Observations on the Biosynthesis of Phytoterpenoid Quinone and Chromanol Nuclei

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1. *p*-Hydroxy[U-¹⁴C]benzoic acid, except for loss of the carboxyl group, is effectively incorporated into the nucleus of ubiquinone and an unidentified prenylphenol by maize roots, maize shoots, french-bean leaves, french-bean cotyledons and *Ochromonas danica*. Plastoquinone, α -tocopherol, γ -tocopherol and α -tocopherolquinone are all unlabelled from this substrate. The high radioactivity of the prenylphenol and its behaviour in a pulse-labelling experiment with maize shoots suggested that it may be a ubiquinone precursor. 2. Members of the 2-polyprenylphenol and 6-methoxy-2-polyprenylphenol series, compounds that are known ubiquinone precursors in *Rhodospirillum rubrum*, could not be detected in maize tissues, but possibly they may occur as their glycosides. 3. [G-¹⁴C]Shikimic acid is incorporated into the nuclei of phyloquinone, plastoquinone, α -tocopherolquinone, γ -tocopherol, α -tocopherol and ubiquinone in maize shoots, showing that in plant tissues the nuclei of these compounds arise via the shikimic acid pathway of aromatic biosynthesis. 4. L-[U-¹⁴C]Phenylalanine and L-[U-¹⁴C]tyrosine are incorporated into plastoquinone, γ -tocopherol, α -tocopherolquinone and ubiquinone. α -Tocopherol, which is absent from shoots incubated with L-[U-¹⁴C]tyrosine, is also labelled from L-[U-¹⁴C]phenylalanine. Degradation studies showed that there is little ¹⁴C radioactivity in the terpenoid portions of the molecules and from this it is concluded that the aromatic portions of these amino acids are giving rise to the quinone and chromanol nuclei. 5. It is proposed that in maize the nucleus of ubiquinone can be formed from either phenylalanine or tyrosine by a pathway involving *p*-coumaric acid and *p*-hydroxybenzoic acid. Plastoquinone, tocopherols and tocopherolquinones are formed from tyrosine by some pathway in which the aromatic ring and C-3 of the side chain of this amino acid gives rise to the nucleus and one methyl substituent respectively of these compounds.

Griffiths, Threlfall & Goodwin (1967) reported on the nature, intracellular distribution and formation of terpenoid quinones and chromanols in maize shoots in relationship to chloroplast development. Biosynthetic studies showed that in this tissue the prenyl side chains of plastoquinone, phyloquinone, ubiquinone, α -tocopherol, γ -tocopherol and α -tocopherolquinone are formed from mevalonic acid (Threlfall, Griffiths & Goodwin, 1967*b*; O.A. Dada, unpublished work), and that their nuclear C- and O-methyl groups are formed by a transfer of intact methyl groups from methionine (Threlfall, Whistance & Goodwin, 1967*a*). It was also concluded that the reactions concerned with the biosynthesis of the intraplastidic quinones and chromanols took place within the chloroplast, whereas the extra-

plastidic quinone (ubiquinone) was formed elsewhere. The present investigations are concerned with the biosynthetic origins of the quinone and chromanol nuclei in photosynthetic tissues and possible intermediates involved in the formation of these compounds.

Since the ability to synthesize plastoquinone and related compounds, members of the tocopherol series and the phytol side chain of phyloquinone appears to be restricted to plant tissues, which in general have been little studied, the only terpenoid quinone nuclei to receive attention were those of ubiquinone and menaquinone (naphthaquinones that have the same nucleus as phyloquinone), which are present in animals and micro-organisms. Glover (1965) and Threlfall (1967) have fully reviewed this field, so that it is only necessary to consider here recent developments relevant to our studies.

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Cox & Gibson (1964, 1966) demonstrated that in *Escherichia coli* [G-¹⁴C]shikimic acid was incorporated into ubiquinone-8 and the aromatic portion of the menaquinone-8 nucleus (where the numerals indicate the numbers of isoprene units in the side chain); *p*-hydroxybenzoic acid and protocatechuic aldehyde, or alternatively compounds derived from them, appeared to be intermediates.

