$$\text{slope 2:} \quad -\frac{2k_1k_2}{k_1 + k_2} \tag{46}$$

Although intercept 2 is equal to the number of binding sites (two in this example) it is apparent from eq 41 that intercept 1 is *not* equal to the binding constant for the first site. Similarly intercept 3 is not equal to the number of sites in class 1 (one in this example); on the contrary, intercept 3 must always be greater than unity (see eq 43). Furthermore, intercept 4 is *not* equal to k_2, the binding constant for the second site, as seems to be often believed.

Examining Figure 5A,B we find that here again the slopes and intercepts are not always what might be expected. Just as in Figure 5C the only parameter directly obtainable in these representations is the total number of binding sites; both intercept 1 in Figure 2 or 5A and slope 2 in Figure 3 or 5B are $1/n_0$. All the other slopes and intercepts represent various combinations of the different parameters.

If the magnitudes of k_1 and k_2 are far enough apart, some of the intercepts may approach the "intuitively" expected values. Table I illustrates this point, in conjunction with demonstrating the limits obtained for a range of relative magnitudes of the two-site binding constants.

Comparison of Site Binding Constants with Stoichiometric Binding Constants. The classical thermodynamic analysis of binding (Klotz, 1946) does not depend on any recognition of specific sites. The stoichiometric binding constants, K_i, so obtained, express the equilibria purely in stoichiometric terms. It has long been recognized, nevertheless, that the stoichiometric (or macroscopic or classical) binding constants must be related to the site (or microscopic) binding constants. For a two-site system, the relationships are analogous to those worked out for bifunctional proton-dissociating molecules (Adams, 1916; Edsall and Wyman, 1958).

$$K_1 = k_1 + k_2 \tag{47}$$

$$K_2 = \frac{k_1k_2}{k_1 + k_2} \tag{48}$$

For a multisite system, general relations between stoichiometric and site binding constants have been described by Fletcher *et al.* (1970).

Comparing the results of eq 47 and 48 with the intercepts of Figure 5C, we see that K_1 is indeed equal to intercept 1. On the other hand, K_2 is *not* equal to intercept 4, although they are related by a constant factor, 4.

Thus it is apparent that various site binding constants and stoichiometric binding constants can be deduced from graphical analyses for two-site (and multisite) systems.

However, the graphical intercepts and slopes are composites of contributions from both of the sites, and the individual values must be segregated out later.

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Biosynthesis of Bacterial Menaquinones (Vitamins K_2)*

I. M. Campbell, D. J. Robins, M. Kelsey, and Ronald Bentley†

ABSTRACT: The biosynthesis of the menaquinones of *Escherichia coli*, *Mycobacterium phlei*, *Corynebacterium diphtheriae*, and *Streptomyces albus* has been studied using isotope tracer methods. To locate label derived from specifically labeled precursors, the menaquinones were chemically degraded by the following scheme: menaquinone → menaquinol diacetate → 1,4-diacetoxy-3-methyl-2-naphthaleneacetic acid → malonic plus phthalic acids. The latter acid was subsequently decarboxylated. The results demonstrate that menaquinones derive from shikimic acid and 2-ketoglutaric acid. 4-(2′-Carboxyphenyl)-4-oxobutyric acid can be incorporated into the menaquinone nucleus. No evidence could be obtained to suggest that preformed naphthalenoid compounds such as 1-naphthol, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, or 2-hydroxy-1,4-naphthoquinone, were involved as intermediates in the menaquinone biosynthetic pathway.

While all of the fat-soluble vitamins (A, D, E, and K) contain major structural features which identify them biosynthetically as isoprenoid compounds, those materials with vitamin E and vitamin K activity contain, in addition, an aromatic component. The benzene ring which constitutes the aromatic portion of the vitamin E series of compounds, *e.g.*, α-tocopherol, derives *in toto* from tyrosine (Whistance and Threlfall, 1968). The naphthoquinone ring system which figures in compounds with vitamin K activity, *e.g.*, phylloquinone and the menaquinones,[1] appears to have by contrast

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† To whom to address correspondence.

[1] In this paper, vitamins K_2 will be referred to as menaquinones following the recommended nomenclature (Folkers *et al.*, 1965). The abbreviations which are used are as follows: menaquinone with a 2-substituent of *n* prenyl units, MK-*n*; a menaquinone with a reduced double bond in the second prenyl unit, counting from the nucleus, MK-*n* (II-H_2).

a multiple origin. In early experiments (Cox and Gibson, 1964, 1966), shikimic acid was implicated as a major contributor to the C_{10} nucleus. This paper presents evidence that all seven carbon atoms of shikimic acid are contributed to the aromatic ring system and that glutamic acid is the metabolite providing the remaining three-carbon atom unit. Preliminary reports of some of this work have appeared already (Campbell *et al.*, 1967; Robins *et al.*, 1970).

Materials and Methods

General. All solvents were redistilled prior to use. Silicic acid for column chromatography was Clarkson Chemical Company's Unisil, mesh 200–325. Layer chromatography was conducted on silica gel Chromagram sheets (Eastman 6060, with fluorescent indicator) using the solvent systems described. Ultraviolet spectrometry was performed in the specified solvents on Coleman Hitachi Model 124 and Zeiss PMQ II spectrophotometers. Mass spectra were obtained by the direct probe method using an LKB 9000 single-focusing mass spectrometer operating at 15 and 70 eV with an electron current of 60 μA, an accelerating voltage of 3.5 kV, and an ion source temperature of 270°.

All the radioactive materials used directly in feedings or as synthetic starting materials were obtained from commercial sources. Samples were assayed for radioactivity using a Packard Tri-Carb Model 3310 scintillation counter. Organic ^{14}C-labeled intermediates were counted in a liquid scintillator (Bray, 1960). The activity of barium carbonate samples was obtained by suspension in a thixotropic gel prepared by adding Cab-O-Sil (700 mg) to 18 ml of the following solution: 2,5-diphenyloxazole (15.2 g), 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.19 g), and toluene (3.8 l.).

Growth of Organisms. Escherichia coli K-12 was maintained on nutrient agar slopes at 37°. Liquid inocula were prepared by growth for 24 hr at 37° as shake cultures in 250-ml erlenmeyer flasks containing 25 ml of the following medium: yeast extract (Difco), 0.6%; beef extract (Difco), 0.5%; glucose, 1.0%; K_2HPO_4, 1.1%; KH_2PO_4, 0.85%. Prior to sterilization the pH was adjusted to 6.9. These liquid inocula were used to seed 2.8-l. fernbach flasks containing 600 ml of the above culture medium. Growth as shake cultures was continued at 37° for a further 24-hr period. The cells were harvested by continuous centrifugation and the cell paste was washed twice with distilled water. The yield of wet paste was approximately 6 g/l. of broth.

