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Biosynthesis of Phytoquinones

INCORPORATION OF L-[*Me*-^{14}C,^{3}H]METHIONINE INTO TERPENOID QUINONES AND CHROMANOLS IN MAIZE SHOOTS

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1. Radioactivity from L-[*Me*-^{14}C,^{3}H]methionine is incorporated into phylloquinone, plastoquinone, γ-tocopherol, α-tocopherol, α-tocopherolquinone and ubiquinone in maize shoots. 2. Comparative studies with other terpenoids (squalene and β-carotene) and chemical degradation of selected quinones (ubiquinone and plastoquinone) established that all the radioactivity is confined to nuclear methyl substituents. 3. In ubiquinone 76% of the radioactivity is in the methoxyl groups and 24% in the ring *C*-methyl group. 4. Taking the phytosterols as an internal reference and accepting the atomic ratio of ^{14}C/^{3}H transferred from L-[*Me*-^{14}C,^{3}H]-methionine to the supernumerary group at C_{24} to be 1:2 the ratio of all the quinones and chromanols examined approached 1:3. After allowing for the fact that for plastoquinone, γ-tocopherol, α-tocopherol and α-tocopherolquinone one nuclear methyl group is formed from the β-carbon of tyrosine, these results show that one nuclear *C*-methyl group for phylloquinone, plastoquinone and γ-tocopherol, two nuclear methyl groups for α-tocopherol and α-tocopherolquinone and one nuclear methyl and two methoxyl groups for ubiquinone are formed by the transfer of intact methyl groups from methionine. 5. From a comparison of the incorporation of ^{14}C radioactivity into these compounds it would appear that the methylation reactions involved in phylloquinone and plastoquinone biosynthesis take place in the chloroplast, whereas those involved with ubiquinone biosynthesis occur elsewhere within the cell.

The terpenoid quinones and chromanols found in maize shoots all contain nuclear methyl groups (Fig. 1). Ubiquinone is atypical in that in addition to a nuclear *C*-methyl group it also possesses two nuclear *O*-methyl substituents.

Whistance & Threlfall (1967) have shown that in the biosynthesis of plastoquinone, γ-tocopherol, α-tocopherol and α-tocopherolquinone the carbon atoms of the nucleus and one methyl group (probably the methyl group *meta* to the polyprenyl side chain) are derived from the nuclear and β-carbon atoms of tyrosine respectively. Only the nucleus of this amino acid is used for ubiquinone formation. In phylloquinone synthesis the nucleus is formed from shikimic acid without the intermediate participation of either phenylalanine or tyrosine. From these results it follows that the non-tyrosine-derived nuclear *C*-methyl group(s) of plastoquinone, γ-tocopherol, α-tocopherol and α-tocopherolquinone, the nuclear methyl group of phylloquinone and the nuclear *C*- and *O*-methyl groups of ubiquinone are most probably formed by a transfer of methyl groups from the biological methylating agent *S*-adenosylmethionine. Mechanistically this would involve the transfer of intact methyl groups.

With L-[*Me*-^{14}C]methionine it has been found that the methyl group of this amino acid can give rise to the ring *C*- and *O*-methyl groups of ubiquinone in rat (Olson, 1967), *Rhodospirillum rubrum* (Rudney & Raman, 1967), *Chromatium* sp. (G. R. Whistance & D. R. Threlfall, unpublished work) and baker's yeast (Spiller, Threlfall & Whistance, 1967), and to the ring *C*-methyl group of menaquinone in *Mycobacterium phlei* (Guerin, Azerad & Lederer, 1965) and *Chromatium* sp. (G. R. Whistance & D. R. Threlfall, unpublished work).

To investigate (*a*) the efficacy of methionine as a methyl donor in phytoterpenoid quinone and chromanol biosynthesis and (*b*) the nature of any such transmethylations we have carried out experiments with a doubly labelled species of methionine. The results obtained have been reported briefly (Threlfall, Whistance & Goodwin, 1967*a*).

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EXPERIMENTAL

Radiochemicals. L-[*Me*-^{14}C]Methionine (25 mc/m-mole) and L-[*Me*-^{3}H]methionine (143 mc/m-mole) were purchased from The Radiochemical Centre (Amersham, Bucks.).

