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# Identification of Vitamin $K_{2(35)}$, an Apparent Cofactor of a Steroidal 1-Dehydrogenase of *Bacillus sphaericus**

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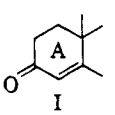
The cells of *Bacillus sphaericus* (MB431) were subjected to alkaline saponification in the presence of pyrogallol. Solvent extraction yielded an orange-colored residue which was subjected to two chromatographic purifications on silica gel and final chromatography over magnesium-aluminosilicate (Decalso). Recrystallizations of appropriate eluates yielded a yellow crystalline product compound which was identified as vitamin $K_{2(35)}$. The identification was based upon analytical and spectral evidence, including the nuclear magnetic resonance spectrum and comparison with synthetic members of the vitamin $K_2$ group. No evidence for the presence of a member of the coenzyme Q group was observed. Vitamin $K_{2(35)}$ has been tested for coenzymatic activity in the microbial 1-dehydrogenation of a $\Delta^4$-3-ketosteroid; activity was observed. Menadione, hexahydrocoenzyme $Q_4$, and 6-phytyl-2,3,5-trimethyl-1,4-benzoquinone were also active. Corresponding coenzymatic activity of coenzyme $Q_{10}$ in steroidal dehydrogenation in mammalian tissue, such as adrenal, may now be considered.

The therapeutic advent of 1-dehydrocortisone and 1-dehydrocortisol was soon followed by the microbial introduction of the $\Delta^1$-double bond into $\Delta^4$-3-ketosteroids by Vischer *et al.* (1955b) using *Didymella lycopersica*, Vischer *et al.* (1955a) using *Calonectria decora*, and Nobile *et al.* (1955) using *Corynebacterium simplex*. In addition, Lindner *et al.* (1956) using *Bacterium subtilis* and Sutter *et al.* (1957) using either *Mycobacterium laticola* or *Septomyxa affinis* accomplished the same type of steroidal transformation.

Stoudt *et al.* (1955), of this laboratory, reported on the selective microbial 1-dehydrogenation with a bacterial culture belonging to the species *Bacillus sphaericus*. In fact, these investigators successfully used ten steroidal substrates (I) and obtained the corresponding $\Delta^{1,4}$ dienone analogues (II) with this organism. The steroids were cortisone, cortisol, corticosterone, deoxycorticosterone, progesterone, 4 - androstadiene - 3,17-dione, and related compounds.



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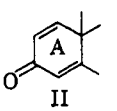

II

Using cell-free extracts from *Bacillus sphaericus*, Hayano *et al.* (1961) reported studies on the mechanism of the reaction and on the nature of the coenzyme involved with the steroidal 1-dehydrogenase. Although menadione was active in catalytic quantities, it was believed that the natural cofactor is probably a quinone of similar structure to menadione. Quinones of the vitamin K and coenzyme Q groups were of interest for evaluation.

We have undertaken an examination of the presence or absence of quinones of the vitamin K and coenzyme Q groups in cells of *Bacillus*

* Coenzyme Q. XXXV.

*sphaericus* to identify the natural cofactor related to the steroidal 1-dehydrogenase.

## Results

An ethanolic solution of the non-saponifiable fraction from cells of *Bacillus sphaericus* exhibited an ultraviolet maximum at 270 mμ which is similar to the peak of a member of the coenzyme Q group. This fraction contained an ultraviolet-absorbing compound with the same papergram mobility (Linn *et al.*, 1959) as that of coenzyme $Q_9$. However, this compound differed from coenzyme Q in its reaction with the leucomethylene blue (Linn *et al.*, 1959) and ferric chloride-bipyridyl reagents (Emmerie and Engle, 1939) (Table I). Consequently, though there was no evidence for a coenzyme Q in the cells, the data suggested the presence of a related compound.

After chromatography of the crude cell extract, using silica gel (Tables II and III) and magnesium aluminosilicate (Decalso) (Table IV), a crystalline product was obtained. Recrystallization from absolute ethanol and then from light petroleum ether (30–60°) afforded 181 mg of pure, crystalline product, m.p. 53–54°; Isler *et al.* (1958) reported 54° for vitamin $K_{2(35)}$.

The positions and relative intensities of the ultraviolet maxima were characteristic for a member of the vitamin $K_2$ group. The molecular weight of the crystalline material was found to be 654, based upon comparison of its ultraviolet absorption at 247.5 mμ with that of synthetic vitamin $K_{2(45)}$; vitamin $K_{2(35)}$ has a molecular weight of 649.02; $K_{2(30)}$, 580.86; $K_{2(40)}$, 717.10.

