The Metabolic Relationships between the Different K Vitamins and the Synthesis of the Ubiquinones

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IT IS WELL KNOWN that a considerable number of compounds exhibit antihemorrhagic properties in biologic tests. One of these is methylnaphthoquinone (Menadione, vitamin K_3). Related compounds which can easily be transformed into methylnaphthoquinone in the body, such as aminomethylnaphthol ("vitamin K_5") are further examples. In addition to these relatively hydrophilic compounds there is a definitely lipophilic group of active substances such as vitamin K_1 (Phylloquinone) and the series of the K_2 vitamins isolated from bacteria. All of these compounds have practically the same activity on a molecular basis, a rather surprising fact when one considers their considerable difference in solubility and other physical properties. It thus appears improbable that these various substances are active as such, that is without undergoing some previous deep-seated change in the body. *A priori*, two explanations are possible: phylloquinone or the bacteria-vitamins lose their side chain in the organism and are transformed into methylnaphthoquinone, which is then responsible for the physiologic effect. Conversely it may be assumed that the organism has the capacity of introducing a long polyisoprene chain into methylnaphthoquinone transforming menadione into phylloquinone or a vitamin of the K_2 series.

As we have shown experimentally some time ago, the second hypothesis is correct.[1] When suitable experimental animals, preferably chickens raised on a vitamin K free diet are fed labeled menadione, it is possible to extract from the lipophylised organs, alongside the unchanged labeled compound, varying amounts of what has been shown to be vitamin $K_{2(20)}$: 2-methyl-3-geranylgeranyl-1,4-naphthoquinone.[2] To prove its nature we have used a method which has given very good results for the identification of microgram quantities of radioactively labeled compounds of this kind. The extract containing the unknown substance was put through a countercurrent distribution over thirty-five steps using glycolmonomethylether and heptane as the two phases. By measuring the radioactivity of each tube a distribution curve is obtained which usually contains one or more clearly distinguishable peaks. The peaks can be ascribed to individual components by comparison with parallel or mixed distribution experiments carried out with known components. Further confirmation of identity is afforded by running analogous distribution experiments with derivatives of the unknowns and of the standards differing as far as possible in their distribution pattern from the original material. A large number of test runs has shown that the results obtained by this method are very reliable.

We consider the fact that methylnaphthoquinone is transformed in the body into vitamin $K_{2(20)}$ as a proof that the latter compound is the biologically active principle.

There now arises the question of how the living animal cell is capable of carrying out a chemical transformation of a compound which in itself has never been observed in nature and is consequently regarded as a synthetic product. The easiest explanation is to assume

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Presented at the Symposium on the Metabolism and Function of the Fat-Soluble Vitamins A, E and K on November 7 and 8, 1960, at The University of Illinois, Urbana, Illinois, under the sponsorship of The National Vitamin Foundation, Inc., New York, New York.