Corynebacterium diphtheriae (CN 2000) was maintained on blood agar slopes at 37°. Inocula from these slopes were used to seed 4 l. of the medium of Scholes and King (1965a). Growth was continued for 2 days at 37° as shake cultures. The cells were harvested and subsequently washed by centrifugation in firmly stoppered vessels. The yield of wet paste was approximately 8 g/l. of broth.

Streptomyces albus (ATCC 3004) was maintained on sporulation agar slopes at 27°. Liquid inocula were prepared by growth for 4 days at 27° as shake cultures in 250-ml erlenmeyer flasks containing 50 ml of the following medium: glucose, 1.0%; Casamino Acids (Difco), 1.0%; NaCl, 0.5%; beef extract (Difco), 0.6%. The liquid inocula were used to seed 2.8-l. fernbach flasks containing 600 ml of the following culture medium: glucose, 2.0%; $MgSO_4$, 1.0%; sodium citrate, 1.0%; NaCl, 0.5%; glycine, 0.5%; yeast extract (Difco), 0.1%; $CaCl_2 \cdot 2H_2O$, 5×10^{-2}%; KH_2PO_4, 5×10^{-2}%; $FeSO_4 \cdot 7H_2O$, 5×10^{-4}%; $ZnSO_4 \cdot 7H_2O$, 4.5×10^{-4}%; $CuSO_4 \cdot 5H_2O$, 4×10^{-4}%; $MnSO_4 \cdot H_2O$, 3×10^{-4}%; $NaMoO_4 \cdot 2H_2O$, 1×10^{-5}%. Growth as shake cultures at 27° was continued for 4 days. Cell harvest was as described above. The yield of wet paste was routinely 40 g/l. of broth.

Mycobacterium phlei (ATCC 354) was grown as described previously (Campbell and Bentley, 1968).

Isolation of Menaquinones from Cellular Paste. All the operations from this point onward to the reductive acetylation of the purified menaquinone[1] were conducted in virtual darkness. This avoids extensive trans–cis isomerization and decomposition of the menaquinone samples (Campbell and Bentley, 1968).

Nonpolar lipids were isolated from the cellular paste of the organisms initially by an acetone homogenization technique (Campbell and Bentley, 1968) and in later experiments by the superior methanol reflux method of Scholes and King (1965b).

In a typical experiment, menaquinone was extracted from the nonpolar lipids (1 g) by fractionation on a column of silicic acid (20 g; 2 × 18 cm) established in benzene. The flow rate was 30 ml/hr and 10-ml fractions were collected. Solvent removal and ultraviolet spectrometry of each fraction in isooctane revealed the characteristic chromophore of a 2,3-disubstituted naphthoquinone in fractions 4–6. Ubiquinone, if present, was routinely encountered in fractions 10–20.[2]

The combined fractions 4–6 were chromatographed on a column of Sephadex LH-20 (20 g; 1.8 × 35 cm; flow rate, 30 ml/hr; swelling time, 24 hr; fraction size, 5 ml) established in the solvent system isooctane–chloroform–methanol (2:1:1, v/v). Ultraviolet spectrometry localized menaquinones in fractions 9–10. Yields of menaquinones were as follows (μ-moles of menaquinone/g wet cell paste): *E. coli*, 0.05–0.1; *C. diphtheriae*, 0.1–0.5; *S. albus*, 0.1–0.2; *M. phlei*, 0.3–1.0.

Characterization of Menaquinones. The small quantities of purified material isolated from the organisms were characterized by ultraviolet (Morton, 1965) and mass spectrometry. The menaquinone fraction of all strains investigated was inhomogeneous with respect to side-chain length. The detailed analysis of the menaquinone content of *E. coli* and *M. phlei* has already been published (Campbell and Bentley, 1968, 1969). Using methods similar to those previously employed, the products formed by *C. diphtheriae* and *S. albus* have been analyzed (D. J. Robins *et al.*, unpublished work). The existing literature (Scholes and King, 1965b; Imhoff and Azerad, 1970; Phillips *et al.*, 1969) has been amplified. *C. diphtheriae* produces principally a mixture of MK-8(II-H_2), MK-7(II-H_2), and an MK-6(H_2). *S. albus* gives rise to a much more complex menaquinone complement; at least 12 different components are present, the major ones being an MK-9(H_6) and an MK-9(H_8).

Isotope Incorporation Experiments. In every case the tracer was added in the prescribed dose to the growth medium simultaneously with the seed inocula. The labeled menaquinone fraction was isolated and purified as described. To obtain sufficient material for degradation the biosynthesized sample was diluted with nonradioactive carrier. In early experiments with *M. phlei*, cold MK-9(II-H_2), the natural prenylog, was used as diluent. In later experiments with *M. phlei* and with all the other organisms studied, phylloquinone

[2] Ubiquinone occurs plentifully in *E. coli* (Daves *et al.*, 1967) and in trace quantities in *S. albus* and *C. diphtheriae*. In the case of *E. coli* the ubiquinone fraction encountered at this stage was further purified by Sephadex LH-20 chromatography. The procedure outlined for menaquinone purification was employed.

was used as carrier. In "swamping" experiments, the nonradioactive swamping substrate was added with seed inocula and tracer.

Degradation of Labeled Menaquinones. PREPARATION OF THE DIACETATES OF THE DIHYDROMENAQUINONES (II, SCHEME I). In a typical experiment the purified menaquinone fraction (2.2 mg), diluted with purified phylloquinone (100.5 mg), was dissolved in acetic anhydride (3 ml) and triethylamine (3 ml), and decolorized by shaking for 5 min with zinc dust (0.7 g). The mixture was refluxed gently on a steam bath for 10 min, cooled, and taken up in ether (50 ml). The ethereal solution was washed with water (four 50-ml portions), 5 N HCl (two 50-ml portions), and then again with water until the washings were neutral (four 50-ml portions). The ether layer was dried ($MgSO_4$), filtered, and evaporated to yield a colorless oil (120 mg).

