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INCORPORATION OF SHIKIMATE AND 4-(2′-CARBOXYPHENYL)-4-OXOBUTYRATE INTO PHYLLOQUINONE

GRAHAM THOMAS and DAVID R. THRELFALL

Department of Plant Biology, University of Hull, Hull HU6 7RX

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Abstract—The patterns of incorporation of D-[G-^{14}C]shikimate and variously labelled ^{14}C-4-(2′-carboxyphenyl)-4-oxobutyrate into the naphthoquinone nucleus of phylloquinone by maize shoots have been investigated. The results show that (a) the alicyclic ring and C-7 of shikimate give rise to Ring A and either C-1 or C-4, and (b) the phenyl ring, 2′-carboxy and C-4, and C-2 and -3 of 4-(2′-carboxyphenyl)-4-oxobutyrate give rise to Ring A, C-1 and -4 and C-2 and -3. Radioactivity from α-[1-^{14}C]naphthol, 1,4-[1,4-^{14}C]naphthoquinone and [*Me*-^{14}C]menadione is not incorporated into phylloquinone to any significant extent.

INTRODUCTION

IT IS now clear that the nuclei of bacterial menaquinones (**6**) and some shikimate-derived simple naphthoquinones (juglone and lawsone) and anthraquinones (alizarin, morindone and purpurin carboxylic acid) of plant origin have many biosynthetic features in common.[1–16] In each group of compounds Ring A and a Ring B carbonyl group, C-4 in the case of menaquinones and C-1 in the case of lawsone,[12,13] are derived from the alicyclic ring and carboxyl carbon atom respectively of shikimate.[1–11] Ring B is completed by a C_3 unit provided by C-2, -3 and -4 of α-oxoglutarate.[8,10,14,15] The key biosynthetic step is believed to be the condensation of either shikimate (**1**) (favoured by Robins *et al.*[15]) or chorismate (**2**) (favoured by Dansette and Azerad[16]) with a C_4 derivative of α-oxoglutarate, possibly succinyl semialdehyde thiamine pyrophosphate (**3**),[15] to form 4-(2′-carboxyphenyl)-4-oxobutyrate,[16] which is then modified to form the appropriate quinone (Scheme