Bentley & Lavate (1965) found that in the moulds *Penicillium stipitatum* and *Penicillium brevicompactum* the nuclei of dihydroubiquinone-10 and ubiquinone-9 respectively are not formed via the acetate pathway. Apart from these two observations, which favour biosynthesis of the ubiquinone and menaquinone nuclei by the shikimic acid pathway, little further direct evidence has been presented for the origins of the nuclei of these compounds.

The most fruitful results have come from the use of *p*-hydroxybenzoic acid, which in micro-organisms and higher plants can arise from shikimic acid (Neish, 1964). It has been found for all organisms examined that this compound is an effective precursor of the ubiquinone ring, e.g. in *Rhodospirillum rubrum* (Rudney & Parson, 1963), yeast (Spiller, 1966), rat (Olson *et al.* 1963), *Tetrahymena pyriformis* (Miller, 1965) and *Phycomyces blakesleeanus* (G. R. Whistance, unpublished work). Parson & Rudney (1965) through the use of *p*-hydroxy[U-¹⁴C]benzoic acid initiated investigations that have led to the elucidation of the biosynthetic sequence for ubiquinone in *R. rubrum* (Olsen *et al.* 1965; Olsen, Daves, Moore, Folkers & Rudney, 1966; Friis, Daves & Folkers, 1966). Two of the more easily isolated intermediates are 2-decaprenylphenol and 2-decaprenyl-6-methoxyphenol (Whistance, Threlfall & Goodwin, 1966a). However, as yet this pathway has not been shown to hold for other organisms.

Olson, Dialameh, Bentley, Springer & Ramsey (1965) showed that in the rat (which cannot synthesize aromatic compounds) the aromatic portions of the amino acids tyrosine and phenylalanine, presumably through the formation of *p*-hydroxybenzoic acid, can be incorporated into the ubiquinone nucleus.

In the present investigation we are concerned with demonstrating whether in oxygen-producing photosynthetic organisms: (a) the shikimic acid pathway is operative in the formation of terpenoid quinone and chromanol nuclei; (b) *p*-hydroxybenzoic acid is an effective precursor of ubiquinone and other quinone nuclei; (c) compounds of the 2-polyprenylphenol type are intermediates in quinone biosynthesis; (d) aromatic amino acids, which are known to give rise to C₈–C₁ acids in plant tissues (Neish, 1964), are incorporated into the quinone nuclei.

EXPERIMENTAL

Radiochemicals

L-[U-¹⁴C]Tyrosine hydrochloride (5.5 mc/m-mole) and L-[U-¹⁴C]phenylalanine (15 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks. [G-¹⁴C]Shikimic acid (3.36 mc/m-mole) was purchased from the New England Nuclear Corp., Boston, Mass., U.S.A.

p-Hydroxy[U-¹⁴C]benzoic acid of high specific activity (4.28 mc/m-mole) was prepared by alkaline fusion of L-[U-¹⁴C]tyrosine hydrochloride (Parson & Rudney, 1964). The product was purified by chromatography in all chromatographic systems listed by these workers. The purity of the product was checked by thin-layer chromatography (Kieselgel G developed with benzene–dioxan–acetic acid, 90:25:4, by vol.) coupled with radioautography. The yield of purified product varied between 5 and 35%.

Biological methods

Biological material. Seeds of *Zea mays* (var. Rhodesian White Double Hybrid) and *Phaseolus vulgaris* (var. Carters Lightning Dwarf French Bean) were purchased from Gunsons (Seeds) Ltd., London, E.1, and Carters Tested Seeds, London, S.W. 20, respectively. The culture of *Ochromonas danica* was a gift from Professor S. H. Hutner, Haskins Laboratory, New York, N.Y., U.S.A.

Cultivation of seedlings. Etiolated 6–7-day-old seedlings of maize were grown as described by Griffiths *et al.* (1967). French-bean seeds were soaked in water for 12 hr., sown in soil (John Innes no. 1) and allowed to germinate for 14 days at 26° with continuous illumination.

