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Characterization of Vitamin $K_9(H)$ from *Mycobacterium phlei*

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A newly characterized naphthoquinone has been isolated from *Mycobacterium phlei*. Spectral data and analytical and papergram results revealed that it is closely related structurally to vitamin $K_{2(45)}$, but differs in that one of the side-chain isoprenoid units is reduced. Nuclear magnetic resonance data also show that the saturated isoprenoid unit is not located at either end of the side-chain. This compound is designated vitamin $K_9(H)$ by the same nomenclature used for the new coenzyme Q_{10}(H-10).

Brodie *et al.* reported in 1958 a new naphthoquinone isolated from extracts of *Mycobacterium phlei* which was active in restoring oxidative phosphorylation to light-inactivated preparations. Their comparisons by paper chromatograms led to the conclusion that the quinone from *M. phlei* is different from all known vitamin K homologs which were available to them and including six members of the vitamin K_1 series, that is, $K_{1(5-30)}$ and vitamin $K_{2(35)}$. Comparison of the infrared spectrum of this quinone from *M. phlei* with the homologs of vitamin K_1 and K_2 showed identity in the position of peaks with the former but marked differences from the latter.

A crystalline naphthoquinone derivative related to vitamin K_2 was isolated from the acetone-soluble fat fraction of *Mycobacterium tuberculosis* (Brevannes) by Noll in 1958. It was shown later by Noll *et al.* (1960) that this compound and vitamin $K_{2(45)}$, synthesized from menadione and solanesol (Shunk *et al.*, 1959), are identical.

On the basis of specific spectral characteristics and quantitative infrared spectroscopy as well as paper chromatography, Noll (1960) concluded that a sample of vitamin K from *M. phlei*, provided by Brodie, and vitamin $K_{2(45)}$ from *M. tuberculosis* are identical.

Our interest in enzyme preparations from *Mycobacterium phlei* for studies concerning the mechanism of oxidative phosphorylation and the apparent involvement of naphthoquinone derivatives in this biochemical process led us to isolate and newly characterize a biologically active[1] vitamin K from *Mycobacterium phlei*. In addition to this interest, we wished to clarify the nature of the vitamin K obtained from *M. phlei*.

Results and Discussion

The nonsaponifiable material obtained from *Mycobacterium phlei* cells was purified by chromatography on a Decalso column with *n*-hexane and ether as eluting solvents (Table I). Eluate fractions contained material having an ultraviolet absorption spectrum characteristic of vitamin K.

After further purification by solvent fractionation followed by chromatography on Decalso columns with isooctane as solvent (Tables II, III), the product was isolated as an orange-colored oil.

Comparison of the ultraviolet absorption spectrum with spectra of authentic vitamins $K_{1(20)}$ and $K_{2(45)}$ (Table IV) revealed that the isolated compound was a member of the vitamin K group. In addition, the strikingly similar quantitative absorptions of the product from *M. phlei* and vitamin $K_{2(45)}$ strongly suggested that the two compounds were of nearly equal molecular weight. The elemental analysis was consistent with the assignment of a vitamin K structure to this com-

TABLE I
CHROMATOGRAPHY ON DECALSO, COLUMN 1

Developing Solvent	Vol. of Fraction (ml)	Color	Weight of Residue (g)
400 ml *n*-hexane	(1) 200	Yellow	0.26
	(2) 200	Yellow	0.46
	(3) 150	Pale yellow	1.07

TABLE II
CHROMATOGRAPHY ON DECALSO, COLUMN 2

Developing Solvent	Volume of Fraction (ml)	Color	Weight of Residue (mg)
2800 ml isooctane	(1) 1000	Colorless	
	(2) 300	First yellow band	24.4
	(3) 800	Second yellow band	138.9
	(4) 700	Pale yellow	242.6
600 ml ether	(5) 600	Yellow	313.8

* Coenzyme Q. XXXVII.

† Oxidative Phosphorylation in Fractionated Bacterial Systems. IX.

[1] Unpublished data.

