VITAMIN K IN BACTERIA

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SUMMARY

1. Bacterial strains of the species *Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Sarcina flava, Staphylococcus aureus, Mycobacterium phlei, Pseudomonas* sp., *Azotobacter vinelandii* and a *Nocardia* species have been investigated for type and quantity of vitamin K.

2. By paper chromatography evidence has been obtained that three different types of vitamin K are synthesized by the different strains.

3. The quantity of vitamin K was estimated by biological test and by measuring the ultraviolet absorption of lipid extracts after previous purification by paper chromatography.

4. Two types of vitamin K have been isolated in crystalline form, one from *Bacillus cereus* and one from *Mycobacterium phlei*. The ultraviolet and infrared spectra of these compounds are discussed.

INTRODUCTION

The discovery of new naphthoquinones in bacteria has been reported by various authors. ISLER *et al.*[1] announced the existence of two kinds of vitamin K_2 in putrefied fishmeal, one with a C_{30}—side chain and another with a C_{35}—side chain. The identity of the isolated compounds was verified by synthesis. SNOW[2], NOLL[3] and BRODIE *et al.*[4] studied the naphthoquinones of mycobacteria and have reported chemical differences of these quinones from vitamin K_2.

In the present study different bacteria are investigated regarding the quantity and type of vitamin K which they produce.

EXPERIMENTAL

Media

The basal medium for most of the bacteria investigated had the following composition: casein–peptone-hydrolysate with 1 % of nitrogen, 400 ml; yeast autolysate with 1 % of nitrogen, 100 ml; tap water, 500 ml; pH was adjusted to 7.0. For *S. aureus* this medium was used without any additions. For the strains of *B. cereus, B. subtilis, Proteus vulgaris* and *Sarcina flava* 0.2 % of dextrose was added. For the *Pseudomonas* strain 6 and for the micrococcus strain 4 1.5 % of NaCl was added, because these organisms were isolated from marine water. The *Mycobacterium phlei* was grown on a synthetic Dorset medium: dextrose, 1 %; glycerol, 3 %; asparagine, 1.4 %; sodium

citrate, 0.09 %; ferric citrate, 0.03 %; K_2HPO_4, 0.18 %; $MgSO_4 \cdot 7H_2O$, 0.15 %; pH 7.0. *Azotobacter vinelandii* was grown on a synthetic, nitrogen-free medium[5]: dextrose, 2 %; K_2HPO_4, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.02 %; $FeSO_4 \cdot 7H_2O$, 0.005 %; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0005 %; $CaCl_2 \cdot 6H_2O$, 0.02 %; pH 7.5. For the *Nocardia* sp. was used a potato-dextrose medium: dextrose, 1 %; peptone, 0.5 %; yeast extract, 0.1 %; K_2HPO_4, 0.2 %; KNO_3, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.2 %; NaCl, 0.1 %; $CaCO_3$, 0.2 %; $FeSO_4 \cdot 7H_2O$, trace; boiled extract of 200 g potatoes per liter.

Growth of bacteria and preparation of lipid extracts

The bacteria were grown in a liquid medium for 10–20 days. The cells were harvested by centrifugation and then dried by lyophilization. The dry culture was extracted overnight with peroxide-free ether–absolute alcohol (1:1). The extract was evaporated in vacuum, and the residue obtained was extracted with peroxide-free ether; then the ether was evaporated and this residue, which contained the vitamin K activity, was dissolved to known volume in *n*-hexane and used for paper chromatography.

Methods for separation and characterization of the K vitamins

The vitamins K were characterized by the paper chromatographic method of GREEN AND DAM[6], using Whatman No. 1 impregnated with Dow Corning Silicone 1107. The solvent for paper chromatography was: ethanol–glacial acetic acid–water (850:25:125). In a few cases *n*-propanol–glacial acetic acid–water (650:25:325) was used. As reference compounds were used: natural vitamin K_1 (Merck & Co.); natural vitamin K_2 (side chain C_{35}); synthetic vitamin K_2 (side chain C_{25} and C_{30}). These three compounds were kindly supplied by Hoffmann-La Roche and Co., Basle.