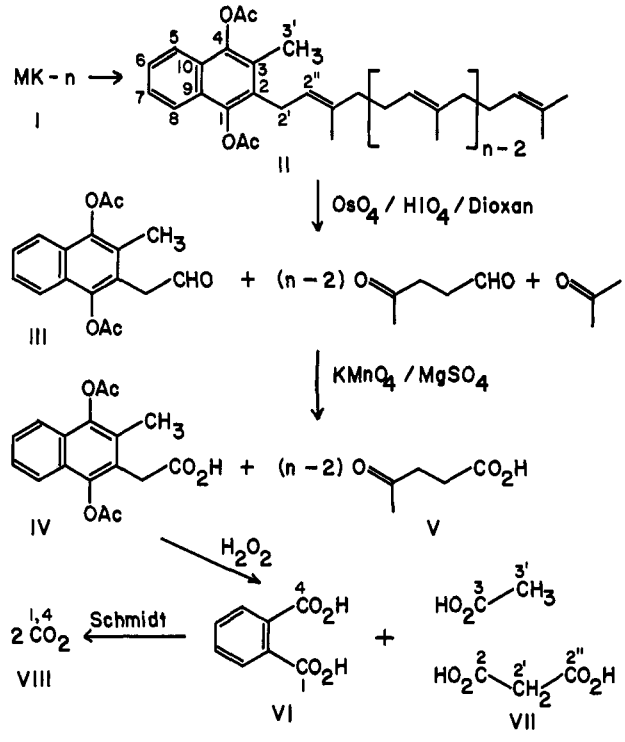
The oil was chromatographed on a column of silicic acid (10 g, 1.8 × 17 cm, flow rate 60 ml/hr) established in benzene; 10-ml fractions were collected. Ultraviolet spectrometry, radioisotope assay, and layer chromatography (solvent system, benzene–chloroform, 1:1, v/v) indicated that the desired product was present in fractions 5–15 (115 mg, 95%). This material was not crystallized lest the menaquinone and phylloquinone derivatives were fractionated one from another.

PREPARATION OF 1,4-DIACETOXY-3-METHYL-2-NAPHTHALENEACETIC ACID (IV). Dioxane was distilled from KOH and passed through a column of neutral alumina immediately prior to running this reaction. The diacetate prepared above (115 mg) was dissolved in purified dioxane (25 ml) and water (5 ml). Osmium tetroxide (three crystals) was added followed by solid periodic acid (0.5 g). This solution was stirred at room temperature until all the starting material had reacted as judged by layer chromatography in the solvent system, benzene–chloroform (1:1, v/v). The time involved was routinely 18 hr. Thereafter, the reaction solution was partitioned between ethyl acetate and water (50 ml of each). The ethyl acetate layer was removed, washed with water (three 50-ml portions), and added to a solution of $KMnO_4$ (200 mg) and $MgSO_4$ (200 mg) in water (25 ml). The biphasic mixture was stirred vigorously at room temperature until layer chromatography (chloroform–methanol, 10:1, v/v) indicated that the intermediate aldehyde (III) had been completely converted into the acid (IV). A reaction time of 3 hr was usually required.

The reaction solution was decolorized with $NaHSO_3$ and just acidified with HCl (congo red). The aqueous layer was removed and the organic layer extracted with Tris buffer (pH 7.8, five 50-ml portions). The buffer extracts were acidified with concentrated HCl and extracted with ethyl acetate (four 100-ml portions). The ethyl acetate extracts were dried ($MgSO_4$), filtered, and evaporated to yield a white solid (40 mg, 59%) which was crystallized from aqueous ethanol, then ethyl acetate: heptane to give the naphthaleneacetic acid as needles, mp 207–209° (Binkley *et al.*, 1940, mp 208–209°); parent molecular ion, *m/e* 316; base peak, *m/e* 186; λ_{max} (EtOH), 286 nm (ϵ 4740) and 277 nm (ϵ 4635); ν_{max} ($CHCl_3$), 1748, 1695 cm^{-1}; δ values (CF_3CO_2H), 2.38 (CH_3-aryl), 2.62 (CH_3-CO_2 aryl), 5.99 (aryl-CH_2CO_2H), 7.68 (4 aryl hydrogens).

RECOVERY OF LEVULINIC ACID FROM THE OSMYLATION OF MK-9(II-H_2). As mentioned heretofore, early experiments with *M. phlei* used cold MK-9(II-H_2) as diluent. Levulinic acid (V) was, therefore, produced in the osmylation and was recovered as follows. The initial stages of osmylation were similar to those described above but after 3-hr reaction water (10 ml) was added followed by the buffered oxidant (400 mg each of $KMnO_4$ and $MgSO_4$). After a further period of 3 hr,

SCHEME I



the reaction solution was acidified with 5 N HCl and extracted with ethyl acetate to give the naphthaleneacetic acid. Lyophilization of the aqueous layer and chloroform extraction thereof gave crude levulinic acid which was converted to its semicarbazone as described by Vogel (1954).

PREPARATION OF PHTHALIC AND MALONIC ACIDS (VI AND VII). Naphthaleneacetic acid (30 mg) was dissolved in 2 N NaOH (8 ml) at 0° under a stream of N_2. H_2O_2 (6 ml of 30%) was added slowly during 30 min. The solution was kept at 0° for 2 hr, then warmed gradually to 50° during 1 hr. The solution was acidified with concentrated HCl, evaporated to dryness, and the resulting white solid extracted with ethyl acetate (four 25-ml portions). This organic extract was dried ($MgSO_4$), filtered, and evaporated to give a white solid (22 mg). Layer chromatography in the system, benzene–dioxane–acetic acid (65:20:15, v/v), and visualization with bromophenol blue revealed the presence of malonic and phthalic acids (R_F 0.15 and 0.45, respectively).

The acid mixture was chromatographed on a column of silicic acid (10 g, 18 × 1.8 cm, flow rate 100 ml/hr) on which had been adsorbed 0.5 N H_2SO_4 (8 ml). The eluting solvents were equilibrated with 0.5 N H_2SO_4. The column was developed with increasing proportions of 1-butanol in chloroform. Phthalic acid was detected in the 5% butanol fractions. The white solid (12 mg, 75%) was crystallized to constant specific activity from water by the addition of HCl gas to give needles mp 205–207° (rapid heating). Malonic acid was detected in the 25% butanol fractions. The oil (3 mg, 30%) was crystallized to constant specific activity from ethyl acetate–*n*-heptane, to give needles mp 134–135°.

DECARBOXYLATION OF PHTHALIC ACID. The method of Phares (1951) was employed; a final bath temperature of 140° gave optimal results. The barium carbonate was washed successively with water, ethanol, and ether before drying; yield, routinely 1 mg of $BaCO_3$/mg of phthalic acid.

TABLE I: Compounds Tested as Precursors of Ring A of Menaquinones.