Biological methods. The cultivation of maize seedlings and their exposure to radioactive substrates were carried out as described by Whistance, Threlfall & Goodwin (1967).

Analytical methods. The extraction, subsequent purifications and spectrophotometric determinations of all compounds examined were carried out as described by Whistance *et al.* (1967).

Degradation of plastoquinone and ubiquinone. ^{14}C radioactivity in the nonaprenyl side chain of plastoquinone was determined by ozonolytic degradation (Whistance *et al.* 1967).

^{14}C radioactivity in the methoxyl groups of ubiquinone was determined by converting them into methyl iodide (Zeisel reaction), which was assayed for radioactivity as tetramethylammonium iodide (Spiller *et al.* 1967).

To determine incorporation of radioactivity from L-[*Me*-^{14}C]methionine into the ring *C*-methyl group(s) of ubiquinone and plastoquinone, the sample plus 26 μmoles of plastoquinone was oxidized to acetic acid by the Kuhn–Roth oxidation (Phares, 1951). The methyl group of the acetic acid represents the ring *C*-methyl group(s) and lateral methyl groups of the nonaprenyl side chain. G. R. Whistance & D. R. Threlfall (unpublished work) have shown that Kuhn–Roth oxidation, though converting all the ring *C*-methyl groups into acetic acid, only converts 5 of the 9 lateral methyl groups in the side chain into acetic acid. Since in this experiment no radioactivity is incorporated into the prenyl portion of the molecule the latter contributes no radioactivity to the acetic acid. The acetic acid was purified by column chromatography (Swim & Utter, 1957), titrated with 0·05 N-NaOH, assayed for radioactivity and

Table 1. *Incorporation of* ^{14}C *from* L-[Me-^{14}C]*methionine into lipids of maize shoots*

Etiolated 7-day-old maize shoots (350) were excised and the cut ends dipped in 100 ml. of water containing 50 μc of L-[*Me*-^{14}C]methionine and 300 μc of L-[*Me*-^{3}H]methionine. The system was then incubated for 24 hr. with continuous illumination (the characteristics of this system have been reported by Griffiths, Threlfall & Goodwin, 1967). At the end of this period the lipid was extracted and chromatographed on a column of Brockmann grade III acid-washed alumina.

Fraction	Wt. (mg.)	$10^{-3} \times$ ^{14}C radioactivity (counts/min.)	Terpenoid constituents
Total lipid extract	586	Not determined	
Column fractions			
0·25% E/P	37·2	27·6	β-Carotene (0·3 mg.), phylloquinone (0·3 mg.) and squalene
1% E/P	6	137	Plastoquinone (2·1 mg.)
3% E/P	9·5	25·6	α-Tocopherol (0·5 mg.)
5% E/P	27·8	54·7	Ubiquinone (0·45 mg.) and γ-tocopherol (0·17 mg.)
8% E/P	20	126	3β-Hydroxy sterols (55 mg.)
12% E/P	37·3	746	
20% E/P	39·5	207·3	α-Tocopherolquinone (0·35 mg.)

Table 2. *Incorporation of radioactivity from* L-[Me-^{14}C]*methionine and* L-[Me-^{3}H]*methionine into quinones, chromanols and 3β-hydroxy sterols*

The corrected ^{14}C/^{3}H ratios are calculated assuming that the ^{14}C/^{3}H ratio in the 3β-hydroxy sterols is 2:4 (see Scheme 1 and the text).

Terpenoid	Specific ^{14}C activity (counts/min./μmole)	Specific ^{14}C activity (counts/min./μg.atom of ^{14}C)	Observed ^{14}C/^{3}H ratio ± S.E.M. (6 determinations)	Atomic ratio: Corrected ^{14}C/^{3}H	Atomic ratio: Expected ^{14}C/^{3}H*
Squalene, β-Carotene	These compounds were completely non-radioactive				
3β-Hydroxy sterols	11 111	5 556	1:2·83 ± 0·005	2:4	2:4
Plastoquinone	4 280	4 280	1:4·18 ± 0·014	1:2·96	1:3
Phylloquinone	5 444	5 444	1:4·22 ± 0·059	1:2·98	1:3
γ-Tocopherol†	21 750	21 750	1:4·28 ± 0·011	1:3·03	1:3
α-Tocopherol†	9 100	4 550	1:4·16 ± 0·039	2:5·88	2:6
α-Tocopherolquinone	9 150	4 575	1:4·09 ± 0·002	2:5·78	2:6
Ubiquinone	30 850	10 283	1:4·63 ± 0·011	3:9·81	3:9