Culture of *O. danica*. *O. danica* was grown in shake culture on the chemically defined medium of Baker *et al.* (1962), supplemented with yeast extract. The cultures were grown for 4 days at 20–24° in the light.

Exposure of tissues to ¹⁴C-labelled substrates. Etiolated maize shoots (200–500) were excised at the node and the cut ends dipped in water (50 ml./100 shoots) containing the ¹⁴C-labelled substrate. The shoots were then exposed to continuous illumination for 24 hr. under conditions described previously (Griffiths *et al.* 1967; Threlfall *et al.* 1967b).

In experiments involving bean leaves and cotyledons (taken from 6-day-old plants), maize shoots (7-day-old) and roots (14-day-old) and tobacco leaves the tissues were sliced into small pieces with a sharp razor blade, put into 50 ml. conical flasks and moistened with 1 ml. of water containing the ¹⁴C-labelled substrate. The flasks were then incubated at 26° with continuous illumination for 3 or 6 hr.

O. danica was harvested by centrifugation, resuspended in a small volume of water containing *p*-hydroxy[U-¹⁴C]benzoic acid and exposed to light for 3 hr.

Analytical methods

Extraction and preliminary purification of lipid. At the end of the experimental period tissues from higher plants were rinsed thoroughly with water and the lipid was extracted by our routine method (Griffiths *et al.* 1967). Cells of *O. danica* were harvested by centrifugation and the lipid was extracted by the procedure described for *Euglena gracilis* by Threlfall & Goodwin (1967).

The lipids were then resolved into various quinone-containing fractions by column chromatography on acid-washed alumina (Brockmann grade III) (Woelm; anionotropic) developed by stepwise elution with 0.25%, 1%, 3%, 5%, 8%, 12% and 20% (v/v) E/P* as described in detail by Griffiths *et al.* (1967) (see also Table 2). The fractions were evaporated to dryness and, after assay for ^{14}C , quinone (except phylloquinone, which cannot be estimated at this stage), 3 β -hydroxy sterol and β -carotene (Threlfall *et al.* 1967b) were purified further. Members of the 2-polyprenylphenol and 2-polyprenyl-6-methoxyphenol series if present would be eluted by 3% and 1% E/P respectively (Whistance *et al.* 1966a).

Purification procedures. The terpenoids were purified by combinations of column, adsorptive thin-layer and reversed-phase thin-layer chromatography. The sequences and systems used differ from those described by Threlfall *et al.* (1967b) and represent more rigorous purification procedures. Kieselgel G (E. Merck A.-G., Darmstadt, Germany) impregnated with Rhodamine 6G was used for all routine adsorptive thin-layer systems (Griffiths *et al.* 1967). Reversed-phase chromatography was carried out on thin layers of Kieselgel G impregnated with paraffin (Threlfall & Goodwin, 1967); after development the quinones were located by spraying the plate with 0.002% (w/v) sodium fluorescein in ethanol and examining under u.v. light (Whittle, Dunphy & Pennock, 1966). Details of recovery and assay of the various compounds have been reported by Threlfall *et al.* (1967b).

(a) Purification of β -carotene, squalene and phylloquinone. The fraction (0.25% E/P) containing these compounds was chromatographed on a Brockmann grade II alumina column. β -Carotene and squalene were eluted by 1% E/P and phylloquinone by 2% E/P. Phylloquinone was purified further by using the systems shown in Table 1. Squalene and β -carotene were resolved from each other and purified by repeated chromatography on thin layers of Kieselgel G developed with light petroleum (b.p. 40–60°) and benzene–light petroleum (3:97, v/v), respectively.

(b) Purification of plastoquinone, ubiquinone and α -tocopherolquinone. The systems and sequences used for the purification of these compounds are shown in Table 1. The ubiquinone-containing fraction (5% E/P), which also contains γ -tocopherol, has previously proved difficult to chromatograph on thin layers owing to the presence of large amounts of a long-chain saturated alcohol that made effective application of the fraction impossible (Threlfall *et al.* 1967b). This difficulty has now been overcome by dissolving the fraction in a minimum volume of warm light petroleum (b.p. 40–60°) and allowing the solution to stand at 0°. The alcohol is precipitated out and is removed by filtration.