For quantitative estimation of the K vitamins the spots were eluted from the paper with *n*-hexane, and the u.v. spectra from 230 to 360 mμ were recorded on a Beckman Spectrophotometer DK 2. The *n*-hexane eluate from a blank strip of the chromatogram was used as a blank. This procedure incurred no difficulties of the kind reported by LESTER AND RAMASARMA[7] concerning paper chromatography of ubiquinones. Owing to too much absorption of the eluate, these investigators were not able to obtain u.v. spectra of the eluates of the chromatograms. The discrepancy between their results and ours may arise from the use of different Dow Corning Silicones for impregnation of the paper.

RESULTS AND DISCUSSION

Table I shows the R_F values for the reference compounds (solvent: ethanol–glacial acetic acid–water (850:25:125)) and the values of $E^{1\%}_{1\,cm}$ at 248 mμ. The extinction coefficients of the three K_2 compounds are those given by ISLER *et al.*[1].

Table II shows the R_F values of the K-vitamins in the bacterial extracts and the calculated quantity of vitamin K expressed as vitamin K_2 with side chain C_{35}. For calculation the absorption peak at 248 mμ was used. The vitamin K content of some of the bacteria was tested biologically on vitamin K-deficient chicks according to the method of DAM *et al.*[8]. In this test the biological activity was compared with Menadione as reference standard. For comparison with the results of the u.v. measurement the data in Table II are presented as the effect of vitamin $K_{2(35)}$ by multiplying

the data directly obtained by the ratio of the molecular weights ($K_{2(35)}$/Menadione). However, WISS *et al.*[9] have pointed out that the vitamin K activity in K-deficient chicks is dependent on the length of the side chain of the vitamin K compound; this must be taken into consideration in judging the accuracy of the figures.

TABLE I

R_F VALUES AND EXTINCTION COEFFICIENTS OF REFERENCE COMPOUNDS

Solvent for paper chromatography: ethanol–glacial acetic acid–water (850:25:125).

Compound	R_F *value*	$E^{1\%}_{1\,cm}$ *(248 mμ)*
K_1	0.75	450
$K_{2(25)}$	0.80	363[1]
$K_{2(30)}$	0.60	320[1]
$K_{2(35)}$	0.45	292[1]

From Table II it is seen that *Sarcina flava* strain 50 has two spots giving a vitamin K-spectrum: one, rather small with R_F value 0.45 like that of vitamin $K_{2(35)}$, and a second, bigger spot with R_F 0.33.

The R_F values of the compounds of *Proteus vulgaris* and *Sarcina flava* were not changed by purification of the extract by chromatography on Decalso*. The *Nocardia*

TABLE II

R_F VALUES AND QUANTITIES OF VITAMIN K IN BACTERIA

Bacterial strain	R_F*-value*	R_F*-value*	*Vitamin K (mg, calculated as mg* $K_{2(35)}$*/g culture)*	
	ethanol–glacial acetic acid–water (850:25:125)	*n-propanol–glacial acetic acid–water (650:25:325)*	*u.v. measurement*	*biological test*
B. cereus strain 35*	0.45	0.70	2.6	2.75
B. cereus strain 44*	0.46			
B. subtilis strain 13*	0.48		0.33	0.38
*Proteus vulgaris**	0.33	0.59	0.46	1.7
Sarcina flava strain 50*	0.45	0.70	0.07	
	0.33	0.58		
S. aureus 209 P (ATCC 6538P)*	0.38		little	
*Mycobact. phlei***	0.22	0.52	2.3	2.3
Pseudomonas strain 6***			nil	
Micrococcus strain 4***			nil	
Azotobacter vinelandii (Kluyver)§			nil	
Nocardia sp. strain 69§§	0.30?		0.15	

* Supplied by A. Snog-Kjær, Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen.

** Supplied by Dr. H. E. Ottosen, The State Veterinary Laboratory, Copenhagen.

*** Supplied by S. B. Halkier, Technological Laboratory, Ministry of Fisheries, Copenhagen.

§ Supplied by Dr. H. L. Jensen, State Laboratory for Soil and Crop Research, Lyngby, Denmark.