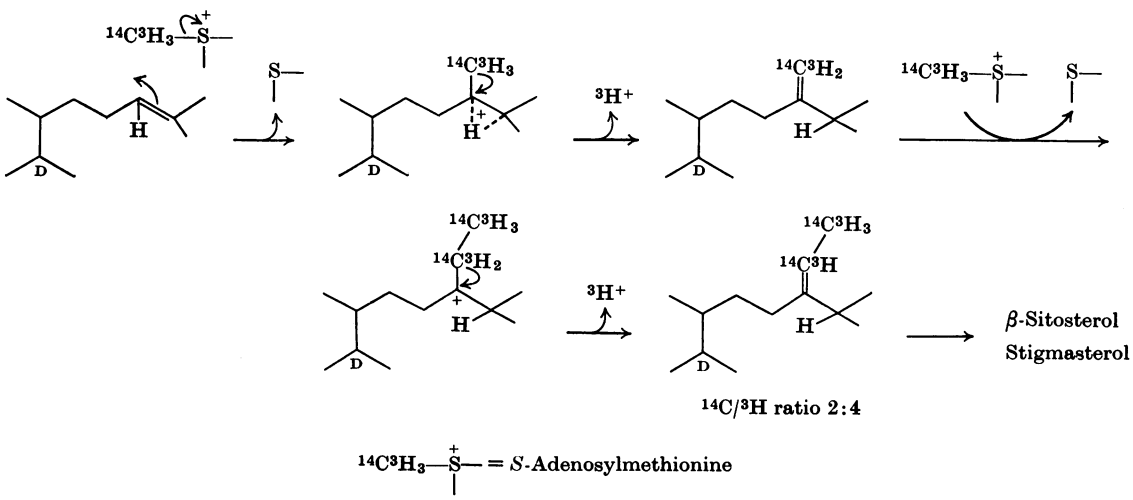
* See Fig. 1 and the text for how these values were arrived at.

† Expressed in terms of the quinone.

Table 3. *Degradation of ubiquinone and plastoquinone samples labelled from* L-[Me-^{14}C]*methionine and* L-[Me-^{3}H]*methionine*

The distribution of ^{14}C radioactivity in the plastoquinone and ubiquinone molecules was determined by using the degradation procedures listed below.

		Sample taken			Results of degradation		% of total radioactivity in molecule	
Sample	Degradation procedure	(μmole)	(counts/min.)	Carrier material	Derivative examined	Radioactivity (counts/min.)	Expected	Found
Plastoquinone	Ozonolysis	0·97	3820	None	2,4-DNP-laevulic aldehyde	0	0	0
	Kuhn–Roth	0·97	3820	Plastoquinone (26 μmoles)	Sodium acetate	3828	100	100
	Schmidt	Further degradation of the sodium acetate obtained on Kuhn–Roth oxidation		Sodium acetate	CO_2	0	0	0
	Persulphate oxidation			None	CO_2	3590	100	94
Ubiquinone	Zeisel	0·19	5615	2-Methoxy-ethanol	Tetramethyl-ammonium iodide	4160	—	74
	Kuhn–Roth	0·19	5615	Plastoquinone (26 μmoles)	Sodium acetate	1573	26	28
	Schmidt	Further degradation of the sodium acetate obtained on Kuhn–Roth oxidation		Sodium acetate	CO_2	0	0	0
	Persulphate oxidation			None	CO_2	1345	26	24



Scheme 1. Scheme to account for the ethylation of phytosterols (after Goad *et al.* 1966).

then degraded by the Schmidt reaction, as described by Phares (1951). Two modifications made were: (i) potassium persulphate (Sakami, 1955) instead of alkaline permanganate was used to oxidize the methylamine, and (ii) the $^{14}CO_2$ formed on cleavage of acetic acid and oxidation of methylamine was trapped in M-Hyamine 10X (hydroxide form) in methanol, which was then assayed for radioactivity in the scintillation counter.