TABLE III
CHROMATOGRAPHY ON DECALSO, COLUMN 3

Developing Solvent	Volume of Fraction (ml)	Color	Weight of Residue (mg)
575 ml isooctane	(1) 150	Colorless	6.0
	(2) 25	Colorless	3.5
	(3) 50	Yellow	15.2
	(4) 50	Yellow	14.1
	(5) 50	Pale yellow	10.8
	(6) 50	Pale yellow	10.9
	(7) 100	Pale yellow	10.1
	(8) 100	Pale yellow	10.0

TABLE IV
ULTRAVIOLET SPECTRA OF VITAMIN $K_9(H)$ AND VITAMINS $K_{1(20)}$ AND $K_{2(45)}$[a]

	$E\%$				
	Positions of Maxima (mμ)				
Compound	242.5	247.5	260	270	325
Vitamin $K_9(H)$	192	200	198	196	37
Vitamin $K_{2(45)}$	196	208	204	200	37
Vitamin $K_{1(20)}$	292	304	300	298	53

[a] Measured in isooctane with a Beckman DU spectrophotometer.

pound. The compound from *M. phlei*, however, did not crystallize from solvents that readily afford crystalline vitamin $K_{2(45)}$, m.p. 58–59°, and vitamin $K_{2(35)}$, m.p. 53–54°; vitamin $K_{2(50)}$ melts at 62°. On the basis of reversed-phase chromatography (Linn *et al.*, 1959) the isolated compound was not vitamin $K_{2(45)}$. Instead, its mobility was the same as that of vitamin $K_{2(50)}$.

Interpretation of the nuclear magnetic resonance (nmr) spectrum, based upon considerations similar to those of previous investigations reported in connection with the structural determinations of coenzyme Q_{10} and solanesol (Wolf *et al.*, 1958; Erickson *et al.*, 1959), confirmed the presence of the naphthoquinone structure of vitamin K in the quinone of *M. phlei*. In addition, area measurements of the characteristic resonances corresponded to a linear side-chain having eight unsaturated isoprenoid units and one saturated unit. The spectrum also showed that the saturated unit was not located at either end of the nine-unit chain.

This small structural difference, *i.e.*, the presence of a single reduced isoprenoid unit in the side-chain, was easily detected by both nmr spectroscopy and paper chromatographic separations on Vaseline-impregnated paper with dimethylformamide-water as solvent.

Catalytic hydrogenation of vitamin $K_{2(45)}$ followed by mild oxidation of the resulting hydroquinone yielded a product having ultraviolet absorption of the type associated with aromatic quinoid structures (Ewing *et al.*, 1939). The nmr spectrum of the reduced compound was compatible with the quinone structure deduced from the ultraviolet data. This established a method by which the length of the side-chain of the naphthoquinone from *M. phlei* could be determined. Small samples of it and of vitamins $K_{2(35)}$, $K_{2(45)}$, and $K_{2(50)}$ were catalytically hydrogenated, and the product from each in the quinone form was chromatographed on Vaseline-impregnated paper. Mobilities of the products from the reference compounds plotted on graph paper against the length of their side-chains gave a smooth curve. The hydrogenated product from the quinone of *M. phlei* had the same mobility as the corresponding product from vitamin $K_{2(45)}$; this fact gave evidence that the two parent compounds had been converted to the same reduced compound.

Therefore, this quinone from *M. phlei* is similar to vitamin $K_{2(45)}$ in every respect except that one of the isoprenoid units in the side-chain is reduced.

Since there is no formal nomenclature to designate this new compound, we have used the same tentative nomenclature for it which we have recently used to name the new coenzyme Q isolated from *Gibberella fujckuroi* (Gale *et al.*, 1963). This coenzyme Q differs from Q_{10} in that the terminal side-chain unit is reduced. This compound was designated Q_{10}(H-10). Similarly, the compound described in this report is designated vitamin $K_9(H)$. The subscript number indicates the total number of isoprenoid units in the side-chain. The parenthetical letter H indicates that one isoprenoid unit is reduced. In the analogous case of coenzyme Q_{10}(H-10), the number after the letter H signifies reduction of the tenth isoprenoid unit, counting from the benzoquinone nucleus. Since the precise location of the reduced unit in vitamin $K_9(H)$ is unknown, no designation follows the letter H.

In the crude vitamin K concentrate which we have now investigated, vitamin $K_9(H)$ is by far the major component. We have not identified vitamin $K_{2(45)}$ in the current study; however, as much as 2% of the total K in our preparation may exist in that form.

One of the original biologically active preparations of vitamin K which had been isolated from *M. phlei* by Brodie at Harvard was still available. We have now compared it with our newly characterized vitamin $K_9(H)$ and with vitamin $K_{2(45)}$. In our papergram system, before hydrogenation, this original preparation migrated with the same R_F as is characteristic of vitamin $K_9(H)$ and also vitamin $K_{2(50)}$ but not $K_{2(45)}$. After hydrogenation, however, the reduced product was identical to that from vitamin $K_9(H)$ but was clearly differentiated from the corresponding product from $K_{2(50)}$. Therefore, this original preparation is also vitamin $K_9(H)$. None of the other original preparations of vitamin K from *M. phlei* is available today. It may be that one of the earlier fermentations did produce a preponderance of vitamin $K_{2(45)}$ and that such a sample was examined by Dr. Noll.