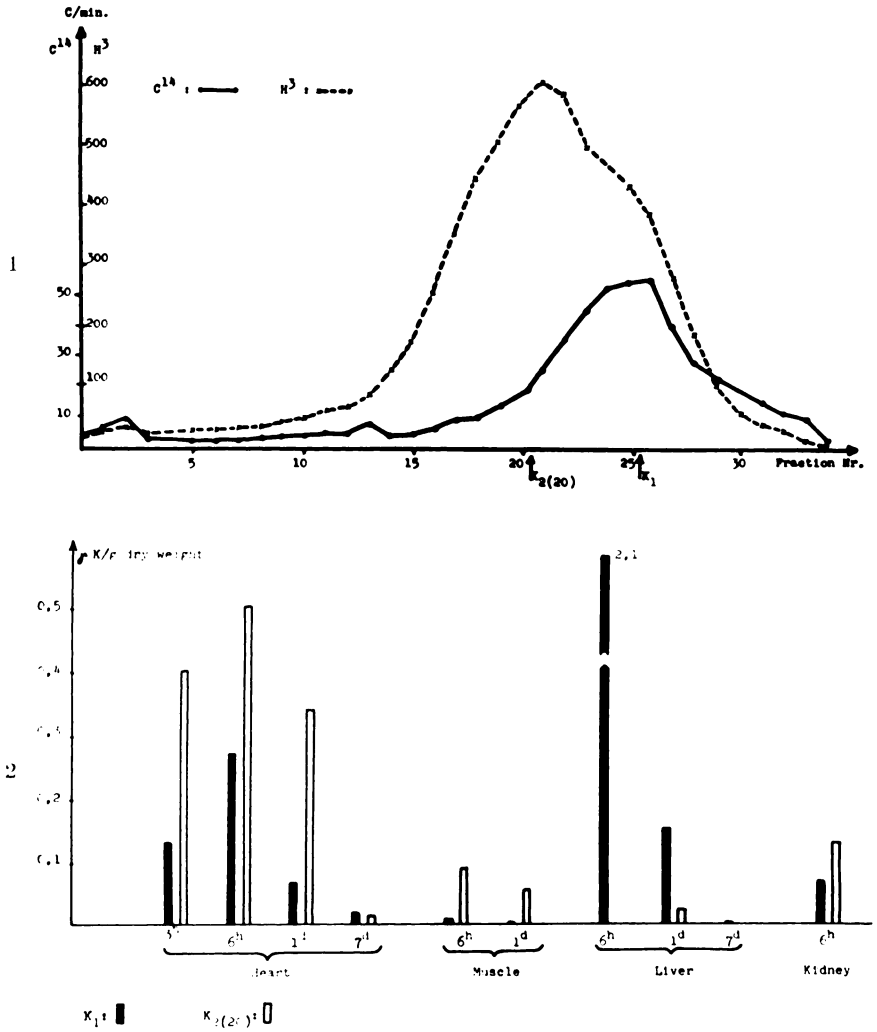

FIG. 1. Countercurrent distribution pattern. Heart extract. Chicken, killed twenty-four hours after application of vitamin K_1.

FIG. 2. Comparison of vitamin K_1 and $K_{2(20)}$ content in heart, muscle, liver and kidney in different time intervals following application of vitamin K_1 (350 μg.). Vitamin K deficient chicken, killed three, six, or twenty-four hours or seven days after application of vitamin K_1.

that methylnaphthoquinone can in fact occur in the cell, for instance as a degradation product of phylloquinone or of other vitamins of the K_2 group. This hypothesis was experimentally tested and proved to be right.[3] Double labeled phylloquinone of high specific radioactivity was synthetized for this experiment. It contained tritium in the methyl group of the nucleus and C^{14} in positions 1′ and 2′ of the phytol side chain. The experimental animals, chickens or pigeons, were fed the labeled compound per os, and were examined after several days. By using the same technic as in the previous experiments it was found that the organs of the animals contained vitamin $K_{2(20)}$ labeled only in the nucleus, together with unchanged starting material (Fig. 1). The original phylloquinone was particularly abundant in the liver, which in turn contained no vitamin K_2. In all other organs, vitamin K_2 was found one hour after feeding, whereas only traces of vitamin K_1 were present (Fig. 2). The metabolism of the vitamin is obviously considerable, as the maximum concentration of labeled vitamin K_2 is reached after six hours. Thereafter the concentration di-

minishes and after one week only traces can be detected, mostly in the skeletal musculature.

These findings lead to the conclusion that only vitamin K_2 will be found in the body. Whether the K_2 vitamins with thirty and thirty-five carbon atoms in the side chain are taken up as such by the body cell or whether also in such compounds the side chain is exchanged against a geranyl-geranyl group, remains to be established.* The only organ in which one may expect to find phylloquinone itself, in all probability introduced through food uptake, is the liver. I consider it likely that in the liver vitamin K_1 supplants vitamin K_2 also functionally.

From the experimental explanation of these relations there arises the question of the mechanism by which the chemical transformations come about. The available experimental evidence stands to indicate that the phylloquinone side chain is split off as a unit. It could be traced in the extracts thanks to its C^{14} label and was found to occur in the form of a strongly lipophilic ester, which upon hydrolysis yields phytol.

Thanks to the work of Lynen,[4] Bloch and others we are well informed about the biosynthesis of polyisoprene chains by animal cells. It appeared plausible that the introduction of an isoprenic side chain in a quinone nucleus would take place by an analogous route as in the synthesis of the chain itself. In other words it may be assumed that the side chain is attached by elimination of pyrophosphate between the quinone (or the hydroquinone) and the pyrophosphoric acid ester of a polyisoprene alcohol (Fig. 3).

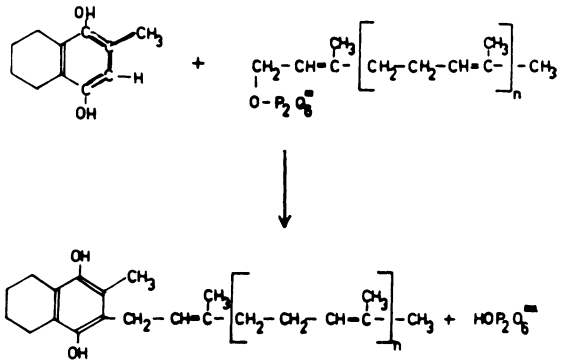

FIG. 3. Scheme for the introduction in methylnaphthohydroquinone of a polyisoprene side chain.