[1] COX, G. B. and GIBSON, F. (1966) *Biochem. J.* **100,** 1.
[2] CAMPBELL, I. M., COSCIA, C., KELSEY, M. and BENTLEY, R. (1967) *Biochem. Biophys. Res. Commun.* **28,** 25.
[3] ZENK, M. H. and LEISTNER, E. (1967) *Z. Naturforsch.* **226,** 460.
[4] LEISTNER, E., SCHMITT, J. H. and ZENK, M. H. (1967) *Biochem. Biophys. Res. Commun.* **28,** 845.
[5] LEISTNER, E. and ZENK, M. N. (1968) *Z. Naturforsch.* **226,** 865.
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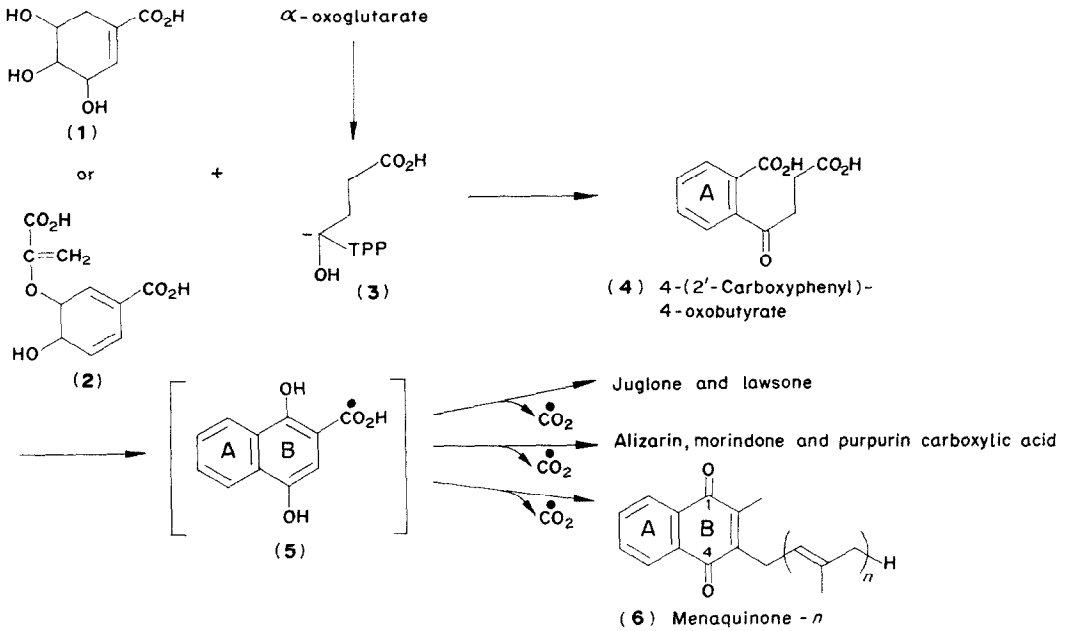
[9] CAMPBELL, I. M., ROBINS, D. J., KELSEY, M. and BENTLEY, R. (1971) *Biochemistry* **10,** 3069.
[10] LEISTNER, E. (1973) *Phytochemistry* **12,** 337.
[11] LEISTNER, E. (1973) *Phytochemistry* **12,** 1669.
[12] GROTZINGER, E. and CAMPBELL, I. M. (1972) *Phytochemistry* **11,** 675.
[13] BALDWIN, R. M., SNYDER, C. D. and RAPOPORT, H. (1973) *J. Am. Chem. Soc.* **95,** 276.
[14] CAMPBELL, I. M. (1969) *Tetrahedron Letters* 4777.
[15] ROBINS, D. J., CAMPBELL, I. M. and BENTLEY, R. (1970) *Biochem. Biophys. Res. Commun.* **39,** 1081.
[16] DANSETTE, P. and AZERAD, R. (1970) *Biochem. Biophys. Res. Commun.* **40,** 1090.

1) (**4**).[9–11,16] In the case of menaquinones, for example, this would involve cyclisation, decarboxylation, isoprenylation and methylation (not necessarily in this order) (Scheme 1). So far there is no evidence to support the suggestion that 4-(2′-carboxyphenyl)-4-oxobutyrate is cyclized to form 1,4-dihydroxy-2-naphthoate (**5**).[16] The oxidized form of the decarboxylation product of 1,4-dihydroxy-2-naphthoate,1,4-naphthoquinone, and its methylated product, menadione (a possible menaquinone precursor) are incorporated into naphthoquinones by some, but not all, organisms:[6,7,9,10,17–19] however, dilution studies and the requirement that in the final products C-1 and C-4 are biosynthetically assymmetrical make it unlikely that they are natural precursors.[7,12,13]



SCHEME 1. PROPOSED PATHWAYS FOR THE BIOSYNTHESIS OF MENAQUINONES BY BACTERIA AND NAPHTHOQUINONES AND ANTHRAQUINONES BY PLANTS.

Although the biosynthesis of the naphthoquinone nucleus of bacterial menaquinones has been actively studied, the formation of the nucleus of phylloquinone (**7**) (an analogue of the menaquinones) by plants has been neglected. The studies that have been carried out have shown that shikimate is incorporated into the naphthoquinone ring system,[20] mevalonate is the precursor of the 3-phytyl group,[21] and the *S*-methyl group of L-methionine gives rise to the 2-methyl group.[22]

In this communication we report on the pattern of incorporation of shikimate and 4-(2′-carboxyphenyl)-4-oxobutyrate into the nucleus of phylloquinone by maize shoots. We also provide evidence that α-naphthol, an aberrant precursor of menaquinones in some bac-

