphthoquinone and are water-soluble by reason

⁶ Although it is commonly believed that the methyl ethers of phenols are not easily hydrolyzed, Westerfeld (201) and Stroud (178) have shown that certain ethers of this type are hydrolyzed by monkeys and by rabbits, respectively.

⁷ The water-soluble compounds can be used orally as well as intravenously. Warner and Flynn (198) have shown that the potassium salt of the disulfuric acid ester of 2-methyl-1,4-naphthohydroquinone is readily absorbed from the intestinal tract of rats without the aid of bile salts. Smith and Owen (171) have found that patients respond to the oral administration of 1 mg. of 4-amino-2-methyl-1-naphthol without the addition of bile salts.

of a free amino group which forms a water-soluble hydrochloride, or an acidic group which forms a salt, or glucose residues which increase the water solubility. On a molecular basis, sodium 1,4-naphthohydroquinone disulfuric acid ester is one-third as active as the quinone itself. Foster *et al.* (95, 134) and Almquist (18) claim that tetrasodium 1,4-naphthohydroquinonediphosphoric acid ester (XVIII) is about 1.5 times as active as 2-methyl-1,4-naphthoquinone on a molecular basis and conclude that the vitamin probably acts through this derivative in the animal body. On the other hand, Ansbacher (32) reports that it is less active than the parent quinone or hydroquinone.

TABLE 6
Highly potent water-soluble compounds

COMPOUND AND REFERENCE	APPROXIMATE WEIGHT OF ONE UNIT
	γ
2-Methyl-1,4-naphthoquinone (XIV) (18, 29, 61, 67, 72, 134, 170, 180, 181, 183, 190).....	0.5
2-Methyl-1,4-naphthohydroquinone (XIII) (18, 31, 67, 170).....	0.5
2-Methyl-1,4-naphthohydroquinone monosuccinate (161).....	0.7
2-Methyl-1,4-naphthohydroquinone disuccinate (61).....	1
4-Amino-2-methyl-1-naphthol hydrochloride (XII) (18, 61, 67, 161)...	1
4-Amino-3-methyl-1-naphthol hydrochloride (161).....	1
Disodium 2-methyl-1,4-naphthohydroquinone disulfate (XVII) (32, 79, 88, 161).....	6
Tetrasodium 1,4-naphthohydroquinone diphosphoric acid ester (XVIII) (18, 32, 94, 95, 134).....	0.6-5
2-Methyl-1,4-naphthohydroquinone diglucoside (162).....	?
2-Methyl-1,4-naphthoquinone sodium bisulfite complex (162).....	?

Since the methods of synthesis of the quinones mentioned in the previous paragraphs and tables are mainly standard procedures which had been described prior to the development of the vitamin K field, a complete review of these methods will not be given. The methods of synthesis of vitamin K₁ have general application for the introduction of other substituted allylic groups into the 3-position in 2-methyl-1,4-naphthohydroquinone and into the 2-position in 1,4-naphthohydroquinone (89). The method of Barbot (34) has been used by Fernholz *et al.* (73) and by Karrer *et al.* (118) for the production of β-substituted tetralins. The ketones were reduced by the Clemmensen method, and the resulting hydrocarbons were dehydrogenated with sulfur to give the desired alkylnaphthalenes. The quinone was made by chromic acid oxidation. Karrer *et al.* (121) prepared 2-phytylnaphthalene by dehydration of the tertiary alcohol

obtained by the reaction between β -naphthylethylmagnesium bromide and 2,6,10-trimethylpentadecanone-14. After the preparation of the dibromide, the compound was oxidized to a 1,4-naphthoquinone and debrominated with zinc. In a second method naphthylacetylene was condensed with the ketone and the triple bond was hydrogenated to give the same tertiary alcohol. Fieser prepared the oxido derivatives by treatment of the quinone with hydrogen peroxide (189). Ring-closure methods were used for the preparation of the tetralones (189). 4-Amino-2-methyl-1-naphthol was prepared by the reduction of the monoxime of 2-methyl-1,4-naphthoquinone. The esters, ethers, and hydrogenation products were obtained by the usual methods. The references given after each compound refer to its preparation and bioassay.

X. CLINICAL WORK WITH ANTIHEMORRHAGIC COMPOUNDS

A. PHYSIOLOGICAL CONSIDERATIONS

Before passing to a brief discussion of the clinical use of vitamin K, it seems advisable to review certain physiological observations upon which the later clinical work was based. Since Wedelius (199) reported the first case of fatal cholemic bleeding in 1683, many investigations have been directed at the problem of the hemorrhagic tendency in obstructive jaundice. The various components of the clotting system were examined for abnormalities, but it was not until 1935 that Quick, Stanley-Brown, and Bancroft (158) devised a satisfactory method for the determination of prothrombin and showed that in obstructive jaundice the prothrombin concentration may be markedly reduced. In the same year, Hawkins and Whipple (103) found a hemorrhagic condition in dogs 3 to 4 months after an operation which established a complete biliary fistula. Besides the spontaneous bleeding, they noted a prolonged clotting time which they thought was due to prothrombin deficiency. Continuing the work, Hawkins and Brinkhous (102) in 1936 showed that the delayed clotting is due to a deficiency of prothrombin.

At about the same time (1936), Greaves and Schmidt (100) found spontaneous bleeding and decreased coagulability in rats with bile fistulas. Oral administration of bile corrected the hemorrhagic condition. Later (1937) they (98, 101) showed that the condition of the rats having bile fistulas could be cured by vitamin K and bile, and that bile was important in the absorption of vitamin K.