§§ Supplied by T. Vincents Nissen, Department of Technical Biochemistry, Polytechnic Institute, Copenhagen.

* "Zerolit" S/F, — 60 + 90 mesh, from United Water Softeners Ltd, London.

strain 69 always gave long spots of not well-defined R_F values, even after chromatography on Decalso.

Azotobacter vinelandii is reported to contain ubiquinone (LESTER AND RAMASARMA[7]). After chromatography we found a spectrum indicating the presence of ubiquinone and no spectrum of vitamin K.

B. cereus strain 35 and *M. phlei* are very rich in vitamin K. These strains were grown in large quantities, and their naphthoquinones were purified. After extraction of the freeze-dried culture (100 g) with acetone, the extract was dissolved in petroleum ether, and dark-coloured impurities were extracted from the solution with methanol 95 %. The petroleum ether layer was evaporated, and the residue dried over P_2O_5. It was then dissolved in petroleum ether (b.p. 60–100°) and chromatographed on Decalso. The naphthoquinone was eluted with petroleum ether, 80 %, plus benzene, 20 %. The naphthoquinones were recrystallized twice from acetone. From *B. cereus* strain 35 was obtained 80 mg of yellow crystals of vitamin $K_{2(35)}$, m.p. 54° (uncorr.). *M. phlei* gave yellow crystals at —20°, m.p. —4° (uncorr.). This oil had an u.v. spectrum similar to that of vitamin K_1 and K_2, and an $E^{1\%}_{1\,cm} = 255$ at 248 mμ, indicating a molecular weight of maximally 740 assuming a molecular extinction of 20,000. The infrared spectra* of the naphthoquinones from the two bacteria were compared with those of vitamin K_1 and $K_{2(35)}$. Similarity was found between the spectrum of $K_{2(35)}$ and that of the vitamin K isolated from *B. cereus* strain 35. The IR-spectrum of the naphthoquinone of *M. phlei* was more like that of vitamin K_1 than that of vitamin $K_{2(35)}$'. The main differences between the spectra of vitamin K_1 and $K_{2(35)}$ were seen in the region 650–900 cm^{-1} (Fig. 1). Vitamin $K_{2(35)}$ had four strong peaks at 687, 715, 793 and 872 cm^{-1}. Two of these, at 687 and 715 cm^{-1}, were also found in vitamin K_1 and with the same relative intensity, but instead of the two strong peaks at 793 and 872 cm^{-1} vitamin K_1 had two much weaker peaks at 785 and 890 cm^{-1}. The absorption characteristics of vitamin K_1 were found in the naphthoquinone of *M. phlei* also, but they were obscured in the preparation to some extent by an absorption at 800–850 cm^{-1} (Fig. 1). Fig. 2 shows the spectrum of vitamin K_1 and that of the *M. phlei*-naphthoquinone from 1,200 to 1,700 cm^{-1}. The quinone structure is illustrated in the spectrum by the peak at 1,660 cm^{-1}, and the benzene structure by the peak at 1595 cm^{-1}. Assuming the same molecular extinction of vitamin K_1 and of the *M. phlei*-naphthoquinone, the following calculations of the molecular weight of the *M. phlei*-naphthoquinone can be made:

(1) From the peak at 1,660 cm^{-1} a molecular weight of maximally 650.

(2) From the peak at 1,595 cm^{-1} a molecular weight of maximally 700.

Calculation from the u.v. spectrum gave a molecular weight of 740 of this compound; BRODIE *et al.*[4] reported the molecular weight of the naphthoquinone of *M. phlei* to be maximally 620.

The absorption peaks at 1,375 cm^{-1} (CH_3-groups) and at 1,440 cm^{-1} (CH_2-groups) are relatively higher in the *M. phlei*-naphthoquinone than in vitamin K_1 (Fig. 2). This may suggest that the molecule of the *M. phlei*-naphthoquinone has more CH_2- and CH_3-groups than vitamin K_1.