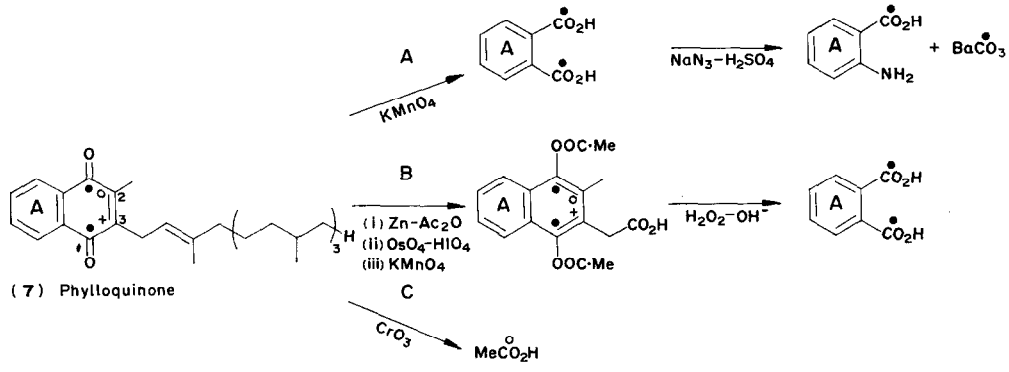
[17] MARTIUS, C. and LEUZINGER, W. (1964) *Biochem. Z.* **340**, 304.
[18] HAMMOND, R. K. and WHITE, D. C. (1969) *J. Bacteriol.* **100**, 573.
[19] HURD, D. M. (1970) B.Sc. Thesis, University College of Wales, Aberystwyth.
[20] WHISTANCE, G. R., THRELFALL, D. R. and GOODWIN, T. W. (1967) *Biochem. J.* **105**, 145.
[21] DADA, O. A., THRELFALL, D. R. and WHISTANCE, G. R. (1968) *European J. Biochem.* **4**, 329.
[22] THRELFALL, D. R., WHISTANCE, G. R. and GOODWIN, T. W. (1968) *Biochem. J.* **106**, 107.

teria,[18,19,23,24] naphthoquinone and menadione cannot be used in the biosynthesis of phylloquinone.

RESULTS

Incorporation of D-[G-^{14}C]*shikimic acid*

As expected, from previous studies,[20] the incorporation of radioactivity from this substrate into phylloquinone by maize shoots was low (Table 1). The purified product, however, contained sufficient radioactivity (4770 dpm) for a meaningful degradation to be carried out by the procedure outlined in Scheme 2, pathway A.



SCHEME 2. CHEMICAL DEGRADATION OF PHYLLOQUINONE.

The results obtained show that of the total radioactivity incorporated into the phylloquinone molecule 84% was present in Ring A and 16% was present in C-1 + C-4 or C-1 or C-4 (the degradation procedure does not distinguish between C-1 and C-4) (Table 2). In this part of the study, due to an oversight, the distribution of radioactivity between the

TABLE 1. INCORPORATION OF ^{14}C-SUBSTRATES INTO PHYLLOQUINONE BY 7-DAY-OLD ETIOLATED MAIZE SHOOTS KEPT IN THE LIGHT FOR 24 hr

Substrate	Sp. act. (μCi/μmol)	Amount administered (μCi)	No. of maize shoots	Phylloquinone* (dpm/μmol)	Dilution factor
D-[G-^{14}C]Shikimate	1·86	10	200	9930	416
4-(2′-Carboxyphenyl) -4-oxo[1-^{14}C]butyrate	0·5	2	100	0	—
4-(2′-Carboxyphenyl) -4-oxo[2,3-^{14}C]butyrate	2·5	3·3	120	602258	9
4-(2′-Carboxyphenyl) -4-oxo[2,3-^{14}C]butyrate	0·5	1·8	190	85400	13
4-(2′-[*Carboxy*-^{14}C]carboxy-phenyl)-4-oxo[2,3,4-^{14}C]butyrate	1·03	0·6	180	102050	22
α-[1-^{14}C]Naphthol	19·6	20	300	427	—
1,4-[1,4-^{14}C]Naphthoquinone	19·6	20	300	40	—
[*Me*-^{14}C]Menadione	9·7	20	300	596	—

* The amount of phylloquinone fell in the range 0·4–0·8 μmol/100 shoots.