(c) Purification of α - and γ -tocopherol. After chromatography on one adsorptive thin-layer system these chromanols were oxidized with gold chloride (Dilley & Crane, 1963) to their corresponding *p*-benzoquinones before further purification (Table 1).

(d) Purification of unesterified 3 β -hydroxy sterols. These were purified either by chromatography on thin layers developed with ethyl acetate–benzene (3:22, v/v) or by

Table 1. Purification sequences for terpenoid quinones and chromanols

Purification step	Phylloquinone		Plastoquinone		Ubiquinone		α -Tocopherol		γ -Tocopherol		α -Tocopherolquinone	
	System	R_F	System	R_F	System	R_F	System	R_F	System	R_F	System	R_F
1	Chromatographed on grade II alumina column											
2		II	II	0.40	III	0.51	I	0.39	III	0.32	IV or V	0.44 or 0.41
3		VII	VIII	0.30	VIII	0.53 (Q-9); 0.45 (Q-10)	V	0.41	V	0.33	VII	0.75
4		II	III	0.69	IV	0.26 0.70	VII or VI	0.75 0.44 or 0.33	VII	0.85 0.35 or 0.31	V	0.41 0.33

The total lipid extract was chromatographed on a column of acid-washed Brockmann grade III alumina and resolved into various terpenoid-containing fractions (Table 2). The quinones and chromanols were isolated and purified by repeated chromatography on thin layers of Kieselgel G developed with a variety of solvent systems: system I, benzene; system II, benzene–light petroleum (b.p. 40–60°) (2:3, v/v); system III, benzene–chloroform (1:1, v/v); system IV, chloroform; system V, ethyl acetate–benzene (3:22, v/v); system VI, di-isopropyl ether–benzene (3:17, v/v); system VII, reversed phase, aq. 90% (v/v) acetone; system VIII, reversed phase, aq. 95% (v/v) acetone. Q-9 and Q-10, Ubiquinone-9 and -10 respectively.

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

precipitation as, and regeneration from, their digitonides (Threlfall *et al.* 1967b).

(e) Detection of prenylphenols. The 1%- and 3%-E/P fractions were examined for the presence of prenylphenols by chromatography on thin layers of Kieselgel G developed with benzene. The phenols are detected by spraying with 2% (w/v) 2,6-dibromo-*p*-benzoquinone-4-chloramine in methanol followed by overspraying with saturated aq. NaHCO_3 solution. The plates are then dried at 90°. 2-Polyprenylphenols (R_F 0.51) and 6-methoxy-2-polyprenylphenols (R_F 0.65) appear as blue and violet-blue spots respectively.

Ozonolytic degradation of plastoquinone and ubiquinone. The ^{14}C radioactivity in the isoprenoid side chains of plastoquinone and ubiquinone was determined by ozonolytic degradation. The samples (about 200 μg . for ubiquinone and 1 mg. for plastoquinone) without carrier were reductively acetylated (Olson *et al.* 1965) and then degraded (Threlfall *et al.* 1967b). The bis-2,4-dinitrophenylhydrazine derivatives of laevulic aldehyde were assayed in the proportional counter.

Spectrophotometry. Ultraviolet and visible spectra were determined in the Unicam SP.800 spectrophotometer; all absorption maxima and readings at specific wavelengths were checked on the Unicam SP.500 spectrophotometer. β -Carotene, phyloquinone, plastoquinone, ubiquinone, α -tocopherolquinone and α -tocopherol were estimated by the procedures of Threlfall & Goodwin (1967). γ -Tocopherol, as γ -tocopherolquinone, was assayed in ethanolic solution by reduction with NaBH_4 . The $\Delta E_{1\text{cm}}^{1\%}$ (262 m μ) value used was 412.

Radioassay. Details of scintillation counting (used for all samples except the bis-2,4-dinitrophenylhydrazine derivative of laevulic aldehyde), scanning of thin-layer chromatograms and radioautography were as described by Threlfall *et al.* (1967b). All counts were corrected for background and machine efficiency.