Radioassay. Samples were assayed for ^{3}H and ^{14}C content in a Packard Tri-Carb liquid-scintillation spectrometer with toluene containing 2,5-diphenyloxazole (0·5%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene

(0·03%, w/v) as the scintillation fluid. Counting was by the discriminator method (Okita, Kabara, Richardson & Le Roy, 1957).

Tetramethylammonium iodide from the Zeisel degradation and sodium acetate from the Kuhn–Roth oxidations were assayed in a proportional counter (Nuclear–Chicago model 4306 automatic gas-flow system; Continental Distributors Ltd., London, S.W. 7) fitted with a window.

RESULTS AND DISCUSSION

Seven-day-old etiolated maize shoots were excised and incubated with a mixture of 50 μc of L-[*Me*-^{14}C]methionine and 300 μc of L-[*Me*-^{3}H]-methionine for 24hr. with continuous illumination. At the end of this period the lipid was extracted and chromatographed on a column of Brockmann grade III acid-washed alumina (Table 1).

The incorporation of ^{14}C radioactivity into the fractions was low (1·19%), but nevertheless it represents a specific incorporation of methyl groups. The distribution of ^{14}C radioactivity between the column fractions was similar to that found when DL-[2-^{14}C]mevalonate was administered (Threlfall, Griffiths & Goodwin, 1967*b*), i.e. the greatest amounts in the fractions containing sterol ester (1% E/P*), sterol precursor (5% E/P) and unesterified sterol (8% E/P + 12% E/P).

Table 2 summarizes the specific activities and ^{14}C/^{3}H ratios found in the purified quinones and chromanols. Squalene, β-carotene and unesterified 3β-hydroxy sterols were isolated as reference compounds.

The absence of radioactivity from squalene and β-carotene means that the methyl group of methionine has not been metabolized to give compounds capable of entering the biosynthetic sequences leading to the formation of the polyprenyl side chains of the quinones and chromanols and the terpenoid skeleton of the 3β-hydroxy sterols. Additional confirmation was obtained by ozonolytic degradation of a sample of plastoquinone, when it was found that the laevulic aldehyde obtained from the nonaprenyl side chain was completely unlabelled (Table 3).

With plastoquinone and ubiquinone the intramolecular distribution of ^{14}C radioactivity was investigated further (Table 3). Kuhn–Roth oxidation of the plastoquinone followed by a Schmidt degradation of the sodium acetate showed that all the ^{14}C radioactivity was in the methyl groups; since those derived from the prenyl side chain are unlabelled it follows that the radioactivity is contained entirely in one or other of the ring *C*-methyl groups. With ubiquinone 26% of the total ^{14}C radioactivity in the molecule was found in the ring *C*-methyl group: Zeisel degradation established