Results from reversed-phase paper chromatography of the substance and reference samples of vitamins $K_{2(30)}$, $K_{2(45)}$, and $K_{2(50)}$ are plotted in Figure 1. A similar comparison of coenzyme Q $R_F$ values is included in Figure 1. The mobilities of the isolated crystalline compound on the several papergrams were in excellent agreement with the values predicted for vitamin $K_{2(35)}$.

The nuclear magnetic resonance spectrum[1] of this quinone from *B. sphaericus* was qualitatively identical with that of synthetic vitamin $K_{2(45)}$. However, replicate area measurements made on the two compounds were in accord with a 35-carbon seven-isoprenoid-unit side-chain for the *B. sphaericus* isolate instead of the nine-unit side-chain of vitamin $K_{2(45)}$.

All these data on the quinone of *Bacillus sphaericus* identify it as vitamin $K_{2(35)}$.

Although the experimental details of an extended study of vitamin $K_{2(35)}$, and many related compounds, in the 1-dehydrogenase system will be reported (Stoudt *et al.*)[2] separately, we wish to summarize the first data on the activity of vitamin $K_{2(35)}$ and three other compounds of

Table I
Comparison of Extract of *Bacillus sphaericus* with Coenzymes $Q_9$ and $Q_{10}$ on a Papergram[a]

| | $R_F$ | Ultraviolet Absorption | Reaction with Leucomethylene Blue[b] | Reaction with Ferric-Bipyridyl[b] |
|---|---|---|---|---|
| *Bacillus sphaericus* Extract | 0.34 | + | | + |
| Coenzyme $Q_9$ | 0.35 | + | + | |
| Coenzyme $Q_{10}$ | 0.25 | + | + | |

[a] $R_F$ values were obtained from circular chromatograms with Whatman No. 1 impregnated with Vaseline. Dimethylformamide containing water and Vaseline was the mobile phase. [b] Spray reagent.

Table II
Chromatography on Silica Gel

| Developing Solvent | Vol. of Fraction (ml) | Color | Weight of Residue (g) |
|---|---|---|---|
| 500 ml Skellysolve B | (1) 700 | Colorless | — |
| 1500 ml 2% ether in Skellysolve B | (2) 1500 | Yellow | 1.55 |
| 1000 ml 50% ether in Skellysolve B | (3) 1000 | Yellow | 2.30 |

Table III
Rechromatography on Silica Gel

| Developing Solvent | Volume of Fraction (ml) | Color | Weight of Residue (mg) |
|---|---|---|---|
| 100 ml Skellysolve B | (1) 200 | Colorless | — |
| 300 ml 1% ether in Skellysolve B | (2) 100 | Yellow | 74.5 |
| | (3) 100 | Yellow | 183.6 |
| 200 ml 2% ether in Skellysolve B | (4) 200 | Yellow | 362.7 |

Table IV
Chromatography on Decalso

| Developing Solvent | Volume of Fraction (ml) | Color | Weight of Residue (mg) |
|---|---|---|---|
| 3800 ml Skellysolve B | (1) 500 | Colorless | — |
| | (2) 200 | Yellow | — |
| | (3) 600 | Colorless | — |
| | (4) 250 | Yellow | 47.9 |
| | (5) 250 | Yellow | 61.6 |
| | (6) 250 | Yellow | 48.0 |
| | (7) 250 | Yellow | 33.1 |
| | (8) 500 | Yellow | 36.0 |
| | (9) 1000 | Yellow | 31.4 |

[1] We are indebted to Dr. N. R. Trenner and Mr. Byron Arison for obtaining and interpreting the spectral data.

[2] Manuscript in preparation.