Expt	Precursor	Organism	Radioact. Added (μCi)	Sp Act. of Precursor (mCi/mmole)	Sp Act. of MK (dpm/μmole)	Incorp (%) $\times 10^3$	Dilution[a] $\times 10^{-3}$	MK:Q[b]
1	[G-^{14}C]Shikimate	*E. coli*	120	1.26	527,789	1600	0.0053	0.85
2	[G-^{14}C]Shikimate	*E. coli*	3	2.56	12,688	100	0.45	0.56
3	[G-^{14}C]Shikimate[c]	*E. coli*	3	2.56	19,492	200	0.29	0.49
4	[G-^{14}C]Shikimate	*S. albus*	50	1.86	273	7	16	
5	[G-^{14}C]Shikimate	*M. phlei*	70	2.56	1,738	20	3.3	
6	[U-^{14}C]Benzoate	*E. coli*	300	20.6	469	3	97	0.05
7	[U-^{14}C]Benzoate	*M. phlei*	500	8.72	68	0.1	280	
8	[1-^{14}C]Phenylacetate	*M. phlei*	500	10.0	9,257[d]	*d*	*d*	
9	[U-^{14}C]Phenylalanine	*M. phlei*	200	7.0	905	4	17	

[a] Dilution value is defined as specific activity of precursor:specific activity of product. [b] MK:Q = specific activity of menaquinone:specific activity of ubiquinone from same experiment. [c] In this experiment, 9.2 mg of unlabeled 3,4-dihydroxybenzaldehyde was added to each of the six culture flasks. [d] Crystallization of this menaquinone sample as the diacetate of its dihydro derivative led to complete loss of the radioactivity.

Synthesis of the Noncommercially Available Precursors. [1,4-^{14}C]1,4-Naphthoquinone (2.28 mg) was prepared by oxidizing [1-^{14}C]1-naphthol (97.7 mg) in dry acetone (25 ml) with chromium trioxide (101.6 mg) for 4 hr at room temperature. Solvent removal, product extraction with benzene, and chromatography on silicic acid in benzene and then on Sephadex LH-20 in ethyl acetate gave pure product which was crystallized to constant specific activity from isooctane. Unreacted starting material could be recovered and recycled.

[1,4-^{14}C]2-Hydroxy-1,4-naphthoquinone (5.2 mg) was prepared by converting [1,4-^{14}C]1,4-naphthoquinone (24 mg) into its oxide (Fieser *et al.*, 1939). The oxide was rearranged to lawsone by treatment at room temperature with concentrated H_2SO_4 for 5 min (E. Grotzinger and I. M. Campbell, unpublished work). Lawsone purification involved buffer extraction (pH 7.9) and crystallization from ethyl acetate–isooctane, then aqueous methanol.

Specifically labeled 4-(2′-carboxyphenyl)-4-oxobutyric acids were prepared according to a modification of the method of Roser (1884) employing either [2,3-^{14}C]- or [1,4-^{14}C]succinic acids as substrates. Yields were routinely 10%.

Isolation and Degradation of Tyrosine. Tyrosine was isolated from cellular protein and degraded as previously described (Hudson *et al.*, 1970).

Results

At the beginning of this undertaking in 1965 verification was sought of the provisional findings of Cox and Gibson (1964) that shikimic acid was involved in the construction of the naphthoquinone ring system of the menaquinones of *E. coli*. It was our hope to obtain samples sufficiently radioactive to allow meaningful chemical degradations to be performed. Such had not been the case in the original work. The incorporation results obtained by feeding (−)-[G-^{14}C]shikimic acid to *E. coli* cultures are shown in Table I, expt 1 and 2. The high incorporation level and low dilution value confirmed that shikimic acid, in part or as a whole, was involved in menaquinone biosynthesis.

Degradation of a menaquinone sample, derived from (−)-[G-^{14}C]shikimic acid, demonstrated that 89% of the total incorporated activity was resident in the phthalic acid portion; *i.e.*, in ring A and the two quinonoid carbon atoms of ring B (Table II, expt 1). Furthermore, the distribution of activity between ring A and these quinonoid carbon atoms was 5.4:1. Since this is in good agreement with the average value of 5.4:1 found for the ratio of ring carbons:carboxyl carbon in generally labeled shikimic acid,[3] support was obtained for the hypothesis that shikimic acid was utilized as a C_6–C_1 unit to provide the whole of the menaquinone ring A and one of the quinonoid carbon atoms.

Similar results with shikimate feeding were obtained in *S. albus* and *M. phlei* (Tables I and II, expt 4 and 5). With both organisms incorporations of radioactivity were lower and dilution values higher than in the *E. coli* experiments. However, the isotope distribution pattern in the menaquinone was in the same sense; the ratio ring A activity:carbonyl activity was 5.1:1 for *S. albus* and 4.6:1 for *M. phlei*.

Having established that shikimate was implicated in naphthoquinone biosynthesis, the next task was to determine whether it was involved directly or was first converted to an aromatic species. Using both *E. coli* and *M. phlei* as test organisms, probable preformed aromatic precursors, benzoate and phenylacetate, were tested (Table I, expt 6–8). Although [1-^{14}C]phenylacetate initially appeared to be a possible precursor, derivatization and further purification led to complete loss of activity from the sample. Benzoate was not effectively incorporated either. Phenylalanine was also examined (Table I, expt 9) and, in harmony with the previous demonstration of utilization of all the shikimate carbon in menaquinone biosynthesis, was not found to be incorporated.

With the probable origin of seven of the ten naphthoquinonoid carbon atoms located, and in the knowledge that the

[3] The shikimic acid used in these experiments was a commercial sample, prepared from *Ginkgo biloba* seedlings exposed to [^{14}C]CO_2 and was designated by the manufacturers as "generally" labeled. Since no claim for uniform labeling was made, samples of this material, one obtained in 1967, and the other in 1969, were decarboxylated. Using the Schmidt method, the ratios for the two samples were, respectively, 5.9:1 and 4.4:1; by copper chromite in quinoline decarboxylation, the values were, respectively, 6.6:1 and 4.7:1. The average value is 5.4:1.

TABLE II: Distribution of Radioactivity in Menaquinone Samples.

	[G-^{14}C]Shikimate						[1-^{14}C]Glucose				[1-^{14}C]Acetate				[2-^{14}C]Acetate			
	Expt 1 *E. coli*		Expt 4 *S. albus*		Expt 5 *M. phlei*		Expt 24 *E. coli*		Expt 25 *E. coli*		Expt 11 *M. phlei*		Expt 12 *M. phlei*		Expt 13 *M. phlei*		Expt 14 *M. phlei*	
Degradation Compound	Sp Act.[a]	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II
II	2270	100	297	100	328	100	539	100	773	100	6380	100	8724	100	21241	100	11872	100
IV	2065	91	260	88	235	71	91	18	140	18	1397	22	1903	22	5700	24	2633	22
V[b]					10	3					511	8			1991	9	1011	9
VI	2028	89	245	83	225[c]	67	54[c]	10	62[c]	8	1310	21	1788[c]	21	2060[c]	10	1508	13
VII	0	0	9	3	9[c]	3	11[c]	2	69[c]	9			61[c]	1			915[c]	8
VIII	334	14	46	16	40	12	0	0	8	1	792	12	628	8	402[c]	2	296	2
C-5 to C-10[d]	75		66		55		10		6		9		13		8		11	
C-1 + C-4[e]	14		16		12		0		2		12[f]		8[f]		2		2	
C-2 + C-2′ + C-2″[g]	0		9		3		2		9		} 1		2		} 14		8[h]	
C-3 + C-3″[i]	2		0		1		6		1				0				1	

[a] As dpm/μmole. [b] Only recovered in the early *M. phlei* experiments where MK-9 (II-H_2) was used in dilution. [c] Corrected for a further dilution. [d] Derived as VI–VIII. [e] Derived from value of VIII. [f] Application of the C-1 or C-4 determining correction, described in detail in Table VI, footnote *a*, gives values of 10.5% and 6% for expt 11 and 12, respectively. [g] Derived from value of VII. [h] Application of the C-2 determining correction, described in detail in Table VI, footnote *a*, gives a value of 10% for C-2 + C-3 + C-3′ in expt 13; a value of 4% for C-2 in expt 14. [i] Derived as IV–VI–VII.