This hypothesis was easily confirmed by experiments in which labeled methylnaphthoquinone and pyrophosphates of polyisoprene alcohols of varying chain length were incu-

* Further experiments have shown that indeed in the rat and the chicken, vitamin $K_{2(30)}$ is converted into vitamin $K_{2(20)}$.

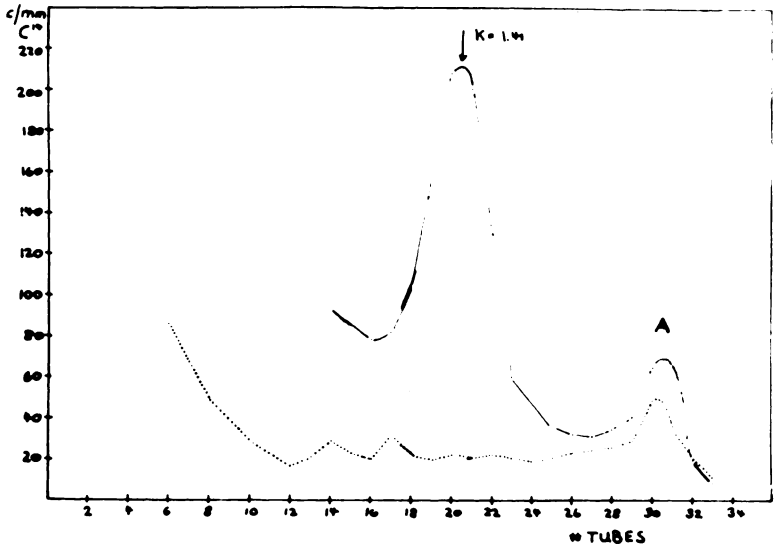

FIG. 4. CCD pattern of liver mitochondria lipid extract. Vitamin K deficient chicken. C^{14}-2-methylnaphthoquinone 2.5×10^{-4} M, geranyl-geranylpyrophosphate 10^{-3} M. System: n-neptane-methylglycol (.——. = lipid extract, ... = lipid extract control [without geranyl-geranylpyrophosphate]).

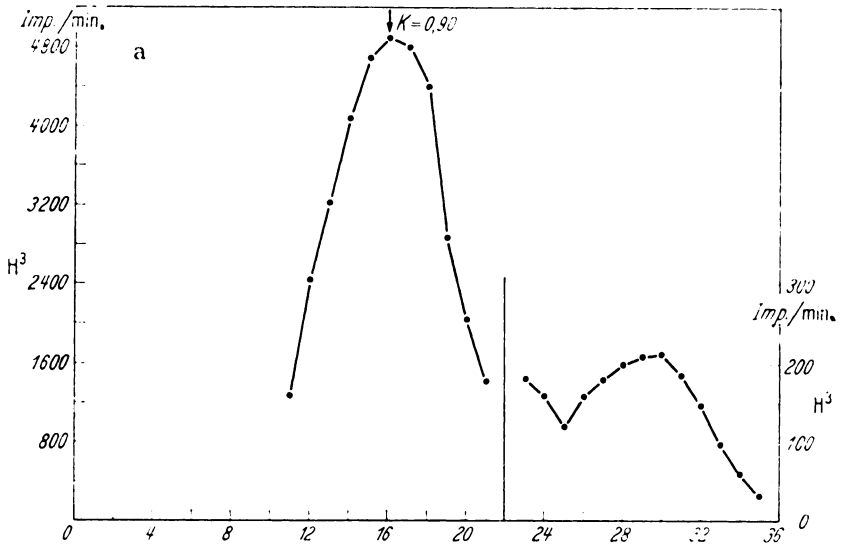

FIG. 5. Countercurrent distribution pattern of a rat liver homogenate lipid extract. H^3-5,6-dimethoxy-2-methylbenzoquinone 10^{-4}M, geranyl-geranyl-pyrophosphate 10^{-3}M. Formation of ubiquinone (20) and ubiquinone (45 and 50) (right curve).