EXPERIMENTAL

Saponification of Cells of Mycobacterium phlei.—To a suspension of 100 g of dried cells of *Mycobacterium phlei*, in 1 liter of 50% ethanol, was added 33 g of pyrogallol and 100 g of sodium hydroxide. The mixture was heated under reflux for ½ hour, then cooled slightly and extracted with three successive 500-ml volumes of *n*-hexane. The extracts were combined and washed with 300-ml portions of water until the washes were no longer alkaline. Evaporation of the solvent layer gave 2.6 g of partially crystalline residue, which was then triturated with three successive 50-ml portions of hexane. The hexane-insoluble fraction weighed 0.3 g.

Chromatography on Decalso.—A column of magnesium-alumino silicate (Decalso) was prepared by slowly pouring 30 g of the adsorbent, 50 mesh and finer, into *n*-hexane contained in a 2-cm diameter glass chromatograph column. The column was then inverted several times to thoroughly mix adsorbent and solvent, and then clamped in an upright position. After excess hexane drained, the hexane-soluble fraction was added to the column and allowed to flow slowly through it. Hexane and ether were eluting solvents (Table I).

Aliquots of the three fractions were diluted with

isooctane, and the ultraviolet absorption curves of the solutions were traced with a Bausch and Lomb Spectronic 505. Maxima at 244, 250, 262, 271, and 326 mμ (broad) were seen with $E^{1\%}_{1cm}$ values 64, 67.5, 73, 69, and 9 for fraction 1; 21, 22, 20, 20, and 4.3 for fraction 2; and 6.9, 7.9, 7.6, 7.5, and 1.2 for fraction 3.

Samples of the three fractions and authentic vitamin $K_{2(35)}$ were papergrammed; Vaseline-impregnated Whatman No. 1 circles were used, with 98:2 dimethylformamide (Merck, reagent)–water, saturated with Vaseline, as mobile phase. A mixture of fraction 1 and vitamin $K_{2(35)}$ gave ultraviolet absorption zones with R_F values 0.32 and 0.67, respectively. A mixture of fractions 2 and 3 and vitamin $K_{2(35)}$ gave zones with R_F values 0.29 and 0.63, respectively.

Residues from fractions 1, 2, and 3 were combined and dissolved in 15 ml of isooctane. A crystalline material which was obtained by chilling the solution in ice was recrystallized twice from 15-ml volumes of isooctane. Evaporation of the combined mother liquors gave 1.1 g of orange-colored residue.

Rechromatography on Decalso.—A solution of the 1.1 g of residue in 50 ml of isooctane was loaded on a column of Decalso prepared as described above. The column was developed with isooctane (Table II).

Ultraviolet absorption curves of aliquots from fractions 2–5 in isooctane were traced. The absorption shown by fraction 3 was characteristic for vitamin K.

Samples of fractions 3, 4, and 5 and authentic samples of vitamin $K_{2(45)}$ and $K_{2(50)}$ were chromatographed by the reversed-phase procedure. Fraction 3 gave a strong ultraviolet absorption zone with R_F 0.31. A mixture of fraction 3, vitamins $K_{2(45)}$, and $K_{2(50)}$ gave zones with R_F values 0.27 (fraction 3 and $K_{2(50)}$) and 0.38 ($K_{2(45)}$). Fraction 4 gave a faint absorption zone at R_F 0.31, and no zone was seen in fraction 5.

A solution of the residue from fraction 3 in 1 ml of isooctane was cooled in an ice bath. The white crystalline product that formed was recrystallized from 0.7 ml of isooctane. The combined mother liquors were evaporated and yielded 106.5 mg of a thick, orange-colored sirup.

Rechromatography on Decalso to Give Vitamin $K_9(H)$.—An 0.8-cm-diameter column of Decalso (6.0 g) was prepared by the procedure previously described. The sirupy residue (106.5 mg) obtained from fraction 3 was dissolved in 10 ml of isooctane and was put on the column. The eluting solvent was isooctane (Table III).

Isooctane solutions of fractions 3–8 gave ultraviolet absorption curves with sharp maxima at 245, 251, 263, and 271 mμ and a broad maximum at 325 mμ. Those fractions were combined; total weight, 68.5 mg. The orange liquid did not solidify or crystallize directly or from methanol, ethanol, acetone, or light petroleum ether solutions during storage at 5° for 2 weeks.

