STUDIES ON α-NAPHTHOL AS A PRECURSOR OF MICROBIAL MENAQUINONE

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1. Introduction

Leistner, Schmitt and Zenk [1] presented evidence to show that α-naphthol is a precursor of menaquinone (vitamin K_2) in microorganisms. These workers found that menaquinone-7 (the numeral refers to the number of isoprene units in the molecule) isolated from a culture of *Bacillus megaterium* grown in the presence of α-[1-^{14}C] naphthol was radioactive. Degradation studies indicated that the carbon skeleton of α-naphthol had been incorporated *in toto* into the naphthoquinone nucleus. We have carried out similar experiments with both Gram-negative and Gram-positive bacteria, including the strain of *Bacillus megaterium* used by Leistner et al., but have obtained no significant incorporation of radioactivity into menaquinone or demethylmenaquinone.

2. Methods

Proteus mirabilis N.C.I.B. 5887 was grown aerobically in 10 l batches on a glucose-glutamate medium [2]. *Bacillus megaterium* N.C.T.C. 9848 was grown for 18 hr in 500 ml batches at 30° with constant shaking. The medium contained (g/l of distilled water): Bactopeptone, 20; NaCl, 5. *Bacillus megaterium* (provided by Dr. M.H. Zenk) was grown for 6–9 hr in 10 l batches at 37° with constant aeration (10 l/min) and agitation (180 rev/min) in a Microferm Laboratory Fermenter. The medium contained (g/l of distilled water): K_2HPO_4, 7; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; sodium citrate, 0.6; glycerol, 6.3; L-phenylalanine, 0.017; L-tyrosine, 0.018; DL-tryptophan, 0.041. The cultures were inoculated by adding 1 l of an aerobic bactopeptone-NaCl culture.

α-[1-^{14}C] naphthol (19.6 mC/m-mole) was purchased from the Radiochemical Centre, Amersham, Bucks. The radiosubstrate was dissolved in water, sterilised by filtration through a membrane, and added to the growth medium just prior to inoculation or to grown cells which had been harvested and resuspended in 0.05 M potassium phosphate buffer, pH 7.0.

After incubation with the radioactive substrate the lipids were extracted from the cells by a routine procedure [2], and chromatographed on a column of Brockmann Grade III acid-washed alumina (Woelm) developed with 0.25%, 1%, 3% and 5% diethyl ether in light petroleum (40–60°) [2]. Menaquinone and demethylmenaquinone (when present) were eluted by 1% diethyl ether in light petroleum and α-[1-^{14}C]-naphthol by 3% and 5% diethyl ether in light petroleum. Menaquinone and demethylmenaquinone were purified by quantitative thin layer chromatography on (i) Rhodamine 6G-impregnated plates with benzene as developing solvent (R_F 0.6); (ii) reversed-phase thin-layer plates with aq. 95% acetone as developing solvent (menaquinone-7, R_F 0.4; menaquinone-8, R_F 0.3; demethylmenaquinone-8, R_F 0.4). The R_F's of α-[1-^{14}C] naphthol in systems (i) and (ii) are 0.2 and 0.95 respectively.

The naphthoquinones were estimated spectroscopically [2] and their radioactivity determined by liquid scintillation counting.

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Table 1

Incorporation by *Proteus mirabilis* N.C.I.B. 5887 (experiments 1 and 2), *Bacillus megaterium* N.C.T.C. 9848 (experiment 3) and *Bacillus megaterium* Z* (experiments 4 and 5) of radioactivity from α-[1-^{14}C] naphthol into menaquinone-7 (MK-7), menaquinone-8 (MK-8) and demethylmenaquinone-8 (DMK-8).

Expt. No.	Experimental conditions	α-[1-^{14}C] naphtol administered (μC)	Period of incubation (hr)	MK and DMK (if present)		
				Quinone(s) present	Amount (μmoles)	Sp. radioactivity ** (counts/min/μmole)
1	α-[1-^{14}C] naphthol added to 10 l of glutamate-glucose medium at the time of inoculation	10	3.75	DMK-8 MK-8	13	37 ***
2	Cells (55.7 g wet wt) suspended in 80 ml of buffer containing α[1-^{14}C]-naphthol and passed through an Apex hydraulic press	5	4	DMK-8 MK-8	4.6	0
3	α-[1-^{14}C] naphthol added to 1 l of bactopeptone-NaCl medium at the time of inoculation	5	15	MK-7	0.11	0
4	α-[1-^{14}C] naphthol added to 10 l of glycerol-citrate medium at the time of inoculation	50	5	MK-7	0.69	0
5	Cells (62.6 g wet wt) suspended in 100 ml of buffer containing α-[1-^{14}C] naphthol	10	13	MK-7	1.68	187

* Organism provided by Dr. M.H.Zenk

** Counts corrected for background and instrument efficiency

*** Not subjected to reversed-phase thin-layer chromatography

3. Results and discussion

These investigations were undertaken with an aim to establishing in the Gram-negative bacterium, *Proteus mirabilis*, the metabolic relationship (if any) of demethylmenaquinone and menaquinone. However, despite the use of a variety of experimental procedures we were unable to obtain any significant incorporation of radioactivity from α-[1-^{14}C]naphthol into menaquinone-9 and demethylmenaquinone-9 (table 1). These results are in agreement with the finding of Ellis and Glover [3] for the Gram-negative bacterium, *Escherichia coli*.

These findings led us to examine the incorporation of radioactivity from α-[1-^{14}C]naphthol into menaquinone in Gram-positive organisms. We first tried *Bacillus megaterium* N.C.T.C. 9848 (table 1) and, having no success, we then tried Zenk's strain of *Bacillus megaterium* (table 1). Again no significant incorporation of radioactivity into menaquinone was obtained. A feature of these experiments was the apparent low recovery of menaquinone-7 from the organisms, i.e. 0.09—0.12 μmole/g dry wt compared to 0.66 μmole/g dry wt quoted in the literature [4]. It was felt that this might be due to our extraction procedure being inefficient, but when we used the methanol extraction procedure of Bishop, Pandya and King [4] we obtained no additional menaquinone.

The question remains why we were not able to repeat the experiment of Leistner et al. [1]. Our experiments had shown that great care is needed to separate menaquinone from α-[1-^{14}C]naphthol and/or its breakdown products. Thus, on column chromatography of the lipid isolated in experiment 5 the radioactivity present in the 0.25%, 1%, 3% and 5% diethyl ether-light petroleum fractions was 2000, 8000, 150000 (α-[1-^{14}C]naphthol) and 2250000 (α-[1-^{14}C]naphthol) counts/min respectively. The apparent specific activity of the menaquinone in the 1% diethyl ether-light petroleum fraction was 11 500 counts/min/μmole. On further purification this fell through 450 to 52 counts/min/μmole. It is difficult to remove the last traces of radioactivity, but it can be achieved by further thin-layer chromatography on Rhodamine 6G-impregnated plates developed with ethyl-acetate-light petroleum (1:9, v/v, R_F 0.35). It should be stressed that similar results are obtained on chromatography on alumina of a mixture of α-[1-^{14}C]naphthol and menaquinone. In view of our experiences it would appear that in the experiment of Leistner et al. [1] the column and thin-layer chromatographic techniques used were insufficient to remove all the α-[1-^{14}C]naphthol or its breakdown products from menaquinone.

In conclusion we would suggest that in Gram-negative and Gram-positive bacteria α-naphthol is not involved in the biosynthesis of the nuclei of menaquinone and demethylmenaquinone.

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