BIOSYNTHESIS OF DIHYDROMENAQUINONE-9 by Mycobacterium phlei.

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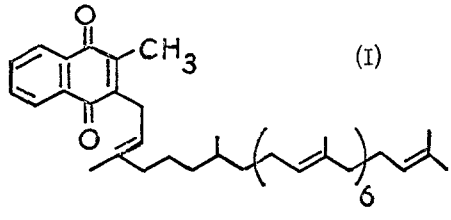
Guérin et al. (1965) have shown recently that whole cells of M. phlei incorporate the methyl group of methionine-$^{14}CH_3$ into the 2-methyl group of the dihydromenaquinone-9* described by Gale et al. (1963). Formula I, based on mass spectrometry has been proposed for this compound (Lederer, 1964) and confirmed quite recently by degradation (Azerad et al., 1967).



(I)

The radioactive menaquinone (I) had been degraded and the incorporation into the methyl group proved by the isolation of inactive phthalic acid and of radioactive acetic acid, having all its label in the methyl group. It has also been shown (Jauréguiberry et al., 1966 a) that all three deuterium atoms of methyl-d_3 methionine are recovered in the 2-methyl group of the menaquinone after incubation with whole cells of M. smegmatis.

The biosynthesis of menaquinone was then studied with a cell-free extract, which was recently shown to be able to methylate oleic acid to tuberculostearic acid (Jauréguiberry et al., 1966 b), and again "radioactive dihydromenaquinone-9" was isolated and we reported thus the "biosynthesis of vitamin K_2 by cell-free extracts of M. phlei" (Azerad et al., 1965, 1966).

We have found, since, that the "radioactive dihydromenaquinone-9" obtained from cell-free extracts is accompanied by a strongly radioactive contaminant, since, after irradiation at 360 mμ, destroying all the mena-

*) Abbreviations : Menaquinone-9 : MK-9 ; Dihydromenaquinone-9:MK-9(H_2); 2-Desmethylmenaquinone-9 : DMK-9.

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Table I

Incorporation of radioactivity from methionine-$^{14}CH_3$ by M. phlei intact cells, sonicated homogenates and 10,000 g supernatants.

	Radioactivity in the MK fraction	
	Before $AgNO_3$/silicagel chromatography (MK + fatty acid methyl esters) dpm/μmole MK	After $AgNO_3$/silicagel chromatography (MK) dpm/μmole MK
Intact cells *	20,400	16,700
8 min. sonicated cells		
- Homogenate**	17,300	920
- 10,000 g supernatant***	33,700	460
3 min. sonicated cells		
- Homogenate**	15,300	1,090

* Conditions of incubation: 7.5 g fresh bacteria/75 ml tris buffer 0.01 M pH 7.4 containing 1.15 % KCl; Na malate, 3.75 mmoles; methionine-$^{14}CH_3$ (10.8 mC/mM), 10 μcuries. Final volume: 90 ml. 2 hours at 30°.

** Conditions as above except that 7.5 g of fresh bacteria were sonicated in 15 ml of tris buffer 0.1 M, pH 7.8 and 1 mmole $MgCl_2$, 100 μmoles ATP and only 250 μmoles of Na malate were added. Final volume: 18 ml.

*** Conditions as above except that the homogenate was centrifuged at 10,000 g for 20 min. and dialysed overnight against tris buffer 0.01 M pH 7.4 containing 1.15 % KCl.

quinone, the radioactivity migrated still at the same Rf on TLC. This radioactive contaminant has, in several solvents, the same Rf values as dihydromenaquinone-9, which is only weakly radioactive, but can be completely separated from it by TLC on silicagel G with benzene, or on silver nitrate impregnated silicagel (solvent : hexane-methylethylketone, 8:2).

This radioactive impurity, formed from methionine-$^{14}CH_3$ and purified on a silver nitrate impregnated silicic acid column, has been found to be a mixture of esters of inactive fatty acids with methanol-^{14}C, giving three radioactive spots on vaseline impregnated paper chromatography

(solvent : acetone-water, 97:3, Rf = 0.62, 0.71 and 0.92). After saponification or $LiAlH_4$ reduction of these esters, methanol-^{14}C was identified as the only radioactive product ; dilution with cold methanol followed by reaction with p-nitrobenzoyl chloride afforded a radioactive p-nitrobenzoate which was recrystallised to constant specific activity. The fatty acid moieties were tentatively identified by VPC of the ^{14}C-methyl esters on QF-1 (10%) column as palmitic, oleic and tuberculostearic acids.