* The IR-spectra (650–4,000 cm^{-1}) were recorded on a Model 21 Perkin Elmer Double-beam Spectrophotometer using the technique of KBr discs (Disc-diameter: 12.5 mm). Concentrations: approximately 1 mg naphthoquinone in 300 mg KBr; vitamin $K_{2(35)}$ from Hoffmann-La Roche: 1.08 mg; vitamin $K_{2(35)}$ from *B. cereus*: 1.08 mg; vitamin K_1 from Merck: 1.22 mg; compound from *M. phlei*: 1.04 mg.

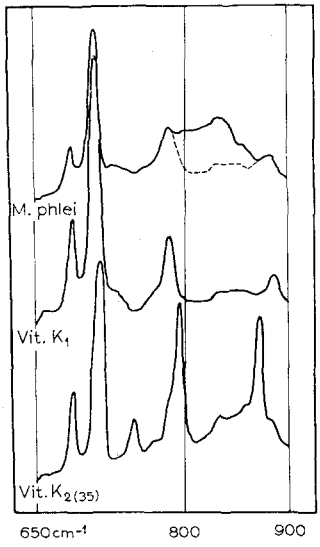

Fig. 1. Comparison of the IR-spectrum of the naphthoquinone of *M. phlei* with those of vitamins K_1 and $K_{2(35)}$. 650–900 cm^{-1}. The ordinate is optical density. The broken line represents the spectrum that would have been obtained if the substance examined had been pure vitamin K_1. Whether the difference between the solid and the broken lines is due to impurities or differences in the molecule is uncertain.

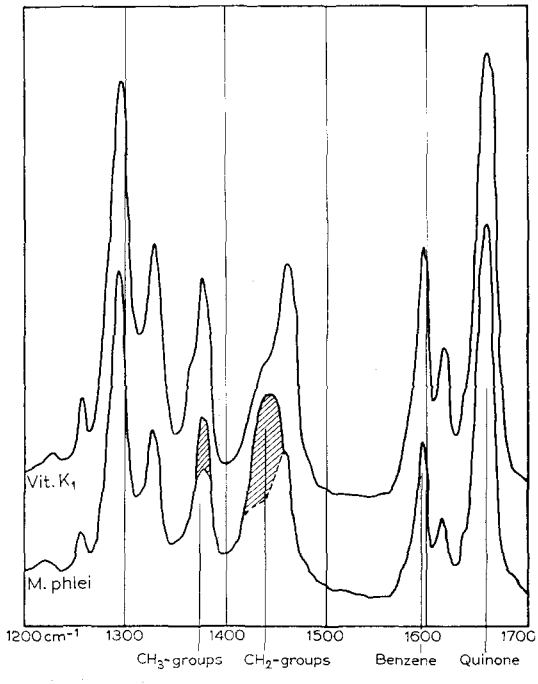

Fig. 2. Comparison of the IR-spectrum of the naphthoquinone of *M. phlei* with that of vitamin K_1. 1,200–1,700 cm^{-1}. The ordinate is optical density. The broken line in the spectrum of the naphthoquinone of *M. phlei* represents the height of the peaks if the compound were pure vitamin K_1.

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THE ACTION OF N-BROMOSUCCINIMIDE ON TRYPSINOGEN AND ITS DERIVATIVES

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SUMMARY

The reactions of trypsin, trypsinogen, acetyltrypsinogen, and an enzymically active fragment of trypsinogen with N-bromosuccinimide have been explored. Under the conditions used, the reagent selectively oxidized the tryptophan residues without significant cleavage of tryptophyl peptide bonds. The marked difference in reactivity of tryptophan in trypsin and trypsinogen is ascribed to differences in their secondary or tertiary structure. Enzymic inactivation (trypsin) or loss of activatability (trypsinogen) was studied as a function of the oxidative modification of tryptophan. Such partially inactivated enzyme preparations still had their DFP phosphorylation sites intact. At least one tryptophan residue may be needed for activity. This demonstrates that an intact phosphorylation site *per se* is not sufficient for enzymic activity.

INTRODUCTION

The selective cleavage of C-tryptophyl peptide bonds in various model peptides, the hormone glucagon, and several proteins, *e.g.*, TMV protein and serum albumins has been reported recently. Selective cleavages varying in yield from 10–40 %, have been