Anal. Calcd. for $C_{56}H_{82}O_2$ (787.22): C, 85.42; H, 10.50. Found: C, 84.80; H, 10.37.

The ultraviolet absorption spectra of the isolated product and reference samples of vitamin $K_{1(20)}$ and vitamin $K_{2(45)}$ were determined with a Beckman DU spectrophotometer. Positions and $E^{1\%}_{1cm}$ values of the maxima are recorded in Table IV.

The product of *M. phlei* and reference samples of vitamins $K_{2(45)}$ and $K_{2(50)}$ were papergrammed. Both the product and vitamin $K_{2(50)}$ gave an ultraviolet absorption zone with R_F 0.20; the R_F for vitamin $K_{2(45)}$ was 0.26.

Nuclear Magnetic Resonance Data.—The nmr spectrum of the new quinone confirmed the naphthoquinone nuclear structure and provided additional information concerning the side-chain.

Resonances were found at 2.02 (multiplet) and 2.42 τ units (multiplet) for aromatic protons of two different types; 4.92 τ units for eight olefinic protons; 6.64–6.75 τ units (doublet) for the two methylenic protons on the first carbon atom of the isoprenoid side-chain; 7.83 τ units for the aromatic ring methyl group protons; 8.02 τ units for the methylene group protons of the isoprenoid units; 8.20, 8.32, and 8.41 τ units for the protons of all of the isoprenoid methyl groups; 8.72 τ units (multiplet) for the methylenic protons of a saturated side-chain unit; 9.12 τ units (multiplet) for the methyl group protons of a saturated side-chain unit. The three frequencies for the methyl group protons of the isoprenoid units are the result of differences between certain of those protons.

Since the area of the 6.64–6.75 doublet corresponds to two protons, it can be determined that the band area of 4.92 corresponds to eight protons or eight unsaturated isoprenoid units in the side-chain. In the same manner, the area of the band of 9.12 corresponds to three protons and the band of 8.72 to about six, which requires the presence of one saturated isoprenoid unit in the side-chain or a total side-chain of 45 carbon atoms.

As for the location of the saturated unit in the side-chain, it was proved only that it is located within the side-chain. The presence of the doublet of 6.64–6.75 precludes its being the first 5-carbon unit (counting from the naphthoquinone nucleus), and the presence of only three protons in the band of 9.12, together with the presence of no more than seven protons in the band of 8.72, rules out its being the terminal side-chain unit.

Catalytic Hydrogenation of Vitamin $K_{2(45)}$.—A 31.5-mg sample of synthetic vitamin $K_{2(45)}$ dissolved in 25 ml of ethyl acetate was reduced catalytically with use of 0.2 g of 5% palladium on charcoal. The product was purified by chromatography on a column (0.5 cm × 22 cm) of Davison silica gel desiccant grade (activated), 100–200 mesh. Isooctane and then 1:9 ether-isooctane were the developing solvents. Evaporation of the ether-isooctane eluate yielded 22.7 mg of light orange-colored liquid as the quinone. The ultraviolet absorption spectrum, measured with a Bausch and Lomb Spectronic 505, showed the following maxima: 244, 250, 264, 272.5, and 325 mμ (broad) with $E^{1\%}_{1cm}$ values 158, 165, 188, 190, and 24. The nmr spectrum indicated that the side-chain had been reduced, but that the aromatic character of the nucleus was present.

Catalytic Hydrogenation of the Product from M. phlei and Vitamins $K_{2(35)}$, $K_{2(45)}$, and $K_{2(50)}$.—Approximately 2-mg samples of vitamins $K_{2(35)}$, $K_{2(45)}$, and $K_{2(50)}$ and the compound obtained from cells of *M. phlei* were catalytically reduced. Samples of the products, without further purification, were chromatographed radially on Vaseline-impregnated Whatman No. 1 paper. Dimethylacetamide saturated with Vaseline was the mobile phase. Products from vitamins $K_{2(35)}$, $K_{2(45)}$, and $K_{2(50)}$, and the product of *M. phlei*, gave ultraviolet-absorbing zones having R_F values 0.22, 0.06, 0.02, and 0.06, respectively. Mixtures of the compounds were chromatographed (descending) for 136 hours on Vaseline-treated paper with glacial acetic acid saturated with Vaseline as mobile phase. Perhydrogenation products from the quinone of *M. phlei* and vitamin $K_{2(45)}$ moved 5.0 cm; the corresponding products from vitamins $K_{2(35)}$ and $K_{2(50)}$ moved 22 cm and 1.2 cm, respectively. Mixtures of those compounds and the quinone from *M. phlei* were easily resolved.