In comparative experiments with M. phlei resting cells, sonicated homogenates and the corresponding 10,000 g supernatants, it was found that these esters were only formed after cell sonication, even during short periods (Table I). Resting cells incorporated most of the radioactivity into MK-9(H_2) as shown previously (Guérin et al., 1965).

The formation of ^{14}C-methanol can be ascribed to a non enzymatic degradation of the S-adenosyl-^{14}C-methionine formed from methionine-$^{14}CH_3$ in the extracts, since boiling abolished the formation of radioactive esters from methionine-$^{14}CH_3$ but not from S-adenosyl-C^3H_3-methionine. Methanol-^{14}C was also incorporated in the same ester fraction by boiled extracts (Table II).

Table II

Incorporation of radioactivity from various precursors in the methyl ester fraction by cell-free extracts of M. phlei.

Additions*	Extract	Total incorporation dpm
15 µcuries methionine-$^{14}CH_3$ (specific activity: 10.8 mC/mM)	native	13,800
" "	boiled	0
5 µcuries S-adenosyl methionine-C^3H_3 (specific activity: 10 mC/mM)	native	5,750
" "	boiled	40,000
10 µcuries methanol-^{14}C (specific activity: 0.03 mC/mM)	native	1,810
" "	boiled	925

* All other conditions as described by Azerad et al. (1965).

The ester formation can be due to the presence in cell-free extract of acylCoA able to react with any methanol liberated.

Desmethylmenaquinones as precursors of menaquinones.

In an attempt to increase the incorporation of radioactivity from methionine-$^{14}CH_3$ into the menaquinone, desmethylmenaquinone-9(DMK-9) a possible precursor of MK-9(H_2), was added. Good incorporation of

Table III

Incorporation of radioactivity from methionine-$^{14}CH_3$ into the menaquinone and the ester fractions by cell-free extracts of M. phlei.

Additions	Extract* ml	Methionine -$^{14}CH_3$ µcuries	Incorporation into menaquinone fraction*** dpm	ester fraction dpm
3.7 µmoles DMK-9 in:				
- micellar lecithin suspension 0.1 ml (10 mg/ml)	15	10	29,500	-
- BCG phospholipid solution 0.1 ml (10 mg/ml) **	15	10	21,400	-
no DMK + 1 ml lecithin suspension	30	15	1,000	785
1,5 µmole DMK-9 in 1 ml lecithin suspension	30	15	4,000	2,700
3 µmoles DMK-9 in 1 ml lecithin suspension	30	15	4,700	1,080
6 µmoles DMK-9 in 1 ml lecithin suspension	30	15	12,100	2,300

* All conditions as described by Azerad et al. (1965), except that the extract was dialysed overnight against 100 volumes of tris buffer 0.01 M pH 7,4, containing KCl 1.15 %.

** kindly donated by Dr. Erna Vilkas.

*** the menaquinone fractions were obtained by repeated thin-layer chromatography on silicagel G with hexane-EtCOMe (9:1) and benzene as solvents. All the menaquinone fractions were then tested for purity by TLC chromatography on $AgNO_3$-impregnated silicagel and radioactivity scanning (Snyder, 1964). In all cases, all the radioactivity was associated only with the DMK + MK-9 spot.

radioactivity into the menaquinone was obtained by using solutions of DMK-9 in micellar suspensions of soy bean lecithin (Sigma) (20mg/ml) obtained by the Fleischer and Klouwen technique(1961). A water solution of BCG phospholipids gave very similar results. The incorporation of the ^{14}C-methyl group of labeled methionine into menaquinone, obtained with these DMK "solutions" is shown in Table III.

The radioactive DMK + menaquinone mixture isolated was chromatographed on silver nitrate impregnated silicagel plates run with hexane-methylethylketone (8:2) (Beau et al., 1966) giving clear-cut separations of MK-9 + DMK-9 and MK-9 (H_2); all the radioactivity was associated with the MK-9 + DMK-9 spot. Vaselin impregnated paper chromatography with acetone-water (95:5) (Beau et al., 1966) showed again that all the radioactivity was associated with MK-9.

Addition of NADH or NADPH to the incubation mixture, maintained under nitrogen pressure to avoid rapid reoxydation of the reduced coenzymes, resulted in the same overall incorporation, but silver nitrate chromatography revealed that under these conditions 10 to 20 % of the total counts were associated with MK-9 (H_2) (I).

Our experiments seem to prove that cell-free extracts of M. phlei can catalyse the following reactions:

$$\text{DMK-9} \longrightarrow \text{MK-9} \longrightarrow \text{MK-9}(H_2) \quad \text{(I)}$$

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