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

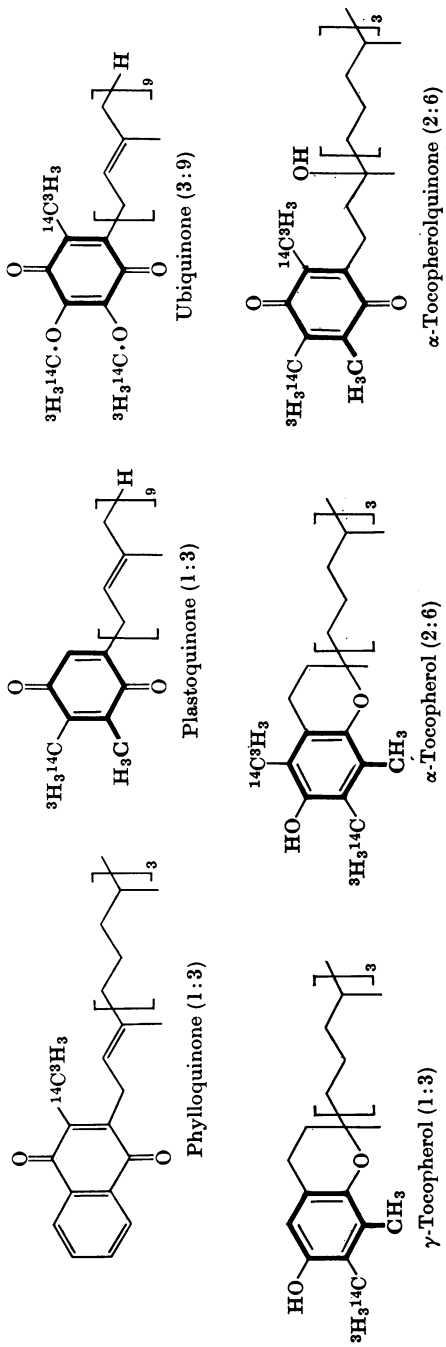
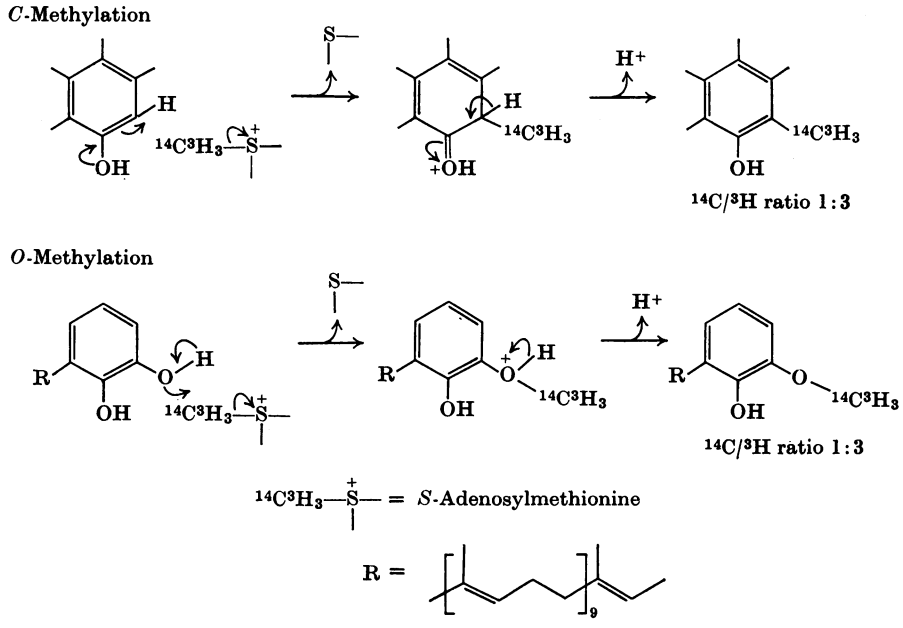


Fig. 1. Structures of the phytoterpenoid quinones and chromanols found in maize shoots. The heavy lines and carbon atoms represent the parts of the molecule derived from tyrosine. The nuclear methyl groups formed by transfer of methyl groups from L-[*Me*-^{14}C,^{3}H]methionine are shown as $^{14}C^{3}H_3$ and the expected ^{14}C/^{3}H atomic ratios are given in parentheses after the names of the compounds.



Scheme 2. Possible mechanisms of *C*- and *O*-methylation reactions. The example taken for *O*-methylation is a step known to occur in the biosynthesis of ubiquinone by *Rhodospirillum rubrum* (Friis, Daves & Folkers, 1967).

that the remainder was in the methoxyl groups. On the basis of these degradations and the finding that squalene and β-carotene were unlabelled it was concluded that the ^{14}C radioactivity was confined to the ring *C*-methyl (and *O*-methyl for ubiquinone) substituents of all the quinones and chromanols examined, and to the side-chain ethyl substituent of 3β-hydroxy sterols.

Tritium was assumed to be confined entirely to the ^{14}C-labelled methyl groups. Indirect evidence for this comes from the finding that no ^{3}H radioactivity was found in squalene or β-carotene. Further, if ^{3}H were present in other positions it would have undergone a large dilution and should not seriously interfere with our calculations.