TABLE V: Distribution of Radioactivity in Menaquinone Samples Biosynthesized in the Presence of Labeled Glutamates and 4-(2′-Carboxyphenyl)-4-oxobutyrate.

	[U-^{14}C]Glutamate						[2-^{14}C]Glutamate				[2,3-^{14}C]4-(2′-Carboxyphenyl)-4-oxobutyrate
	Expt 28 *E. coli*		Expt 29 *M. phlei*		Expt 30 *C. diphteriae*		Expt 31 *E. coli*		Expt 32 *C. diphtheriae*		Expt 35 *E. coli*
Degradation Compound	Sp Act.[a]	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II	Sp Act.	% of II
II[b]	4850	100	3800	100	1900	100	236	100	302	100	2160	100
IV	2790	58	1090	29	1192	63	66	28	122	40	2030	94
VI	1340	28	638	17	480	25	72	31	126	42	45	2
VII	767	16	245	7	344	18	3	1	3	1	967	45
VIII	906	18	208	6	452	24	62	26	116	38	5	0

[a] As dpm/μmole. [b] For significance, see Table II.

SCHEME II

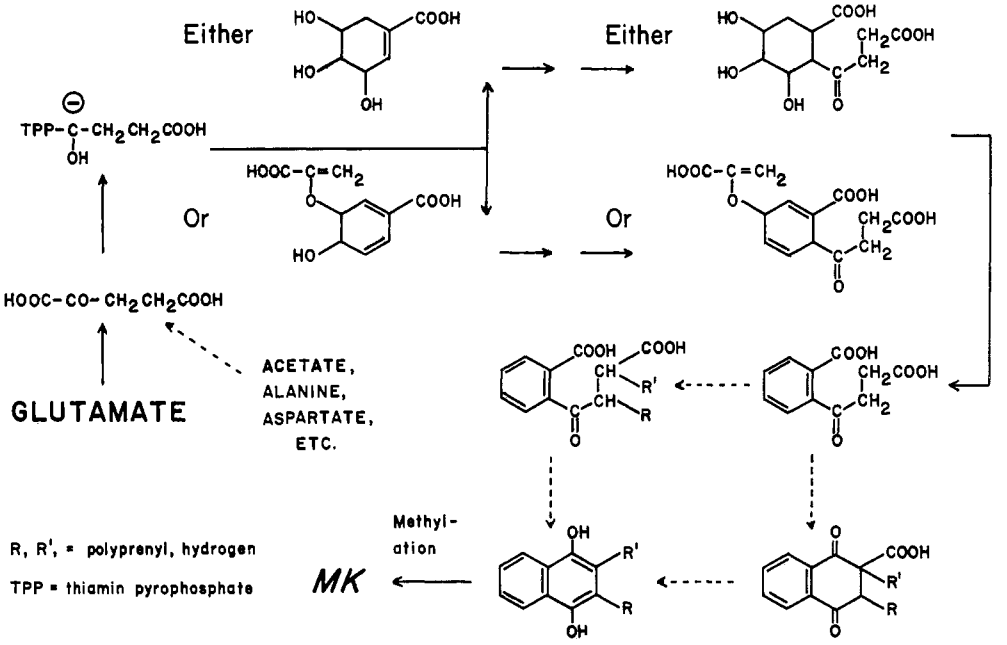


TABLE III: Compounds Tested as Precursors of Ring B of Menaquinones.

Expt	Precursor	Organism	Radioact Added (μCi)	Sp Act. of Precursor (mCi/mmole)	Sp Act. of MK (dpm/μmole)	Incorp (% × 10^3)	Dilution (× 10^{-3})	MK:Q
10	[1-^{14}C]Acetate	*E. coli*	500	29.0	4,287	2	15	1.14
11	[1-^{14}C]Acetate	*M. phlei*	500	29.0	64,119	90	1	
12	[1-^{14}C]Acetate	*M. phlei*	1000	1.46	76,447	50	0.042	
13	[2-^{14}C]Acetate	*M. phlei*	1000	33.0	162,000	120	0.45	
14	[2-^{14}C]Acetate	*M. phlei*	500	27.4	163,639	220	0.37	
15	[2-^{14}C]Malonate diethyl ester	*E. coli*	500	6.1	236	0.1	57	1.24
16	[1,3-^{14}C]Malonate	*M. phlei*	500	3.9	19,160	26	0.45	
17	[U-^{14}C]Serine	*E. coli*	100	139.0	4,070	5	76	0.8
18	[U-^{14}C]Alanine	*E. coli*	100	90.0	7,797	6	25	1.2
19	[1,3-^{14}C]Glycerol	*E. coli*	100	23.6	8,158	2	6.3	1.15
20	[1,3-^{14}C]Glycerol	*M. phlei*	500	21.0	60,606	32	0.77	
21	[1-^{14}C]Pyruvate	*E. coli*	100	9.3	1,147	3	18	1.08
22	D-[1-^{14}C]Ribose	*E. coli*	50	20.0	1,390	3	31	0.35
23	D-[U-^{14}C]Ribose	*E. coli*	25	20.0	1,355	8	32	0.82
24	D-[1-^{14}C]Glucose	*E. coli*	250	41.5	26,047	40	3.5	1.22
25	D-[1-^{14}C]Glucose	*E. coli*	500	41.5	62,849	6	1.5	0.88
26	D-[1-^{14}C]Glucose[a]	*E. coli*	500	41.5	56,358	6	1.6	0.82
27	D-[1-^{14}C]Glucose	*M. phlei*	500	3.0	198,250	270	0.033	

[a] In this experiment, 250 mg of unlabeled shikimic acid was added to each of the six culture flasks.

2-polyprenyl and 3-methyl substituents derived from mevalonate and methionine, respectively (Azerad *et al.*, 1967; Hammond and White, 1969a,b; Threlfall *et al.*, 1967), a search for the source of the remaining three carbon atoms was begun.