The bis-2,4-dinitrophenylhydrazine derivative of laevulic aldehyde was assayed in the proportional counter (Nuclear-Chicago model 4306 Automatic Gas-Flow System; Continental Distributors Ltd., London, S.W. 7). The counts in addition to correction for background and machine efficiency were when necessary corrected to infinite thinness.

Solvents. Light petroleum (b.p. 40–60°), benzene (A.R.) and diethyl ether were dried over sodium wire and redistilled; ether was also distilled over reduced iron immediately before use.

Ethanol and cyclohexane were of spectroscopic grade.

All other solvents were of A.R. or equivalent grade.

RESULTS

Experiments with p-hydroxy[U- ^{14}C]benzoic acid

Maize shoots. Threlfall & Griffiths (1965) found that, on incubation of excised etiolated maize shoots with *p*-hydroxy[U- ^{14}C]benzoic acid in the light, ^{14}C radioactivity was incorporated into ubiquinone but not into plastoquinone, phytosterols or squalene. The ^{14}C radioactivity recovered in the ubiquinone in this experiment failed to account for all the radioactivity in the original ubiquinone-containing fraction (5% E/P), and some radio-

activity was also present in the α -tocopherol-containing (3% E/P) and α -tocopherolquinone-containing (20% E/P) fractions (D. R. Threlfall, unpublished work). It was suggested that the failure to obtain incorporation of *p*-hydroxybenzoic acid into plastoquinone reflected either a specific requirement for ubiquinone biosynthesis, or the failure to penetrate the chloroplast membrane and reach the proposed site of plastidic terpenoid quinone synthesis.

The experiment of Threlfall & Griffiths (1965) was repeated with *p*-hydroxy[U- ^{14}C]benzoic acid of much higher specific activity. This would allow the detection of a very low incorporation into plastoquinone. In addition, it would be possible to carry out a fuller investigation of the α -tocopherol-containing and α -tocopherolquinone-containing fractions, the former of which along with the 1%-E/P fraction would contain if present members of the 2-polyprenylphenol (3% E/P) and 6-methoxy-2-polyprenylphenol (1% E/P) series (Whistance *et al.* 1966a).

Etiolated maize shoots (300) were excised and exposed with continuous illumination to 2 μC of *p*-hydroxy[U- ^{14}C]benzoic acid (4.28 mc/m-mole). [The characteristics of this experimental system are synthesis of β -carotene, phyloquinone, plastoquinone and α -tocopherolquinone, the ubiquinone, α -tocopherol and phytosterol concentrations remaining relatively unaffected (Griffiths *et al.* 1967).] After 24 hr. the lipid was extracted and chromatographed on a column of alumina. Table 2 summarizes the distribution of ^{14}C radioactivity in the fractions and also the concentrations and distribution of the various terpenoids examined.

The distribution of ^{14}C radioactivity suggested that *p*-hydroxybenzoic acid, apart from being incorporated into ubiquinone, might have been incorporated into tocopherols, α -tocopherolquinone and phenols of the 2-polyprenyl and 6-methoxy-2-polyprenyl type.

Ubiquinone was purified by repeated thin-layer chromatography. It was found during this process that, as judged by the specific activity (170 000 counts/min./mg.), thin-layer radioscanning (Fig. 1) and radioautography, in all adsorptive systems tried ^{14}C radioactivity migrated with ubiquinone, which suggested that this was the only labelled component in the fraction. However, on reversed-phase chromatography a sharp fall in specific activity (116 500 counts/min./mg.) indicated the presence of other labelled components containing some 30% of the total radioactivity. Radioscanning (Fig. 1) and radioautography showed the presence of one other labelled component (compound W), which ran ahead of ubiquinone-9. On spraying the plates with reagents specific for quinones or quinols and phenols (Threlfall & Goodwin, 1967) a com-

Table 2. Preliminary chromatography of the total lipid extract from maize shoots incubated with *p*-hydroxy[^{14}C]benzoic acid

Etiolated 7-day-old maize shoots (300) were excised and exposed with continuous illumination to $2\mu\text{C}$ of *p*-hydroxy-[^{14}C]benzoic acid (4.28mc/m-mole) for 24 hr. Total lipid was extracted and chromatographed on a column of Brockmann grade III acid-washed alumina (Woelm) developed by stepwise elution with light petroleum (b.p. $40\text{--}60^\circ$) containing increasing amounts of ether (see the Experimental section for full details). Values in parentheses show the quantities ($\mu\text{g./100}$ maize shoots) of the various components in a typical experiment (for a full discussion of the characteristics of this experimental system, see Griffiths *et al.* 1967).

