ORIGIN OF THE AROMATIC NUCLEUS IN BACTERIAL MENAQUINONES

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On the basis of scanning thin layer chromatograms for radioactivity, it has been concluded that shikimate-U-C^{14} is converted to menaquinone-8 (MK-8, vitamin K_2(40)) by E. coli (Cox and Gibson, 1964). A role for 3,4-dihydroxybenzaldehyde in this conversion was postulated, beyond the branch point of chorismate, since addition of this material (but not p-hydroxybenzoate) was inhibitory. More recently, partial degradation of such radioactive MK-8 has suggested that the benzenoid ring (ring A) of MK-8 was derived from shikimate but failed to show whether shikimate was employed as a C-6 or C-7 unit (Cox and Gibson, 1966). Furthermore, this conclusion can only be considered tentative, in view of the very low levels of activity used.* Whistance et al. (1966) have claimed that shikimate-U-C^{14} is converted to phylloquinone in etiolated maize shoots. In this latter work, again with marginal levels of precursor activity, it was noted only that K was "active."

To obtain less ambiguous results, and in continuation of our interest in isoprenoid quinone biosynthesis, we have studied, with relatively high levels of activity, the incorporation of shikimate-U-C^{14} both into MK-8 of E. coli and also into dihydromenaquinone-9 (MK-9(H_2)) in Mycobacterium phlei. We have also observed that 3,4-dihydroxybenzaldehyde and its acid are not incorporated into these menaquinones.

*The highest reported activity for MK-8 used in chemical degradation was 1400 cpm/millimole; i.e., about 2 cpm/mg.

Media for growth of E. coli K12 and M. phlei, respectively, were similar to those of San Pietro (1955) and Brodie et al. (1958). Shake cultures were grown at 37° for 24 hours, and radioactive precursors were added at the beginning of this period. Quinones, from the centrifuged cellular paste (Brodie, 1963) were purified by column and repeated thin layer chromatography. We have observed that even after such purification procedures the quinones are appreciably contaminated with, inter alia, mixtures of fatty acid esters. It is essential that the quinones be converted to a derivative and further purified before reliable values of specific activities can be obtained.

The isolated quinones, after appropriate dilution, were reductively acetylated with zinc, acetic anhydride and triethylamine. The resulting quinol diacetates (I), were cleaved with osmium tetroxide-periodic acid in aqueous dioxan (Kupchan et al., 1962); the aldehydic products being oxidized to the corresponding acids with potassium permanganate under neutral conditions. The levulinate from MK-9 (H_2) was converted to its semicarbazone for counting purposes. After a further dilution step, the naphthylacetate derivative (II) was oxidized with alkaline hydrogen peroxide (Bentley et al., 1965) to phthalate and malonate. One equivalent of carbon dioxide, recovered as $BaCO_3$, was subsequently removed from the phthalate by the Schmidt procedure (Phares, 1951). All radioactivity measurements were made by liquid scintillation counting in appropriate solutions; $BaCO_3$ was counted as a suspension in toluene-phosphor solution with Cab-O-Sil gel.

The results of these degradations and the calculated distributions of radioactivity are shown in Tables 1 and 2. The incorporation values were 0.5% and 0.02%, respectively, for E. coli and M. phlei. It is clear that shikimate-U-C^{14} is, indeed, incorporated into the naphthalene nucleus of the menaquinones in both cases, albeit appreciably more efficiently in E. coli. Ring A and the two quinone carbons contain 89% of the total MK

Table 1. Degradation of MK biosynthesized from shikimate-U-C^{14}

Compound	MK-9 (H_2) from M. phlei[a]		MK-8 from E. coli[b]	
	dpm/μmole	% total	dpm/μmole	% total
MK as isolated	1,738		191,500	
MK quinol diacetate	328	100	2,270	100
Levulinate semicarbazone	ca. 10	ca. 3	- [c]	-
Naphthylacetate	235	71	2,065	91
Phthalate	225[b]	67	2,028	89
$BaCO_3$ ex phthalate	20	6	167[d]	7
Malonate	ca. 10	ca. 3	0	0

[a] With M. phlei, 70 μC of shikimate-U-C^{14} was added to 6 × 700 ml. of medium. The isolated MK-9 (H_2), purified by thin layer chromatography (twice), was diluted with cold MK-9 (H_2). After two more thin layer chromatograms, the material was reductively acetylated; the diacetate was purified by column chromatography and recrystallization (three times).