When two and three carbon donors such as acetate, malonate, alanine, serine, pyruvate, glycerol, and ribose, were examined as potential precursors in *E. coli*, only poor incorporations were obtained (Table III). Furthermore, the ratio of activity in menaquinone to that in ubiquinone (MK:Q ratio) was in the range of 0.35–1.24 for the precursors tested; a value considerably in excess of 1.0 would be expected for the preferential incorporation of a precursor into menaquinone. This follows from the fact that ubiquinone biosynthesis requires shikimate, *S*-adenosylmethionine, and mevalonate (Bentley, 1970) while menaquinone biosynthesis involves these precursors plus a C_3-unit donor. Little was achieved by

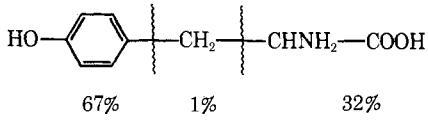
TABLE IV: Incorporation of Labeled Glutamates and Naphthalenoid Precursors into Menaquinones.

Expt	Precursor	Organism	Radioact Added (μCi)	Sp Act. of Precursor (mCi/ mmole)	Sp Act. of MK (dpm/ μmole)	Incorp (% × 10^3)	Dilution × 10^{-3}	MK:Q
28	L-[U-^{14}C]Glutamate	*E. coli*	500	187	11,200	15	0.34[b]	35
29	L-[U-^{14}C]Glutamate	*M. phlei*	500	187	8,287	20	0.008	
30	L-[U-^{14}C]Glutamate	*C. diphtheriae*	200	187	11,800	14	35[b]	
31	DL-[2-^{14}C]Glutamate	*E. coli*	500	4.26	3,180	8[a]	2.6[b]	20
32	DL-[2-^{14}C]Glutamate	*C. diphtheriae*	300	4.26	2,210	4[a]	4.2[b]	
33	DL-[2-^{14}C]Glutamate	*S. albus*	50	3.16	150	2[a]	0.7[b]	
34	[1-^{14}C]4(2′-Carboxy-phenyl)-4-oxobutyrate	*E. coli*	7.5	0.18	0	0	∞	
35	[2,3-^{14}C]4(2′-Carboxy-phenyl)-4-oxobutyrate	*E. coli*	30.6	0.75	264,000	1800	0.0063	
36	[1-^{14}C]1-Naphthol	*E. coli*	100	16.3	609	2	58	0.57
37	[1-^{14}C]1-Naphthol	*M. phlei*	50	16.3	399	12	9.1	
38	[2-^{14}C]2-Methyl-1,4-naphthoquinone	*E. coli*	95	10.0	7,790			
39	[2-^{14}C]2-Methyl-1,4-naphthoquinone	*M. phlei*	100	10.0	2,242			
40	[1,4-^{14}C]1,4-Naphtho-quinone	*M. phlei*	5.57	0.173	30	4	13	
41	[1,4-^{14}C]Lawsone	*M. phlei*	3.66	0.178	23	4	17	

[a] This incorporation value is based on the use of only the L enantiomer. [b] The growth media for *C. diphtheriae*, *E. coli*, and *S. albus* contain peptone, yeast, and beef extracts (see Methods and Materials). The possible presence of glutamate or glutamyl derivatives therein was not taken into account in calculating these dilution values. The values, therefore, represent upper limits. [c] Virtually all activity lost on reductive acetylation of the menaquinone.

these experiments in *E. coli*, except that the incorporation of at least some activity from alanine and glycerol provided a stimulus for further experiments.

Experiments with similar precursors in *M. phlei* proved only marginally more helpful. No specific source of the C_3 unit was found. While both [1-^{14}C]- and [2-^{14}C]acetate were well incorporated into the menaquinones of this organism (Table III, expt 11–14), degradation of the samples provided the immediate paradox that acetate activity was venturing into ring A (Table II, expt 11–14). To reconcile this observation with the proposed shikimate hypothesis, it was necessary to demonstrate conversion of acetate into shikimate. For this purpose, the shikimate-derived amino acid, tyrosine, was isolated from the cell paste and degraded chemically, leading to the activity distribution pattern



The finding of considerable activity in the aromatic portion of tyrosine clearly shows that acetate activity is leaking into the shikimate pool and from there is being incorporated into tyrosine and the menaquinones.

Degradation of the acetate-derived menaquinone samples also suggested there was an association of the carboxyl carbon of acetate with C-1 and/or C-4 of ring B, and of the methyl carbon of acetate with C-2 and/or C-3. While the former association could be rationalized in terms of [1-^{14}C]acetate providing label in the carboxyl group of shikimic acid, the latter association had to be more carefully analyzed.

Results similar to the *M. phlei* [2-^{14}C]acetate findings were encountered in *E. coli* when [1-^{14}C]glucose was administered, and the biosynthesized menaquinone was purified and degraded (Tables II and III, expt 24 and 25). There was the same tendency for label to accumulate in ring B carbon atoms C-2 and/or C-3. [1-^{14}C]Glucose can give rise to [2-^{14}C]acetate through glycolysis.

These acetate findings, coupled with the observation that alanine and glycerol were appreciably incorporated into the menaquinones of *E. coli* and *M. phlei* (Table III, expt 18 and 20), suggested that citric acid cycle intermediates, or their derivatives, could be involved as C_3 donors. This hypothesis was tested first by feeding to the plant *Impatiens balsamina*, the amino acids alanine, aspartate, and glutamate. Transamination of these amino acids yields pyruvate, oxalacetate, and 2-ketoglutarate, respectively. *I. balsamina* produces lawsone (2-hydroxy-1,4-naphthoquinone) and the involvement of shikimic acid as a seven-carbon unit in its biosynthesis had already been demonstrated (Leistner and Zenk, 1968). The preliminary plant experiments established conclusively that glutamate, or more likely its deamination product, 2-ketoglutarate, was indeed the source of the "missing" three carbon atoms (Campbell, 1969). As shown in Table IV, expt 28–33, L-[U-^{14}C]glutamate and DL-[2-^{14}C]glutamate were likewise well incorporated into menaquinones by *E. coli*, *M. phlei*, *Corynebacterium diphtheriae*, and *Streptomyces albus*, and the good incorporations were accompanied, in general by low dilution values. For utilization of L-[U-^{14}C]glutamate by *M.*

TABLE VI: Specific Atom Radioactivity in Menaquinones Derived from Labeled Glutamates and 4-(2′-Carboxyphenyl)-4-oxobutyrate.