[23] LEISTNER, E., SCHMITT, J. H. and ZENK, M. H. (1967) *Biochem. Biophys. Res. Commun.* **28**, 845.
[24] THRELFALL, D. R. (1971) *Vitamins and Hormones* **29**, 153.

alicyclic ring and carboxyl carbon atom of the D-[G-^{14}C]shikimic acid was not determined. However, other workers using D-[G-^{14}C]shikimic acid samples from the same commercial source have quoted an average distribution ratio of 5·4:1,[9] i.e. 15·6% of the radioactivity in the carboxyl carbon atom.

TABLE 2. DEGRADATION OF ^{14}C-PHYLLOQUINONES BY THE PROCEDURE OUTLINED IN SCHEME 2, PATHWAY A

	D-[G-^{14}C]Shikimate expt.		4-(2′-[*Carboxyl*-^{14}C]carboxyphenyl)-4-oxo[2,3,4-^{14}C]butyrate expt.	
	(dpm/μmol)	% Radioactivity in phylloquinone	(dpm/μmol)	% Radioactivity in phylloquinone
Phylloquinone	9930 (83)*	100	102050 (677)*	100
Phthalic acid [= Ring A + C-1 and -4]	82 (17)	99	352 (81)	52
Anthranilic acid [= Ring A + (C-1 and -4)/2]	--	--	39	25
$BaCO_3$ [=(C-1 and -4)/2]	1·4	8	Phylloquinone − phthalic acid	
	2 × $BaCO_3$ = C-1 and -4	16	= C-2 and -3†	48
	Ring A (by diff.)	84	2 × (Phthalic acid − anthranilic acid) = C-1 and -4‡	54

* Figures in parenthesis are the specific activity values after dilution with carrier material.

† Chemical degradation of phylloquinone labelled from 4-(2′-carboxyphenyl)-4-oxo[2,3-^{14}C]butyrate established that C-2 and -3 are equally labelled (Table 4).

‡ Since radioactivity was distributed equally between the carboxy group, C-2, -3 and -4 of the substrate and C-2 and -3 of phylloquinone (see previous footnote), it follows that C-1 and -4 are equally labelled.

TABLE 3. INCORPORATION OF 4-(2′-CARBOXYPHENYL)-4-OXO[2,3-^{14}C]BUTYRATE (2·5 μCi/μmol) INTO PHYLLOQUINONE BY 7-DAY-OLD ETIOLATED AND GREEN MAIZE SHOOTS

Shoots		Conditions of incubation			Phylloquinone			
Type	No.	Time (hr)	Illumination	Substrate (μCi)	(μmol)	(dpm)	(dpm/μmol)	Dilution factor
Green	50	18	+	1·5	0·51	236900	465000	12
Etiolated	50	18	−	1·5	0·11	43700	397000	14
Etiolated	50	18	+	1·5	0·20	128000	640000	9

* In this incubation the specific activities (dpm/μmol) of plastoquinone-9 and 4-demethylsterols were found to be 3567 and 320 respectively.

Incorporation of 4-(2′-*carboxyphenyl*)-4-*oxobutyric acid*

To test the ability of maize to utilize this acid for phylloquinone biosynthesis, 4-(2′-carboxyphenyl)-4-oxo[2,3-^{14}C]butyric acid was administered to etiolated maize shoots kept in the dark and to etiolated and green maize shoots kept in the light (Table 3). It was found that, (a) the acid was incorporated with little dilution into phylloquinone in the three experimental systems used (Tables 1 and 3), and (b) the amount of acid incorporated was more or less directly proportional to the terminal pool size of the phylloquinone (Table 3). It is of note that both the plastoquinone-9 and 4-demethylsterols isolated in the course of this experiment were found to be radioactive (Table 3), indicating that some degradative metabolism of the acid had taken place.

In view of the above findings it was decided for the rest of the study to use etiolated shoots exposed to light, since this system gave a good incorporation of radioactivity into phylloquinone and the purification procedures were not hampered by the presence of large quantities of carotenoids.