[b] With E. coli, 100 μC of shikimate-U-C^{14} was added to 6 × 700 ml. of medium. The isolated MK-8 (two thin layer chromatograms) was diluted with phylloquinone prior to reductive acetylation. The diacetate was purified by column chromatography (twice).

[c] Not obtained since the major component cleaved was phylloquinone.

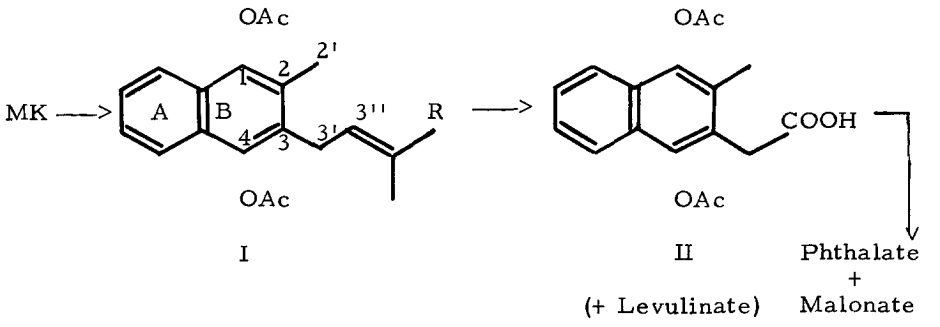
[d] Corrected for further dilution.

quinol diacetate activity in the case of E. coli, and 67% with M. phlei. Moreover, from the phthalate decarboxylation, it can be deduced that shikimate is utilized as a C-7 unit.

Not only was shikimate-U-C^{14} incorporated less efficiently into the M. phlei menaquinone, but as indicated above, about 30% of the incorporated activity was located in the isoprene side chain. This finding suggests some degree of interaction between acetate and shikimate metabolism in the actinomycete. We have, in fact, observed considerable acetate incorporation into ring A of MK-9 (H_2).

To test the proposal that 3,4-dihydroxybenzaldehyde was in some way involved in menaquinone biosynthesis, samples of the aldehyde and corresponding acid, labelled with C^{14} in C-7, were synthesized by the

Table 2. Distribution of C^{14} in MK quinol diacetates.



	% of MK quinol diacetate activity	
	M. phlei	E. coli
Ring A (Phthalate - 2 × $BaCO_3$)	55	75
C-1 + C-4 (2 × $BaCO_3$)	12	14
C-3 + C-3' + C-3'' (Malonate)	3	0
C-2 + C-2' (II - (phthalate + malonate))	1	2
Remainder of isoprene chain	29	9

method of Neish (1959). Neither substance provided radioactive menaquinone when added to E. coli or M. phlei cultures. It can, therefore, be deduced, either that the aromatic nucleus but not the carbonyl group is incorporated - a conclusion at variance with the observed utilization of shikimate as a C-7 unit - or that the involvement of these compounds in menaquinone biosynthesis is indirect.

With knowledge of the roles of shikimate and methionine (Azerad et al., 1967) in biosynthesis of the naphthoquinone nucleus, the general outlines of menaquinone formation are becoming apparent. The source of three carbon atoms of the naphthalene nucleus remains to be determined. Some incorporation of acetate-1,2-C_2^{14} into ring B was observed in E. coli (Cox and Gibson, 1966) and our own preliminary experiments suggest a similar incorporation with M. phlei.

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