Ratio[a]	[U-^{14}C]Glutamate			[2-^{14}C]Glutamate		[2,3-^{14}C]4-(2′-Carboxyphenyl)-4-oxobutyrate
	Expt 28 *E. coli*	Expt 29 *M. phlei*	Expt 30 *C. diphtheriae*	Expt 31 *E. coli*	Expt 32 *C. diphtheriae*	Expt 35 *E. coli*
$\frac{\text{C-1}}{\text{C-1} + \text{C-2} + \text{C-3}}$	39	33	40	100	100	0
$\frac{\text{C-2}}{\text{C-1} + \text{C-2} + \text{C-3}}$	30	25	27	0	0	49
$\frac{\text{C-3}}{\text{C-1} + \text{C-2} + \text{C-3}}$	30	42	33	0	0	51

[a] The extent of labeling of carbon atoms C-1, C-2, and C-3 of ring B was computed as described below. As mentioned in the text, C-1 and C-4 are indistinguishable by the presently used degradation methods. For ease of presentation only, C-1 is cited above. (i) C-1. The activity in C-5 to C-10 must arise from random labeling of shikimic acid. If this random labeling can be assumed to be approximately uniform, the carboxyl group of shikimate will donate one-sixth of ring A radioactivity to the quinonoid carbons, *i.e.*, C-1 = VIII–$^1/_6$(VI–VIII). (ii) C-2. An approximation of the extent to which malonate activity is due to C-2′ and C-2″ can be obtained by assuming the polyprenyl chain is uniformly labeled and then calculating the activity of two chain atoms from a knowledge of the activity of that part of the side chain degradatively removed. In *M. phlei*, for instance, the major prenylog is MK-9(II-H$_2$) with a 45-carbon side chain, 43 of which are removed in degradation. The activity (II–IV) therefore represents the sum of the activities of 43 carbon atoms; the activity of 2 is therefore VII − x(II–IV) where x = 2/43. The x value for *S. albus* is similarly 2/43; that for *E. coli* is 2/38 (major prenylog, MK-8) and that for *C. diphtheriae* is 2/35.5 (MK-7 and MK-8 are equally prominent). (iii) C-3. As mentioned in the text, C-3′ emanates from methionine; we therefore assume that C-3 ≈ C-3 + C-3′ = IV–VI–VII.

phlei, the dilution value was 8, second only to the value of 5.3 obtained for an incorporation of activity from shikimate in *E. coli* (Table I, expt 1). Furthermore, in the *E. coli* experiments, where the MK:Q ratio could be measured, gratifyingly high values were obtained, 35 and 20 for L-[U-^{14}C]glutamate and DL-[2-^{14}C]glutamate, respectively.

Degradation of samples of labeled menaquinones biosynthesized from glutamate gave results in keeping with specific utilization of that material as a source of the quested C_3 unit. As shown in Tables V and VI (expt 28–32), and within the limits of experimental accuracy, uniformly labeled glutamate contributes equal proportions of activity to the menaquinone carbon atoms C-1 and/or C-4, C-2, and C-3, while the [2-^{14}C]glutamate specifically labels C-1 and/or C-4. This label distribution is in harmony with the biosynthetic hypotheses shown in Scheme II and is discussed later.

4(2′-Carboxyphenyl)-4-oxobutyric acid, a substance which Azerad *et al.* (1970) have shown recently is an intermediate in menaquinone and lawsone biosynthesis, was also fed to *E. coli*. In agreement with the conclusions of the French group and with the requirements of Scheme II, [1-^{14}C]4(2′-carboxyphenyl)-4-oxobutyric acid contributed none of its activity to the menaquinone fraction, while the 2,3-^{14}C-labeled material was effectively incorporated into menaquinone ring positions, C-2 and C-3 (Tables V and VI, expt 35).

In an attempt to localize preformed naphthalenoid substances which could function as menaquinone precursors and might prove biosynthetic intermediates, labeled 1-naphthol, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and lawsone were fed to *M. phlei* and *E. coli* (Table IV, expt 36–41). Incorporation values were uniformly low. In the case of 2-methyl-1,4-naphthoquinone, the crude menaquinone extracts proved very radioactive but gel filtration and derivatization rendered the purified menaquinone virtually inactive.

Discussion

Methods. Although this work utilized standard isotopic tracer technology, a number of special problems were encountered. Foremost among these was the question of the purity of the menaquinone samples. The small quantities of menaquinone(s) present in the bacteria had to be handled by chromatographic techniques. At an early stage in our studies, mass spectrometry of material judged homogeneous by thin-layer chromatography, revealed substantial contamination by a variety of other lipids. Esters of fatty and aromatic acids were particularly troublesome; the presence of the latter initially gave rise to a completely misleading conclusion in the case of phenylacetate (Table I, expt 8). Similar problems were encountered by Guérin *et al.* (1968) using benzoic, phenylacetic, and phenylbutyric acids as precursors with *M. phlei*.

These experiences indicate the need for exercising extreme caution in evaluating results of tracer experiments which are based solely on recovery of radioactivity from "appropriate" areas of layer chromatograms. Gel filtration on Sephadex LH-20 proved effective in removing contaminants (Campbell and Bentley, 1968), and, in our opinion, is the method of choice for menaquinone purification in tracer studies. The preparation of a menaquinone derivative, *e.g.*, the dihydro diacetate, as a check on menaquinone activity levels is also advocated.

Although an ozonolysis of the isoprene side chain of the

quinol diacetate was used in the classical work leading to elucidation of the structure of vitamin K, this technique proved unreliable in our hands on a 100-mg scale. Either with the original conditions (Binkley *et al.*, 1940), or with the low-temperature ozonolysis developed for work on ubiquinone biosynthesis (Bentley *et al.*, 1965) yields of the desired naphthaleneacetaldehyde (III) or naphthaleneacetic acid (IV) were either zero or very low. However, hydroxylation of the side-chain double bonds with osmium tetroxide, followed by cleavage with periodate (Kupchan *et al.*, 1962), proved a reliable and reproducible degradation. The crude aldehyde was most conveniently handled by oxidation to the acid under conventional conditions with permanganate.

For further degradation of the naphthaleneacetic acid, cleavage with alkaline hydrogen peroxide was used. Of the three major products of this oxidation, phthalic acid and malonic acid were consistently isolated in yields of 75 and 30%, respectively. The yield of acetic acid was highly variable, and since it emanated from the introduced acetate groups and from malonic acid degradation as well as from C-3 + C-3′, the C-3 + C-3′ activity was generally calculated by difference.

Precursors of the Naphthalenoid Ring System. The data presented in this paper conclusively establish a utilization of all seven carbon atoms of shikimic acid for menaquinone biosynthesis. The six-membered hydroaromatic ring of shikimate becomes ring A of the naphthoquinone nucleus, while the carboxyl group provides a carbonyl group of ring B. Which of the two carbonyls is involved has not been ascertained as yet. A degradation scheme that specifically liberates C-1 is being developed to settle this question and the related issue of whether at any stage in the biosynthesis symmetrical naphthalenoid precursors are involved.