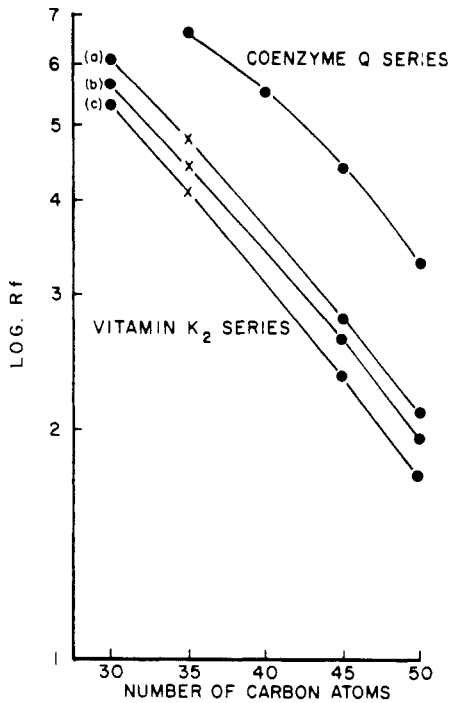


FIG. 1.—Comparison of papergram mobility vs. number of carbon atoms in the side-chain. The $R_F$ values of the isolated vitamin $K_{2(35)}$ on the three papergrams, (a), (b), and (c), are represented by X. Whatman No. 1 circles were prepared for use by being passed once through a 5% (w/v) solution of petroleum jelly (Vaseline) in petroleum ether and then air-dried. The samples, dissolved in 98:2 dimethylformamide (Merck, Reagent)–water solution saturated with Vaseline, were applied to the paper. Chromatograms were developed radially using the same solvent. After air-drying, zones were located by viewing the papergrams illuminated by ultraviolet light.

special interest. A cell-free preparation of *B. sphaericus* (MB431) was demonstrated to possess a steroid 1-dehydrogenase system as evidenced by the conversion of hydrocortisone to prednisolone. The addition of cofactor levels of $K_{2(35)}$ or menadione improved the conversion rate by three- to five-fold, depending on conditions selected for non-inactivated systems. In addition, the cell-free preparation could be essentially completely photo-inactivated, with full original activity being restored by $K_{2(35)}$ or menadione. The kinetics of the inactivation are similar to those of K-compounds, and the $K_{2(35)}$ used in these experiments was actually isolated from the culture. Hexahydrocoenzyme $Q_4$ and 6-phytyl-2,3,5-trimethyl - 1,4 - benzoquinone were approximately three times and one-third as active, respectively, as vitamin $K_{2(35)}$ in restoring activity to the photo-inactivated system.

## DISCUSSION

On the basis of structure-activity relationships, Hayano *et al.* (1961) thought that a quinone related to menadione was responsible for the demonstrated activity in the steroidal 1-dehydrogenase system of *Bacillus sphaericus*. The papergram mobility of the isolated quinone was the same as that of coenzyme $Q_9$, in our comparison, and the quinone absorbed ultraviolet light and had the same mobility as previously reported. However, we observed that leucomethylene blue spray reagent, which is readily oxidized by as little as 3 μg coenzyme $Q_9$, did not reveal the presence of any member of the coenzyme Q group in the *B. sphaericus* extract. Furthermore, both vitamin $K_1$ and this isolated quinone gave weakly positive reactions with ferric-bipyridyl reagent, whereas coenzyme $Q_9$ does not react with the reagent.

The marked similarity of the ultraviolet absorption spectrum to that of a vitamin $K_2$ provided proof that the *B. sphaericus* product belongs to the vitamin K group.

Comparison of the papergram mobilities of the substance and vitamins $K_{2(30)}$, $K_{2(45)}$, and $K_{2(50)}$ definitely established the length of the side-chain. A similar comparison in the coenzyme Q series showed that the mobility vs. chain-length relationship was represented by a curved rather than a straight line. As had also been observed when members of the coenzyme Q group were papergrammed by this system, the relationship between the $R_F$ values of the several compounds was very consistent for a given paper chromatogram, though there were some differences between papergrams.

Since vitamin $K_{2(35)}$ was found effective in restoring 1-dehydrogenase activity to *Bacillus sphaericus*, and it is present in the cells, it appears that $K_{2(35)}$ is the natural cofactor.

One may now give new consideration to a corresponding coenzymatic role for coenzyme $Q_{10}$ in steroidal dehydrogenation since this quinone is in human adrenal tissue (Gale *et al.*, 1961), and was found also in mitochondrial and microsomal fractions from porcine adrenal glands (Leonhäuser *et al.*, 1962).

## EXPERIMENTAL

*Saponification of B. sphaericus Cells.*—A thick cellular paste obtained by centrifuging 150 gallons of whole culture broth weighed 4585 g. To approximately one-third of this semisolid paste in a 5 - liter round - bottomed flask was added 400 ml of water, 1500 ml of ethanol, 125 g of pyrogallol, and 400 g of sodium hydroxide. The mixture was heated under reflux for one half hour, then cooled slightly and extracted with four successive 1-liter portions of Skellysolve B. The combined solvent extract was washed in a separatory funnel with 500-ml volumes of water until the washes were no longer alkaline, and then the extract was concentrated *in vacuo* to about 400 ml. The two remaining portions of cell paste were processed in the same way. Evaporation of the solvent from the three com-

bined solutions yielded 4.3 g of partially solid, orange-colored residue.