Fraction (% E/P)	^{14}C radioactivity (counts/min.)	Terpenoids present
0.25	0	Squalene, β -carotene ($100\mu\text{g.}$) and phyloquinone ($66\mu\text{g.}$)
1	0	Plastoquinone ($700\mu\text{g.}$) and sterol esters
3	8780	α -Tocopherol ($220\mu\text{g.}$) and a trace of ubiquinone
5	73750	Ubiquinone ($200\mu\text{g.}$), γ -tocopherol ($50\mu\text{g.}$) and sterol precursors
8	2045	—
12	350	3β -Hydroxy sterols (20mg.)
20	675	α -Tocopherolquinone ($166\mu\text{g.}$)

pound giving a positive quinol/phenol reaction was found in the region corresponding to that of the unknown radioactive compound. The u.v. spectrum of a recovered sample showed absorption maxima at 276 and $283\text{m}\mu$ in cyclohexane. Unfortunately, such small quantities were available that little progress has been made towards identification, although experiments with [$2\text{-}^{14}\text{C}$]mevalonic acid have established its isoprenoid nature. However, compound W is not ubichromenol (which has similar properties on reversed-phase chromatography but is easily separated from ubiquinone by column chromatography), or a prenylphenol of the 2-polyprenyl or 6-methoxy-2-polyprenyl type (which are readily separable from ubiquinone by column and adsorptive thin-layer chromatography).

The radiochemical purity of the ubiquinone recovered from reversed-phase thin layers was verified by preparing the quinol (Dilley, 1964) and running on reversed-phase thin layers (system VII, Table 1), when all the ^{14}C radioactivity migrated with the quinol (Fig. 1).

Examination of the 0.25%, 1%, 3%, 5%, 12% and 20% E/P fractions showed that phyloquinone, plastoquinone, α -tocopherol, γ -tocopherol, unesterified 3β -hydroxy sterols and α -tocopherolquinone were completely unlabelled. Further, on

Fig. 1. Radioscans of the isolation and purification by thin-layer chromatography of ubiquinone from maize shoots incubated with *p*-hydroxy[^{14}C]benzoic acid. The ubiquinone-containing fraction (5% E/P) from column chromatography of the total lipid extract was chromatographed on several adsorptive systems (scan A was obtained with system IV, see Table 1) followed by chromatography on a reversed-phase system (scan B, system VIII, see Table 1). The radiochemical purity of the ubiquinone-9 recovered from the latter system was checked by reduction to the quinol, which was then chromatographed on a reversed-phase system (scan C, system VII, see Table 1). The instrument settings used were: collimator width, $\frac{1}{8}$ in.; time constant, 40 sec.; scanning speed, $1\frac{1}{2}$ in./hr.; count rate 300 (A and B) or 1000 (C) counts/min. The chromatograms were presented for scanning as Neaton strips. Q-9 and Q-10, Ubiquinone-9 and -10; W, compound W (see the text).

examination of the 1% and 3% E/P fractions by thin-layer chromatography followed by radioautography and spraying of the plates with a reagent specific for prenylphenols (see the Experimental section) no fogging or staining of the plates was observed in the region where 2-nonaprenylphenol or 6-methoxy-2-nonaprenylphenol would run (Whistance *et al.* 1966a).

The absence of ^{14}C radioactivity from phytoosterols, the 'skeleton' and supernumerary groups of which are derived from the same precursors as the prenyl side chain and ring and *O*-methyl groups of ubiquinone respectively, leads to the conclusion that *p*-hydroxy[^{14}C]benzoic acid has been incorporated *in toto* (apart from probable loss of the carboxyl group) into the nuclear portion of ubiquinone.