Coincident with our initial report (Campbell *et al.*, 1967) on shikimate utilization for menaquinone biosynthesis in *E. coli* and *M. phlei*, Leistner *et al.* (1967) observed a similar process in *Bacillus megaterium*. At the same time, these authors observed no labeling of the quinone carbon atoms when DL-[1,2-^{14}C]shikimate functioned as precursor. Recently, using a direct oxidation of the menaquinone with potassium permanganate in aqueous pyridine, Leduc *et al.* (1970) confirmed that the MK-9(II-H_2) synthesized from DL-[1,2-^{14}C]-shikimate by *M. phlei* was labeled at C-9 and C-10. In addition, radioactivity from DL-[1,2-^{14}C]shikimate has been incorporated into both MK-8 and 2-demethyl-MK-8 in *E. coli* E106 (Ellis and Glover, 1968) and from [G-^{14}C]shikimate into phylloquinone using etiolated maize shoots (Whistance *et al.*, 1967). All these findings are consistent with the biosynthetic pathway we propose in Scheme II.

No place is given in Scheme II to 3,4-dihydroxybenzaldehyde. This substance was reported by Cox and Gibson (1966) to decrease the incorporation of shikimic acid into the menaquinone fraction of *E. coli*. As a result, they postulated a "branch point" for menaquinone biosynthesis at chorismic acid, with 3,4-dihydroxybenzaldehyde as an intermediate beyond this branch. A number of attempts by various workers to verify this possibility have proved fruitless. In several bacteria and in maize shoots, labeled 3,4-dihydroxybenzaldehyde or the corresponding acid, did not lead to radioactive menaquinone or phylloquinone (Campbell *et al.*, 1967; Leistner *et al.*, 1967; Whistance and Threlfall, 1968; Guérin *et al.*, 1970). Moreover, there has been no unambiguous confiirmation of the reported "swamping" effect. Leistner *et al.* (1967) reported only a small effect in *E. coli*, while we have found a slight *increase* in shikimate incorporation when cold 3,4-dihydroxybenzaldehyde was added with the tracer (Table I, expt 2 and 3). We conclude from all of these observations that there is no direct role for 3,4-dihydroxybenzaldehyde or the corresponding acid as intermediates in menaquinone biosynthesis.

Our results also clearly implicate glutamate as the distal provider of the remaining three naphthoquinone ring carbon atoms. The manner in which we envision the coupling taking place is shown in Scheme II. It is in keeping with the observed labeling pattern from glutamate; moreover the synthesis of the succinyl semialdehyde complex from 2-ketoglutarate is a well authenticated step in the ketoglutarate dehydrogenase reaction. Whether this thiamin pyrophosphate (TPP) complex couples directly with shikimate or with chorismate as proposed by Dansette and Azerad (1970) remains to be seen.

The observed association of activity from [2-^{14}C]acetate with C-2 and/or C-3 presumably arose from a conversion of this precursor to ketoglutarate with predominant labeling in C-4. A similar explanation rationalizes the [2-^{14}C]malonate finding reported by Leistner and Zenk (1968), and used by Leduc *et al.* (1970) as evidence for an *o*-homophthalate–malonate condensation in naphthoquinone ring biosynthesis. [2-^{14}C]Malonate activity must enter C-2 and/or C-3 of the menaquinones by intermediacy of [2-^{14}C]acetate and [4-^{14}C]-2-ketoglutarate.

Confirmation of Scheme II as originally proposed came from the finding of Dansette and Azerad (1970) that 4-(2′-carboxyphenyl)-4-oxobutyrate was very effectively incorporated into the menaquinones of *M. phlei*, *E. coli*, *A. aerogenes*, and plant naphthoquinones and anthraquinones. As shown in Table IV and V (expt 34 and 35), we have confirmed an excellent utilization of [2,3-^{14}C]4-(2′-carboxyphenyl)-4-oxobutyrate in *E. coli*. Furthermore, the same material with label in the carboxyl group of the oxobutyrate side chain did not give rise to labeled menaquinone in *E. coli*. Hence, this carboxyl group is clearly lost during biosynthesis, exactly as required for the derivation of a three-carbon unit from the five-carbon precursor. Any possibility is thus excluded that the oxobutyrate carboxyl group could in part be reduced to provide the menaquinone 3-methyl substituent as is the case in barnol and javanicin (Mosbach and Ljungcrantz, 1964; Gatenbeck and Bentley, 1965). It remains to be shown that the oxobutyrate is an obligatory intermediate.

As far as the latter stages of naphthoquinone biosynthesis are concerned, little is known for certain. Mechanistically, cyclization of 4(2′-carboxyphenyl)-4-oxobutyrate to 2-carboxyl-1,4-naphthoquinol seems feasible and the latter material is presently being prepared. Decarboxylation of this naphthoquinol, and subsequent methylation and prenylation would give the required menaquinol. However, we have been unable to demonstrate utilization of 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone for menaquinone biosynthesis in *M. phlei* or *E. coli* (Table IV, expt 36–39). We do not attribute these failures to cell membrane impermeability since unreacted tracer was found in large quantity in the cells after harvest, nor can we conceive that the cells were unable to equilibrate the quinone with its quinol. We are, therefore, forced to conclude that these materials do not figure in menaquinone biosynthesis. Independently, Guerin *et al.* (1970) have come to a similar conclusion.

Pursuing the possibility that the decarboxylation of the 2-carboxyquinol could be oxidative, the anticipated product, lawsone, was prepared and fed to *M. phlei*. No incorporation was observed even though tracer accumulated intracellularly. Since neither the unsubstituted naphthoquinol nor its 2-methyl or 2-hydroxy derivatives were utilized for mena-

quinone biosynthesis, we deduce that prenylation precedes methylation and may take place prior to formation of ring B. Thus, Scheme II proposes that the oxobutyrate or the 2-carboxynaphthoquinol is the substrate of the polyprenyl transferase. A search for such prenylated materials is presently being undertaken. That prenylation precedes methylation is supported by the observation of Azerad *et al.* (1967) that cell-free preparations of *M. phlei* can convert demethyl-MK-9 to MK-9.

Following the original observation that 1-naphthol was incorporated into the menaquinone of *B. megaterium* (Leistner and Zenk, 1968), several authors have maintained that 1-naphthol is involved in menaquinone biosynthesis (Hammond and White, 1969a,b). We have been unable to repeat the original experiment with *B. megaterium* and also have failed to incorporate 1-naphthol into the menaquinones of *E. coli* and *M. phlei*. Moreover, the oxidation level of 1-naphthol is incompatible with direct involvement in a biosynthetic scheme involving a glutamate–shikimate coupling. We therefore dismiss 1-naphthol as an obligatory biosynthetic intermediate and view its incorporation as the result of an aberrant naphthol detoxification process. Support that such processes involve oxidation comes from the demonstration that in higher plants 1-naphthol is converted to 1,2-naphthoquinone (Ugrekhelidze and Kavtaradze, 1970).

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