BIOSYNTHESIS OF A VITAMIN K_2 BY CELL-FREE EXTRACTS OF MYCOBACTERIUM PHLEI [*]

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Received February 10, 1965

Very little is known of the de novo biosynthesis of vitamin K_2 by microorganisms. Cox and Gibson (1964) have described the incorporation of ^{14}C-shikimic acid into vitamin $K_2(35)$ in whole cells of E. coli. We have recently found that whole cells of M. phlei incorporate the radioactivity from L-methionine-$^{14}CH_3$ into vitamin $K_2(45)H$[**] (Lederer, 1964; Azerad et al., 1965). We now report the incorporation of radioactivity from L-methionine-$^{14}CH_3$ into vitamin K_2 (45)H by a cell-free supernatant obtained from M. phlei.

METHODS. M. phlei Legroux cells were grown in Sauton medium for 7-8 days. Twenty g. of washed cells were suspended in 20 ml of a KCl-tris buffer (1.15 % KCl in 0.05 M tris, pH 7.8) and were sonicated for 8 minutes at 0°C in a Raytheon 10 kc ultrasonicator. The supernatant obtained after centrifugation for 10 minutes at 10,000 x g was used for the incorporation experiments, the conditions being detailed in Table I. ATP and $MgCl_2$ were added to facilitate the formation of S-adenosyl-methionine; further additions of ATP were made at 1 and 2 hours during the 3-hour incorporation period. The reaction was stopped by precipitation of proteins with acetone (3 vol./vol. of supernatant) and the subsequent filtrate was evaporated to dryness prior to extraction with

[*] Supported in part by a grant (AI-02838) from the National Institute of Allergy and Infectious Diseases (US Public Health Service) and by the CNRS, Paris ("RCP 21" on oxidative phosphorylation). One of us (R. B. H.) thanks F. Hoffmann LaRoche and Co., Basle, for a research fellowship.

[**] M. phlei contains a vitamin $K_2(45)H$, i. e., a 2-methyl 1,4-naphtoquinone with a C_{45} isoprenoid side chain in position 3; one of the isoprenoid units is saturated (Gale et al., 1963); we have confirmed by mass spectrometry the molecular formula proposed by these authors (Lederer, 1964; mass spectrum by Dr. M. Barber, A. E. I., Manchester).

TABLE I

Aerobic incubation of M. phlei supernatant with L-methionine-$^{14}CH_3$ and L-tyrosine-U-^{14}C for 3 hours at 30°C.

Composition of incubation medium*	Dose μcuries	Specific activity dpm/mg of vit. $K_2(45)H$
L-methionine-$^{14}CH_3$(sp. act. 9.3 mcuries/mmole)		
" + cell-free supernatant, 45 ml	45	12,250
" "	22.5	13,000
" "	22.5	11,500
" + boiled supernatant, 45 ml	45	<200
L-tyrosine-U-^{14}C(sp. act. 72.6 mcuries/mmole) + ammonium acetate, 90 μmoles + DL-methionine**, 90 μmoles		
" + cell-free supernatant, 45 ml	10	300
" + boiled supernatant, 45 ml	10	<300

*All incubation media contained: $MgCl_2$, 4.5 mmoles; ATP, 225 μmoles (+90 μmoles added at 1 and 2 hours during incubation) in KCl-tris (1.15 % in 0.05 M, pH 7.4); final volume: 8.5 ml.

**DL-methionine was dissolved in the KCl-tris buffer made slightly acidic with HCl.

hexane. The vitamin $K_2(45)H$ in the hexane extract was purified by repeated thin layer chromatography on Kieselgel G (Merck) in the solvent system hexane-ethyl methyl ketone (97:3), until constant specific activity was reached. In addition, the purity of vitamin $K_2(45)H$ was checked by UV spectrum, reversed phase thin layer chromatography (Kieselgel G plates treated with 10 % vaseline in $CHCl_3$; solvent, acetone-water (95:5), R_f = 0.4), and reversed phase ascending paper chromatography (Whatman No. 1 impregnated with vaseline 5 % in hexane; solvent, acetone-water (95:5), R_f = 0.25).

Vitamin $K_2(45)H$ was determined quantitatively by reduction with $NaBH_4$ in buffered alcohol, pH 5 (Lester et al., 1964) using

$E^{1\%}_{1cm}$ ox. (271 mμ)-$E^{1\%}_{1cm}$ red. (271 mμ) = 166; radioactivity was measured with a Packard 3003 Tricarb Scintillation Spectrophotometer.

RESULTS. Three successive purifications on thin layer plates of the hexane-extracted vitamin K_2 sufficed to obtain a product assessed pure by the criteria described in the preceeding section.

In three experiments as shown in Table I, an average of 12,250 dpm/mg of vitamin K_2(45)H were incorporated from L-methionine-$^{14}CH_3$ during a 3-hour incubation period. That maximum incorporation was obtained under these conditions is indicated by the specific activities determined when L-methionine-$^{14}CH_3$ was used at two concentrations. When the supernatant from M. phlei was boiled for 5 minutes, prior to its addition to the incubation system, radioactivity was not incorporated into vitamin K_2(45)H; preformed vitamin K was not destroyed during the boiling process.

L-tyrosine-U-^{14}C, added to the cell-free supernatant system, was not incorporated into vitamin K_2(45)H (Table I); boiled supernatant also served as a control in this experiment.

DISCUSSION. In this paper, we describe a cell-free supernatant from M. phlei that incorporates radioactivity of L-methionine-$^{14}CH_3$ into vitamin K_2(45)H. This finding confirms our previously reported results (Lederer, 1964; Azerad et al., 1965) on the incorporation of radioactivity from L-methionine-$^{14}CH_3$ into vitamin K_2(45)H by whole cells of M. phlei.

The 2-methyl group of ubiquinone 50 is derived from methionine, as shown by Rudney and Parson (1963) (see also Olson et al., 1963). Work is in progress to locate the labelled carbon in the radioactive vitamin K obtained in the experiments described above and to investigate the biosynthesis of the naphthalene nucleus of this vitamin by means of our cell-free system. Tyrosine does not appear to be a precursor in the biosynthetic pathway to vitamin K_2(45)H in this system.

Addendum. After writing this paper, we have learned that Martius and Leuzinger (1964) have described the incorporation of the radioactivity

of $^{14}CH_3$-methionine into vitamin $K_2(45)$ by whole cells of the anaerobic Fusiformis nigrescens, starting from 1,4-naphtoquinone.

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