The residue was examined for the presence of coenzymes $Q_9$ and $Q_{10}$ by direct comparison with authentic quinones on a papergram. Dimethylformamide (Merck Reagent)–water, 98:2, was used as mobile phase. Zones were located by holding the air-dried papergram over an ultraviolet lamp and spraying with leucomethylene blue or ferric chloride–bipyridyl reagents. Coenzyme $Q_{10}$ had an $R_F$ of 0.26. The residue contained an ultraviolet-absorbing compound having, like coenzyme $Q_9$, an $R_F$ of 0.38. However, it gave no blue zone with leucomethylene blue and, unlike coenzymes $Q_9$ and $Q_{10}$, reacted with the ferric chloride–bipyridyl reagent to give a pink zone with $R_F$ 0.38. The ultraviolet absorption spectrum in ethanol was measured with a Beckman DU spectrophotometer. Maximum absorption occurred at 270 mμ.

*Chromatography on Silica Gel.*—A column (3.5 × 20 cm) of Davison silica gel activated desiccant, 100–200 mesh, was prepared by slowly pouring 125 g of adsorbent into a glass chromatographic column containing Skellysolve B. After the solid had settled, excess solvent was removed and a solution of the 4.3 g of crude concentrate in 200 ml Skellysolve B was added. The solution was allowed to flow slowly through the column. Skellysolve B and ether–Skellysolve B mixtures were used as eluting solvents (Table II).

Fractions 2 and 3 and authentic coenzyme $Q_9$ were papergrammed by the reversed-phase technique described above. Fraction 2 contained an ultraviolet absorbing component with $R_F$ 0.35. No ultraviolet-absorbing material was detected in fraction 3.

*Rechromatography on Silica Gel.*—A column (2 × 30 cm) of 20 g of Davison silica gel was prepared as described above. A solution of fraction 2 in 25 ml of Skellysolve B was loaded onto the column. After the solution had slowly percolated through the adsorbent, the column was developed with Skellysolve B and mixtures of ether and Skellysolve B (Table III).

Fractions 2, 3, and 4 and coenzyme $Q_9$ were papergrammed. Coenzyme $Q_9$ had an $R_F$ of 0.51. Fraction 3 gave a faint ultraviolet absorption zone with $R_F$ 0.51, and fraction 4 gave a strong zone at the same location.

*Chromatography on Decalso.*—A 2-cm diameter chromatographic column of magnesium-aluminosilicate (Decalso) containing 40 g of the adsorbent, 50 mesh and finer, was prepared in the same manner as the silica gel columns. Material from fraction 4, dissolved in 20 ml Skellysolve B, was added. The column was developed with the same solvent (Table IV). Nine fractions were collected, and each eluate was evaporated to dryness.

*Crystallization and Identification.*—The yellow-orange, oily residues from the Decalso fractions 4–9 were stored overnight at 5°. Each residue crystallized. A solution of fractions 4–8 in 25 ml of hot absolute ethanol, when cooled to 25°, deposited yellow crystals. After the mixture had been further cooled to 5°, 210 mg of crystalline material, m.p. 47–52°, was obtained. A solution of this crystalline material in 1.5 ml of petroleum ether (30–60°) was chilled with ice-salt. Rosettes of the yellow crystalline product formed and were separated. After another recrystallization from the same solvent, the product melted at 53–54°; yield, 181.5 mg. *Anal.* Calcd. for $C_{46}H_{64}O_2$ (649.02): C, 85.13; H, 9.94. Found: C, 85.22; H, 9.67.

The ultraviolet absorption spectrum in iso-octane was determined with a Beckman DU spectrophotometer. Maxima at 242.5 mμ, $E^{1\%}_{1\ cm}$ 283; 247.5 mμ, $E^{1\%}_{1\ cm}$ 295; 260 mμ, $E^{1\%}_{1\ cm}$ 290; 270 mμ, $E^{1\%}_{1\ cm}$ 285, and a broad peak at 325 mμ, $E^{1\%}_{1\ cm}$ 54, were observed. A sample of synthetic vitamin $K_{2(45)}$ dissolved in iso-octane gave ultraviolet maxima at the same wave lengths with $E^{1\%}_{1\ cm}$'s 223, 246, 240, 235, and 46 respectively.

The crystalline product and reference samples of vitamins $K_{2(30)}$, $K_{2(45)}$, and $K_{2(50)}$ were papergrammed by the procedure described above. Results from three such papergrams were graphed in Figure 1.