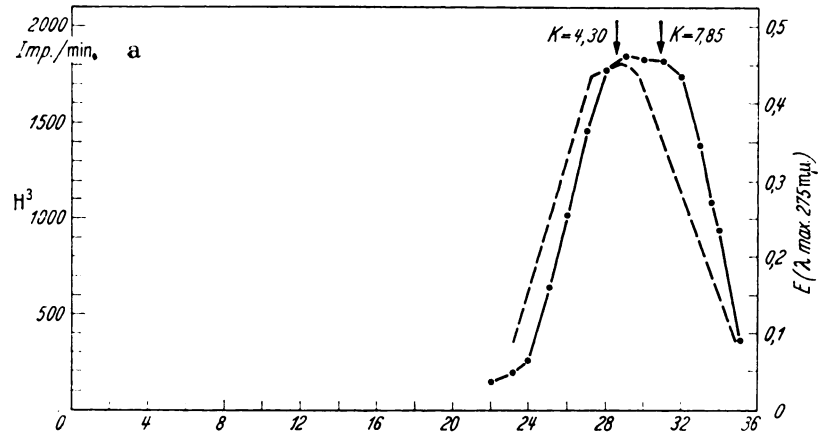

FIG. 6. Identical experiment as described in the previous figure but substituting geranyl-geranyl-pyrophosphate by solanesylpyrophosphate. Formation of ubiquinone (45) and (50). (.——. = radioactivity; --------- = extinction of authentic 5,6-dimethoxy-2-methyl-3-solanesylbenzoquinone).

bated with liver homogenates.[5] Examination of the mixtures after various periods in a countercurrent apparatus according to our usual technic led to the identification of vitamins of the K_2 group. Rat liver gives only low yields of the K_2 vitamins, whereas chicken liver again proved useful. The enzyme system responsible for the condensation is connected with the mitochondria fraction. No addition of microsome fraction or of cytoplasma is required. Disrupture of the mitochondria in a sonic oscillator sets the enzyme or enzyme system free: the supernatant liquid obtained by centrifugation in an ultracentrifuge contains a large fraction of the activity originally bound to the cellular particles. The enzyme does not have a high specificity with regard to the available pyrophosphate.

The alkylation was demonstrated with the pyrophosphates of geraniol, farnesol and geranyl-geraniol. There were thus obtained vitamin $K_{2(20)}$ (Fig. 4), the only product so far observed *in vivo*, and also the lower homologue with a C_{10} and a C_{15} side chain. We also made

the remarkable observation that phytol pyrophosphate does not react with methylnaphthoquinone under our experimental conditions.

In the course of our work we came across some results which led us to extend our study to ubiquinone (coenzyme Q).[5] In our incubation experiments with geranyl-geranyl pyrophosphate, for instance, we found beside geranyl-geranyl-naphthoquinone another labeled compound. It occurred only in small amounts and was considerably more lipophilic. In the distribution experiments it behaved as a K_2 vitamin with a side chain of forty-five or fifty carbon atoms. The same substance was formed also when no pyrophosphate of any of the side chain alcohols had been added. It followed therefore that the side chain can either be synthesized by or is present in the mitochondria. Isoprene chains of this length are present in ubiquinone, which is known to be formed in the body. We concluded that by adding labeled methylnaphthoquinone we had supplanted a natural unit of the synthesis of ubiquinone. We therefore repeated our experiments with labeled Q_0, that is with 2,3-dimethoxy-5-methyl-1,4-benzoquinone and found, as expected, that this compound reacted with all the polyisoprenyl pyrophosphates we added, yielding the corresponding ubiquinones with side chains of C_{10} to C_{45} (Figs. 5 and 6). The enzyme system of the liver mitochondria is considerably more active with dimethoxymethyl-benzoquinone as substrate than with menadione. The yields attain 25 per cent of the quinone. Mitochondria of rat liver are also found to be active.

We cannot as yet say whether the same enzyme is responsible for the formation of vitamin K_2 and of ubiquinone: some of our preliminary experiments point to identity of the enzyme, others are more easily interpreted by assuming that two different enzymes are at work.

We have extended our experiments to other quinones as well and found that toluquinone and trimethylbenzoquinone do not react, whereas 2-monomethoxy-5-methyl-1,4-benzoquinone is alkylated in position six by the enzyme.

These results indicate that the synthesis of ubiquinone in the body occurs along the same lines and perhaps even under the influence of the same enzyme as the formation of the "biological" K_2 vitamins from menadione or phylloquinone. The fundamental difference of the two processes is only that the quinone part in ubiquinone is also produced in the body by a mechanism that is not yet clearly understood, whereas in vitamin K_2 the cells depend on exogenous sources for the naphthoquinone nucleus, which they are unable to synthesize.

In my opinion, this close metabolic relationship of the two active principles finds its parallel in the more important functional one.