To determine the manner of the incorporation of this acid into phylloquinone, three species of ^{14}C-4-(2′-carboxyphenyl)-4-oxobutyric acid were administered to maize shoots. In the experiment in which 4-(2′-carboxyphenyl)-4-oxo[1-^{14}C]butyric acid was administered no radioactivity was incorporated into the phylloquinone molecule (Table 1), thus establishing that the 1-carboxy group is lost. In the remaining two experiments, the phylloquinone, as expected, was radioactive (Table 1). Chemical degradation of the phylloquinone sample labelled with radioactivity from 4-(2′-carboxyphenyl)-4-oxo-[2,3-^{14}C]butyric acid by the procedures outlined in Scheme 2, pathways B and C, showed that the greater part of the radioactivity in the molecule was equally distributed between C-2 and -3 (Table 4). The phylloquinone sample labelled from 4-(2′-[*carboxy*-^{14}C]carboxyphenyl)-4-oxo[1,2,3-^{14}C]butyric acid was not subjected to a full degradation procedure, since the degradation just described had established that the [2,3-^{14}C]butyrate portion of the substrate labels C-2 and -3. Instead it was subjected to the partial degradation outlined in Scheme 2, pathway A: the results of which, when considered in conjunction with the previous degradation, demonstrated that the radioactivity was distributed equally between C-1, -2, -3 and -4 (Table 2).

TABLE 4. DEGRADATION OF ^{14}C-PHYLLOQUINONE LABELLED FROM 4-(2′-CARBOXYPHENYL)-4-OXO[2,3-^{14}C]BUTYRATE BY THE PROCEDURES OUTLINED IN SCHEME 2, PATHWAYS B AND C

	(dpm/μmol)	% Radioactivity in phylloquinone
Procedure B:		
^{14}C-Phylloquinone + carrier	4875	100
Naphthalene acetic acid	4490	92
Phthalic acid	195	4
Malonic acid	—*	—*
Naphthalene acetic acid–phthalic acid = C-2 and -3		88
Procedure C:		
^{14}C-Phylloquinone + carrier	2380 (58950 dpm)	100
Acetic acid (= C-2)	— (27950 dpm)†	47
Naphthalene acetic acid–acetic acid = C-3		41

* Insufficient for purification.

† Schmidt-degradation showed all the radioactivity to be present in the carboxyl group, i.e. C-2 of phylloquinone.

Incorporation of ^{14}C-naphthalenic compounds

Over the past four years various colleagues of one of the authors (D.R.T) have administered a range of ^{14}C-naphthalenic compounds to maize shoots.[19,25] To complete this report their findings are presented in Table 1. They show that none of the compounds tested, α-naphthol (an aberrant precursor in some organisms), naphthoquinone and menadione, was incorporated to a significant extent. It is of note that, in agreement with the experience of workers using other organisms,[26] large amounts of radioactivity were found to be associated with the naphthoquinone-containing fractions making the purification of

[25] HALL, R. A. (1970) B.Sc. Thesis, University College of Wales, Aberystwyth.

[26] BROWN, B. S., WHISTANCE, G. R. and THRELFALL, D. R. (1968) *FEBS Letters* **1**, 323.

the phylloquinone extremely difficult. Indeed, it may be that the inclusion of extra purification steps would have resulted in non-radioactive phylloquinone samples.

DISCUSSION

Whistance *et al.*[20] reported that shikimate is a precursor of the naphthoquinone nucleus of phylloquinone. In this investigation we have extended this observation and shown that shikimate is either a precursor of Ring A and one of the carbonyl groups of Ring B or a precursor of Ring A and a contributor to both carbonyl groups of Ring B (Table 2). It was not possible, since a G-^{14}C substrate was used, to establish the absolute relationships between the carbon atoms of shikimate and the nucleus of phylloquinone. However, in view of the fact that the ratio of ^{14}C in the ring and carboxyl group of shikimate (5·4:1) was similar to the ratio of ^{14}C in Ring B and the carbonyl groups of phylloquinone (5·1:1), it is reasonable to assume that shikimate is incorporated as a C_6–C_1 unit: the alicyclic ring giving rise to Ring A and the carboxyl group giving rise to either C-1 or, as in menaquinone biosynthesis,[12] C-4.