In addition to plastoquinone and ubiquinone, γ-tocopherol, α-tocopherol, α-tocopherolquinone and phylloquinone were found to be labelled, showing that these compounds possessed one (γ-tocopherol and phylloquinone) or more (α-tocopherol and α-tocopherolquinone) methyl groups derived from methionine. The finding of radioactivity in phylloquinone shows that the ring *C*-methyl group arises by transmethylation from methionine, as it does in the menaquinone series (see the introduction), and not as a consequence of naphthaquinone ring formation.

The $^{14}C/^{3}H$ ratios (Table 2) for the various compounds are, except for the 3β-hydroxy sterols, all similar. Even though in some cases we were working with extremely small amounts of material, the S.E.M. for any ratio was such that the mean values can be interpreted with confidence.

The $^{14}C/^{3}H$ atomic ratios were calculated by using the 3β-hydroxy sterols as an internal reference. These sterols, consisting predominantly of β-sitosterol and stigmasterol with traces of campesterol, contain ethyl substituents (methyl in campesterol) at positions 24 that are formed by transmethylation from *S*-adenosylmethionine. During these methylation reactions two protons (one for campesterol) are lost from the first methyl group transferred to give sterols with a $^{14}C/^{3}H$ atomic ratio 1:2 (Scheme 1). Experimental support for the above proposals comes from the work of Goad, Hamman, Dennis & Goodwin (1966) and Smith, Goad, Goodwin & Lederer (1967).

Fig. 1 gives the structures of the quinones and chromanols examined and indicates the methyl groups we would expect to be labelled from the methyl group of methionine. With plastoquinone, γ-tocopherol, α-tocopherol and α-tocopherolquinone one methyl group is derived from the β-carbon of tyrosine (Whistance & Threlfall, 1967). The methyl shown as coming from tyrosine is based on the finding that the only monomethyltocopherol found in Nature, δ-tocopherol, has a tyrosine-derived methyl group *meta* to the polyprenyl side

chain. The expected atomic ratios (Table 2 and Fig. 1) are calculated on the basis that *C*-methylation and *O*-methylation involve the transfer of intact methyl groups from methionine, in the form of *S*-adenosylmethionine, to phenolic intermediates (Scheme 2).

The results in Table 2 show that the observed atomic ratios are, except for ubiquinone, in close agreement with the predicted values, confirming that the transmethylation reaction involved in the formation of these compounds involves the transfer of intact methyl groups. The ubiquinone value is somewhat high at 3:9·81, compared with the predicted value 3:9. However, despite several cross-checks on the counting technique this value could not be modified.

The determined $^{14}C/^{3}H$ ratios (1:4, approx.) for compounds formed by transfer of intact methyl groups were lower than the ratio (1:6) of the administered methionine: however, since the methionine was prepared by mixing predetermined volumes from sealed vials supposedly containing known amounts of radioactivity and was never directly assayed for its ^{14}C and ^{3}H content, the latter ratio is probably meaningless.

Finally, it was decided to examine the specific activities of the various compounds in terms of counts/min./μg.atom labelled (Table 2). First, the incorporation of ^{14}C radioactivity into plastoquinone, phylloquinone, ubiquinone and 3β-hydroxy sterols parallels that found with [2-^{14}C]mevalonate, and thus differs markedly from the incorporation pattern with $^{14}CO_2$ (Threlfall *et al.* 1967*b*). On the arguments employed by Threlfall *et al.* (1967*b*) the ^{14}C radioactivity pattern indicates that the methylation reactions associated with phylloquinone and plastoquinone formation take place in the chloroplast, whereas those associated with ubiquinone and 3β-hydroxy sterol synthesis occur elsewhere within the cell. Secondly, the specific activity data suggest that γ-tocopherol may be a precursor of α-tocopherol, and that α-tocopherol and α-tocopherolquinone are in equilibrium with each other. However, this aspect clearly requires further experimentation. Whittle, Audley & Pennock (1967), using [*Me*-^{14}C]methionine, have provided evidence that, if sequential methylations are involved in tocopherol biosynthesis, then in *Hevea* latex tocotrienols rather than tocopherols may be the true intermediates.

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