Paper Chromatography of Vitamin $K_9(H)$, Vitamin $K_{2(45)}$, and an Original Preparation of Vitamin K from M. phlei.—Aliquots containing 100 μg of vitamin $K_{2(45)}$ and $K_9(H)$ and 400 μg of an original vitamin K prepara-

tion were spotted upon Vaseline-impregnated Whatman No. 1 paper and chromatographed. They gave zones with R_F values of 0.25, 0.18, and 0.19, respectively.

Catalytic Hydrogenation of an Original Preparation of Vitamin K from M. phlei.—A 2.4-mg sample of an original preparation from *M. phlei* was catalytically hydrogenated. A sample of the product, in the quinone form, equivalent to 300 μg of the preparation before reduction, was spotted upon Vaseline-impregnated Whatman No. 1 paper. Samples of the corresponding products from vitamin $K_9(H)$ and $K_{2(50)}$ were also placed upon the paper. The paper chromatogram was developed (descending) for 13 days with glacial acetic acid saturated with Vaseline as solvent. A mixture of the vitamin $K_{2(50)}$ and $K_9(H)$ reduction products gave zones 3.6 cm and 7.4 cm, respectively, from the origin. The product from the original preparation from *M. phlei* gave a slightly elongated zone 7.0 cm from the origin.

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Enzymatic Formation of Testololactone*

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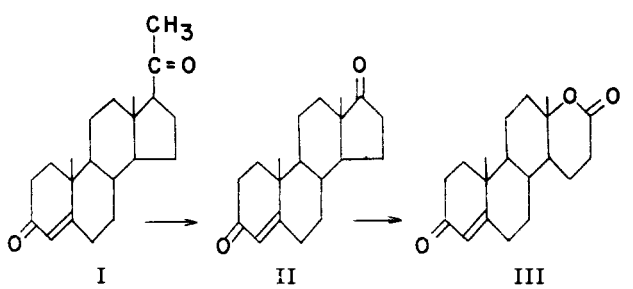
The conversion of testosterone to testololactone has been studied in soluble and partially purified enzyme systems derived from *Penicillium lilacinum*. This transformation is carried out by two steroid-induced enzyme systems: a diphosphopyridine nucleotide–linked 17β-hydroxysteroid dehydrogenase that catalyzes the reversible interconversion of testosterone and 4-androstene-3,17-dione; and a lactonizing enzyme system that converts 4-androstene-3,17-dione to testololactone. The lactonization reaction shows an absolute requirement for reduced triphosphopyridine nucleotide and for molecular oxygen. Tracer studies with oxygen^{18} have shown that the single oxygen atom incorporated during the formation of the lactone is derived from molecular oxygen and is located in the ethereal and not the carbonyl group linked to C-17.

In 1953 two groups of workers (Fried *et al.*, 1953; Peterson *et al.*, 1953) reported that several species of *Penicillia* and *Aspergilli* converted various C_{19} and C_{21} steroids to testololactone (III), a six-membered steroid ring D lactone. The formation of testololactone was discovered incidental to certain other microbiological oxidations, particularly the degradation of the side-chain of C_{21} steroids and the introduction of a Δ^1-double bond into ring A. In a typical reaction, progesterone (I) was converted via 4-androstene-3,17-dione (II) to testololactone (III).



I II III

The testololactone formed during the course of these reactions was identical to a compound prepared by Jacobsen (1947) by the peracetic acid oxidation of a 17-ketosteroid. In 1953, the precise chemical structure of testololactone and related steroid lactones was uncertain, and it was not known whether the lactone oxygen atom was located between C-13 and C-17, or between C-16 and C-17. This question was subsequently resolved unequivocally in favor of the structure shown in formula III (Wendler *et al.*, 1955; Murray *et al.*, 1956).

The discovery of the microbiological conversion of 4-androstene-3,17-dione to testololactone in high yield offered the interesting opportunity of studying the mechanism of a novel biochemical reaction. The possibility was also considered that lactonization of the steroid ring D might constitute a general pathway for the microbial degradation of the steroid skeleton. Recent work by Bradshaw *et al.* (1959) and by Conrad *et al.* (1961, 1962) has demonstrated that the microbial oxidation of camphor proceeds via the lactonization of cyclic ketones. There are chemical similarities between the lactonizations of steroids and camphor, and these transformations are formally analogous to the Baeyer-Villiger (1899) reaction.

This paper describes the conversion of testosterone

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