The source of the C_3 unit required to complete Ring B was not investigated. There seems no reason for doubt, however, that as in the case of menaquinones it will be derived from C-2, -3 and -4 of α-oxoglutarate.[9,10,14,15]

In keeping with the findings of other workers studying the biosynthesis of shikimate-derived naphthoquinones,[9,11,16] 4-(2′-carboxyphenyl)-4-oxobutyrate was found to be a most effective precursor of the naphthoquinone ring system of phylloquinone (Table 1). Chemical degradation of ^{14}C-phylloquinone samples labelled from different species of ^{14}C-4-(2′-carboxyphenyl)-4-oxobutyrate, established that the 1-carboxyl group of 4-(2′-carboxyphenyl)-4-oxobutyrate is lost and that the phenyl ring, the 4-carboxyl and 2′-carboxyl groups, and C-1 and -3 of this compound give rise to Ring A, C-1 and -4, and C-2 and -3 respectively of phylloquinone.

None of the naphthalenic compounds tested, α-naphthol, 1,4-naphthoquinone and menadione, was incorporated into phylloquinone to a significant extent. These findings were not unexpected, since there is a wide variation in the abilities of plants and bacteria to incorporate these compounds into shikimate-derived naphthoquinones.[6,7,10,17–19] Indeed, in the light of recent studies,[7,12,13] the poor incorporation of 1,4-naphthoquinone and menadione is probably an indication that they are not natural precursors.

The above findings provide the first evidence that the pathway for the biosynthesis of the naphthoquinone nucleus of phylloquinone is similar to the pathways for the biosynthesis of bacterial menaquinones and some shikimate-derived simple naphthoquinones and anthraquinones of plant origin.

EXPERIMENTAL

Radiochemicals. [2,3-^{14}C]Succinic acid (22 mCi/mmol, [1,4-^{14}C]succinic acid (21 mCi/mmol), α-[1-^{14}C]naphthol (19·6 mCi/mmol) and [*Me*-^{14}C]menadione (9·7 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. D-[G-^{14}C]Shikimic acid (1·86 mCi/mmol) and [*carbonyl*-^{14}C]phthalic anhydride (10·5 mCi/mmol) were purchased from NEN Chemicals GmbH, Dreieichenhain, West Germany.

4-(2′-Carboxyphenyl)-4-oxo[1-^{14}C]butyric (0·5 mCi/mmol), 4-(2′-carboxyphenyl)-4-oxo[2,3-^{14}C]butyric (0·5 and 2·5 mCi/mmol) and 4-(2′-[*carboxyl*-^{14}C]carboxy-phenyl)-4-oxo[4-^{14}C]butyric (1·56 mCi/mmol) acids were synthesized from the appropriate mixtures of labelled and unlabelled species of succinic acid and phthalic anhydride by the method of Roser[27] as modified by Leistner.[10] 4-(2′-[*Carboxy*-^{14}C]Carboxyphenyl)-4-oxo-

[27] ROSER, W. (1884) *Chem. Ber.* **17**, 2770.

[2,3,4-^{14}C]butyric acid (1·03 mCi/mmol) was prepared by mixing 4-(2'-[*carboxy*-^{14}C]carboxy-phenyl)-4-oxo[4-^{14}C]butyric acid with 4-(2'-carboxyphenyl)-4-oxo[2,3-^{14}C]butyric acid.

1,4-[1,4-^{14}C]Naphthoquinone (19·6 mCi/mmol) was prepared from α-[1-^{14}C]naphthol by the method of Guérin *et al.*[7]

Exposure of maize shoots to ^{14}C-substrates. Shoots of etiolated 7-day-old maize seedlings (*Zea mays* var. South African White Horse Tooth; grown in the manner described by Threlfell and Whistance[28]) were excised at the node and the cut ends dipped into an aqueous solution of the radiosubstrate (30 ml/100 shoots). They were then exposed to continuous illumination (800 lm/ft^2) for 24 hr at 28°. In the last series of experiments the ^{14}C-naphthalenic compounds, because of their poor solubilities in H_2O, were administered in aq. 1% dimethylsulphoxide.