The nuclear magnetic resonance spectrum clearly indicated a compound having the naphthoquinone structure of a vitamin K and an isoprenoid side-chain. Comparison of the spectrum with that for synthetic vitamin $K_{2(45)}$ revealed that the two compounds were qualitatively identical. The side-chain of the isolated crystalline material contained 7.2 (±0.2) olefinic protons, based upon nine replicate area determinations, while the vitamin $K_{2(45)}$ nine-isoprenoid-unit side-chain was found to have the expected nine olefinic protons; calculated 9.2 (±0.5) based upon 18 replicate measurements.

These data are consistent with those calculated for vitamin $K_{2(35)}$. Consequently, that structure was assigned to the compound isolated from *B. sphaericus*.

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# Isolation and Characterization of Human Urinary Metabolites of Aldosterone. III. Three Isomeric Tetrahydro Metabolites

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After the oral administration of a large quantity of tritium-labeled aldosterone to normal human subjects, three isomeric urinary metabolites reduced in ring A were isolated and characterized. These compounds were 3$\alpha$,11$\beta$,21-trihydroxy-18-oxoallopregnan-20-one; 3$\beta$,11$\beta$,21-trihydroxy-18-oxopregnan-20-one, and 3$\alpha$,11$\beta$,21-trihydroxy-18-oxopregnan-20-one.

The isolation and characterization of human urinary metabolites of aldosterone has been the subject of previous reports from this laboratory (Ulick and Lieberman, 1957; Kelly *et al.*, 1962). This report concerns the isolation and proof of structure of two new tetrahydrometabolites from the urine of subjects who had ingested a large quantity of $H^3$ - *d* - aldosterone - 21 - monoacetate. These compounds are 3$\alpha$,11$\beta$,21-trihydroxy-18-oxoallopregnan - 20 - one (3$\alpha$,5$\alpha$ - tetrahydroaldosterone, M10) and 3$\beta$,11$\beta$,21 - trihydroxy-18-oxopregnan - 20 - one (3$\beta$,5$\beta$ - tetrahydroaldosterone, M11). The chemical reactions by which they were characterized are shown in Figure 1.

Ulick and Lieberman (1957) isolated the major metabolite of aldosterone and tentatively assigned to it the structure 3$\alpha$,18,21-trihydroxypregnane-11,20-dione. Recently, Ulick *et al.* (1961, and in preparation) determined that as originally isolated this metabolite was a mixture of 3$\alpha$,18,21-trihydroxypregnane - 11,20 - dione (18 - hydroxy-THA) and 3$\alpha$,11$\beta$,21 - trihydroxy - 18 - oxopregnan - 20 - one (3$\alpha$,5$\beta$ - tetrahydroaldosterone, M12), and that only the latter is a metabolite of aldosterone. In the present study the correct structure proposed by Ulick *et al.* (1961, and in preparation) was confirmed, and the metabolite was further characterized through the preparation of several new derivatives as shown in Figure 2.

## Experimental

Melting points were determined on a Kofler block and were corrected. Infrared spectra were obtained on a Perkin-Elmer Model 221 spectrometer. A Packard Liquid Scintillation Spectrometer was used for the assay of radioactivity, and simultaneous counting of tritium and $C^{14}$ was done according to the discriminator ratio method of Okita *et al.* (1957). The determination of the number of acetylatable hydroxyl groups and the partition chromatography were carried out as previously described (Kelly *et al.*, 1962). The chromatography systems employed are listed in Table I.

*$H^3$-Aldosterone-21-monoacetate.*—One mg of *d*-aldosterone-21-monoacetate was randomly labeled with tritium in the laboratory of the New England Nuclear Corporation by exposure to tritium gas according to the procedure of Wilzbach (1957), and the labile tritium was exchanged for hydrogen by equilibration with methanol. The sample was returned to this laboratory for purification. About 20 mg of carrier was added to the radioactive material and three crystallizations were carried out, during which there was a small decrease in specific activity of the crystals. However, the specific activities of the mother liquor were much greater than those of the crystals, and increased with successive crystallizations. After the third crystallization the specific activity of the mother liquor was more than twice that of the crystals. The mother liquors were combined for further purification. The crystals were chromatographed in system D on a 50 g column, and the specific activity of each radioactive fraction was determined with the blue tetrazolium reaction (Recknagel and Litteria, 1956) used to estimate the weight of the aldosterone acetate. The mean specific activity of the fractions comprising the peak was $10.9 \times 10^6 \pm 1.0 \times 10^6$ cpm/mg. The fractions were combined and crystallized twice. The final crystals weighed 2.598 mg and had a specific activity of $10.6 \times 10^6$ cpm/mg. To this