In the meantime, the study of vitamin K was progressing and Schönheyder (167), one of Dam's associates, showed in 1936 that the hemorrhagic syndrome of chicks is due to a deficiency of prothrombin. In 1937 Greaves and Schmidt (101) showed that administration of vitamin K relieved the prothrombin deficiency in rats having bile fistulas and stated that, although

bile alone sufficed, it was effective because of its capacity to promote the absorption of vitamin K.

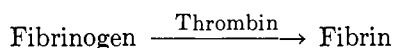
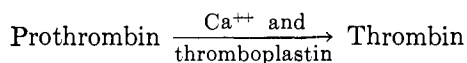
At this time (1937) Quick (153) correlated the different observations in a short note and suggested that vitamin K should prove useful in the hemorrhagic condition which frequently accompanies obstructive jaundice. In this clinical condition there is a deficiency of prothrombin, and in experimental animals such a deficiency can be corrected by the administration of the vitamin K and bile. Though Quick did not immediately test his hypothesis on patients, it was only a few months later that reports on the therapeutic use of vitamin K appeared almost simultaneously from three different groups of investigators: Warner, Brinkhous, and Smith (197) in January, 1938; Butt, Snell, and Osterberg (45) on February 2, 1938; Dam and Glavind (60) on March 26, 1938.

B. DETERMINATION OF PROTHROMBIN

Since it has been found that the clotting time may be within normal limits when the concentration of prothrombin has been reduced to one-third of the normal value, it is obvious that the determination of prothrombin constitutes a more delicate index than gross clotting time of the danger of impaired clotting. In obstructive jaundice the clotting time may be normal when the prothrombin value indicates a close approach to the hemorrhagic condition.

A discussion of the methods of determining prothrombin would take us too far afield; consequently the reader will be given only a brief statement on this point. Almost simultaneously, Quick, Stanley-Brown, and Bancroft (158) and Warner, Brinkhous, and Smith (196) published methods for the quantitative determination of prothrombin. These methods and modifications of them (2, 97, 108, 110, 114, 122, 124, 125, 151, 154, 175, 202) have been used extensively in establishing the desirability of vitamin K therapy in patients and in the control of the efficacy of the treatment.

The methods are based on the time required for blood or plasma to pass from a fluid to a gel state under optimal conditions. This time bears an established relationship to the concentration of prothrombin. The gel is produced by the action of thrombin, an enzyme (?) which is formed from prothrombin, on fibrinogen. The fibrinogen is converted to the insoluble fibrin.



C. TREATMENT OF OBSTRUCTIVE JAUNDICE

Following Quick's suggestion as to the use of vitamin K in obstructive jaundice and the appearance of the three papers previously mentioned, several groups of workers added information on the therapeutic value of vitamin K. The importance of antihemorrhagic compounds in obstructive jaundice seems to be thoroughly established (1, 24, 25, 42, 96, 109, 113, 115, 128, 130, 148, 152, 159, 160, 166, 172, 173, 176, 177, 179, 192). It should be pointed out that impairment of absorption in the intestine or severe damage to liver function (26, 39, 43, 44, 172, 200) may prevent a response to the administration of vitamin K; in the former case the difficulty can be overcome by the injection of the synthetic water-soluble antihemorrhagic compounds.

In an experiment by Zuckerman *et al.* (203), conducted on a patient with a total biliary fistula, slight bleeding from gums occurred after 2 weeks on a low fat, vitamin-K-free, and bile-free diet, and a week later bleeding from the tongue and vagina. A vitamin K concentrate was fed for 4 days without an effect on bleeding; feeding of the patient's own bile for 5 days was without effect, but when the bile and vitamin K were administered together the prothrombin and clotting times approached normal values and bleeding ceased.

In addition to impaired absorption of vitamin K, owing to the absence of bile from the intestine, certain intestinal conditions,—e.g., obstruction and severe diarrheal diseases, such as ulcerative colitis, sprue, and celiac disease,—may cause hypoprothrombinemia (47, 70, 112, 116). Intravenous therapy with one of the simple water-soluble antihemorrhagic compounds should be effective.

Although deficiency in vitamin K of dietary origin in the human should be rare on account of the widespread distribution of antihemorrhagic compounds, Kark and Lozner (114) have reported four cases of mild deficiency. The prothrombin values were slightly low before treatment, but after the administration of a vitamin K concentrate without added bile the values were restored to normal.

D. HEMORRHAGIC DISEASE OF THE NEWBORN

Another important therapeutic use of vitamin K merits consideration. In 1937, Brinkhous, Smith, and Warner (41) found a low prothrombin value in the hemorrhagic disease of the newborn. This was confirmed by Waddell and his collaborators (193, 194), who reported two newborns with prothrombin times in excess of 6 min. Within 2 hr. after the administration of vitamin K, the values had fallen to less than 1 min. Since from 25 to 40 per cent of the mortality of the newborn is due to the hemorrhagic syndrome, Waddell undertook the study of the effect of adminis-

tration of vitamin K to the mother before labor. This work has been extended by Hellman and Shettles (105), who have reported a comparison of values of the newborn with the values of the mothers and have shown that values of the newborn can be increased by medication of the prospective mother with vitamin K. A number of additional contributions (40, 104, 106, 123, 129, 131, 132, 140, 147, 150, 155, 156, 157, 165, 169, 191, 195) have been made to the study of the hemorrhagic disease of the newborn.

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The attention of the reader is directed to the following publications, which cover certain aspects of vitamin K more adequately than this review:

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