Extraction and purification of phylloquinone, plastoquinone-9 and 4-demethylsterols. The extraction and subsequent purification of these compounds was carried out as described by Whistance and Threlfall.[28,29]

Chemical degradation of phylloquinone. The ^{14}C-phylloquinone samples were degraded by procedures that have been well documented.[9,28–30] The choice of the procedure used for any given sample was determined by the nature of the expected labelling pattern. (a) In the first procedure (Scheme 2, pathway A) the ^{14}C-phylloquinone sample was mixed with 54 μmol of phylloquinone and oxidized with $KMnO_4$.[30] The ^{14}C-phthalic acid formed in this reaction was isolated, assayed for radioactivity, diluted by the addition of 100 μmol of phthalic acid and converted to anthranilic acid by the Schmidt-degradation. Three modifications made to the published procedure were, (i) phthalic acid (R_f, 0·5) was purified by TLC on silica gel H in EtOH–NH_3–H_2O (100:12:27), (ii) anthranilic acid (R_f, 0·3) was purified by TLC on silica gel G in $CHCl_3$–HOAc (19:1), and (iii) $BaCO_3$ formed in the Schmidt-degradation was assayed for ^{14}C-activity by suspension in a thixotropic gel prepared by adding 700 mg of Cab-O-Sil to 20 ml of our standard scintillation fluid. (b) In the second procedure (Scheme 3, pathway B) a mixture of ^{14}C-phylloquinone and 220 μmol phylloquinone was reductively acetylated and then degraded to 1,4-diacetoxy-2-methyl-3-naphthaleneacetic acid by treatment with, (i) osmium tetroxide-periodic acid in dioxane, and (ii) neutral $KMnO_4$.[9] The next step in this procedure is alkaline H_2O_2 oxidation of the naphthalene acetic acid to phthalic acid and malonic acid. In our hands, however, the yield of malonic acid was so low as to preclude its purification and subsequent assay for ^{14}C-activity. (c) In the third procedure (Scheme 3, pathway C) ^{14}C-phylloquinone was mixed with 25 μmol phylloquinone and the radioactivity in C-2 determined by a combination of a Kuhn–Roth oxidation and a Schmidt-degradation.[28,29]

Spectrophotometric determination of phylloquinone, plastoquinone-9, phthalic acid, anthranilic acid and 1,4-diacetoxy-2-methyl-3-naphthaleneacetic acid. Phylloquinone and plastoquinone-9 were assayed by the procedures described by Threlfall and Whistance.[28,29] Phthalic acid (λ_{max} 285 nm, in EtOH), anthranilic acid (λ_{max} 335 nm in aq. 80% MeOH) and naphthalene acetic acid (λ_{max} 286 nm, in EtOH) were assayed using ε values of 1928 (determined experimentally), 4002[30] and 4740[9] respectively.

Radioassay. Samples were assayed for radioactivity in a Beckman Liquid Scintillation Spectrometer. Lipid samples were taken up in 10 ml toluene containing 0·05 g 2,4-diphenyloxazole and 0·003 g 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene. Sodium acetate was dissolved in H_2O and a 0·2 ml sample added to 10 ml toluene–methoxyethanol (10:3) containing 0·046 g 2,5-diphenyloxazole, 0·0023 g 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene and 0·00077 g naphthalene. All counts were corrected for background and instrument efficiency.

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[28] THRELFALL, D. R. and WHISTANCE, G. R. (1971) in *Methods in Enzymology* (MCCORMICK, D. B. and WRIGHT, L. D., eds.), Vol. XVIII, p. 369, Academic Press, New York.

[29] THRELFALL, D. R. and WHISTANCE, G. R. (1971) in *Methods in Enzymology* (MCCORMICK, D. B. and WRIGHT, L. D., eds.), Vol. XVIII, p. 559, Academic Press, New York.

[30] LEISTNER, E. and ZENK, M. H. (1971) in *Methods in Enzymology* (MCCORMICK, D. B. and WRIGHT, L. D., eds.), Vol. XVIII, p. 547, Academic Press, New York.