Several years ago, as you may know, I put forward the idea that vitamin K has nothing to do directly with the production of blood clotting proteins. It should act as an oxidation reduction catalyst in the phosphorylating respiratory chain along with other naturally occurring quinones. This hypothesis is based on the following experimental facts: (1) Liver mitochondria of highly K free chicken show a lowered P/O ratio compared with mitochondria from normal animals.[6] (2) This lowered capacity of oxydative phosphorylation can be restored *in vitro* by the addition of vitamin K_1 or K_2.[7] (3) All substances, which *in vivo* act as antagonists to vitamin K_1, the best known representative being dicumarol, act as very potent uncouplers of respiration and phosphorylation.[8,9]

According to its relatively negative ox/red potential the site of action of vitamin K in the respiratory chain should be between the pyridin nucleotides and cytochrome c.[10] We have tried for several years to detect an enzyme which could link the vitamin K with the pyridine nucleotides, a vitamin K reductase, and we were finally fortunate enough to isolate this enzyme in pure state.[11] It represents a so far unknown yellow enzyme with FAD as the prosthetic group. This latter can be reversibly split off and identified by paper chromatography or the d-amino acid oxydase test. The enzyme is reduced by the reduced forms of DPN and TPN as well. In the reduced state it can be reoxidized by a number of quinones, the most active being methyl naphthoquinon with a turn over number of 700,000. This high turnover number decreases if side chains are introduced in position three of the molecule. Vitamin $K_{2(20)}$ shows a turnover number of not more than 350 per minute. This

however may be due to the fact that in order to bring this water insoluble substance in solution one has to add detergents like Tween or others. Such substances seem to envelope and mask the quinon which as a consequence does not show its natural behavior which it may display when properly bound and fixed in the mitochondrial matrix.

Maybe the most interesting feature of this enzyme is the high rate of inhibition which one observes with dicumarol and all other anticoagulants. 10^{-8} M. dicumarol gives a ninety-three per cent inhibition and 10^{-10} M. dicumarol gives a 14 per cent inhibition of a 2×10^{-1} M. solution of the pure enzyme. This is to my knowledge the highest inhibition rate so far observed in any enzyme system. Thus, it seems to me very reasonable to assume that this high and specific inhibition of the enzyme, the mode of action of this enzyme itself as a hydrogen carrier and the known anticoagulant action of the inhibitors *in vivo* must have something to do with each other.

The enzyme vitamin K reductase is present in all kinds of animals, mammals, birds, fishes and amphibians. It is most abundant in the liver, kidney and heart muscle. When, however, pigeons were investigated it turned out that this species usually contains no detectable amount of vitamin K reductase and only in a few cases the reductase could be unambiguously determined.[12] Now it is interesting to note that pigeons in sharp contrast to other birds like chickens, geese or ducks are not dependent on a supply of vitamin K in the food. The question arises what consequence this lack of vitamin K reductase and apparent independence of vitamin K may have on the oxydative phosphorylation. According to my previously mentioned hypothesis, the vitamin K should be necessary for the first of the three phosphorylating steps during cell respiration. Using pigeon liver mitochondria we have investigated the P/O ratios which one gets with succinate and with β-hydroxybutarate as hydrogen donors. With succinate the normal value of about 2 (1.8) was observed; with β-hydroxybutyrate as substrate, however, we likewise obtained a P/O ratio of not more than 2.[1,9] This seems to me to indicate that in the pigeon liver only the energy potential between the succinate level and the oxygen is being used. Therefore, vitamin K and vitamin K reductase are not needed in this animal.

SUMMARY

Methylnaphthoquinone is converted in the animal organism into vitamin $K_2(_{20})$, which is the actual active form of the K vitamins. Vitamin K_1 (phylloquinone) as well as vitamin $K_2(_{30})$ (the K vitamin of bacteria) first lose the side chain and are thus converted into methylnaphthoquinone, which then yields vitamin $K_2(_{20})$. The side chain can be introduced *in vitro* in the presence of an enzyme system which occurs in liver mitochondria. The action of the enzyme system consists of condensing the pyrophosphates of isoprenalkohols with the quinone with elimination of pyrophosphoric acid and formation of the corresponding 3-substituted naphthoquinones.

It has been shown that the synthesis of the ubiquinones (coenzyme A) takes place along the same lines, starting from 2,3-dimethoxy-5-methyl-benzoquinone.

The properties of a new flavoprotein (vitamin K reductase) are described. This reductase transfers hydrogen from $DPNH^+$ and $TPNH^+$ to various quinones and is strongly inhibited by vitamin K antagonists (e.g., dicumarol). The importance of this enzyme for the interpretation of the mechanism of action of vitamin K is discussed.

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