The incorporation of *p*-hydroxy[^{14}C]benzoic

Table 4. *Incorporation of p-hydroxy[U-¹⁴C]benzoic acid into ubiquinone by maize shoots and roots, bean leaves and cotyledons and O. danica*

Higher-plant tissues, with the exception of the etiolated maize shoots, were cut into small pieces, moistened with a few millilitres of water containing the ¹⁴C-labelled substrate and illuminated. The etiolated maize shoots were used intact (see Table 2). *O. danica* was harvested by centrifugation and resuspended in 10 ml. of water containing the ¹⁴C-labelled substrate. All incubations were carried out at 28°. N.D., Not determined.

Biological material	Age (days)	Wet wt. of tissue (g.)	<i>p</i> -Hydroxy[U- ¹⁴ C]-benzoate (4.28 mc/m-mole) used (μc)	Conditions of incubation	Incorporation of ¹⁴ C into ubiquinone+ compound W (% of dose)	Ubiquinone	
						Sp. activity (counts/min./mg.)	Homologue isolated
Etiolated maize shoots	7	300 shoots	2.00	24 hr. in light	1.9	116 500	9
Green maize shoots	7	8	0.10	3 hr. anaerobically in light	0	0	9
Maize roots	14	12	1.00	3 hr. in light	2.2	N.D.	9
Green bean leaves	14	6	0.35	6 hr. in light	3.0	N.D.	10
Bean cotyledons	4	16	0.50	6 hr. in light	2.0	181 500	10
<i>O. danica</i> (light-grown)	4	Cells from 200 ml. of medium	0.50	3 hr. in light	0.4	N.D.	10

Table 3. *Effect of pulse-labelling on the incorporation of p-hydroxy[U-¹⁴C]benzoic acid into ubiquinone by maize shoots*

Etiolated maize shoots (2×200) were excised and exposed to *p*-hydroxy[U-¹⁴C]benzoic acid (4.28 mc/m-mole) for 12 hr. with continuous illumination. At the end of this period one batch (200 shoots) was taken for analysis, and the other batch (200 shoots) was removed from the ¹⁴C-labelled substrate, the bases of the shoots were rinsed and then dipped into unlabelled 1 mM-*p*-hydroxybenzoic acid, and the batch was illuminated for a further 6 hr. before analysis.

	¹⁴ C radioactivity (counts/min.)	
	Shoots exposed to <i>p</i> -hydroxy[U- ¹⁴ C]-benzoate (0.75 μc) for 12 hr.	Shoots exposed to <i>p</i> -hydroxy[U- ¹⁴ C]-benzoate for 12 hr. followed by 6 hr. with unlabelled compound
Ubiquinone+ compound W	14 610	15 590
Ubiquinone	12 400	15 590
Compound W (by difference)	2 210	0

acid into a second compound, apparently of a terpenoid nature, suggested that this might be a precursor of ubiquinone. To examine this further a pulse-labelling experiment was carried out, the details and results of which are summarized in Table 3. The values obtained for the total radioactivities of ubiquinone and compound W both before and after administration of unlabelled

p-hydroxybenzoic acid to shoots that had previously been incubated with *p*-hydroxy[U-¹⁴C]benzoic acid are consistent with the view that compound W is a precursor of ubiquinone; the total radioactivity in compound W falls as the total radioactivity in ubiquinone rises.

Other plant tissues. In addition to maize shoots the incorporation of *p*-hydroxy[U-¹⁴C]benzoic acid into terpenoid quinones by maize roots, bean leaves and cotyledons and *O. danica* was examined. The conditions of incubation and incorporation of ¹⁴C radioactivity into the various fractions examined are given in Table 4. With sliced tissues of higher plants it was possible to obtain incorporations of 2–3% into ubiquinone- (and compound W-) containing fractions within 3–6 hr. With *O. danica* the incorporation was only 0.4%, and this may reflect some permeability barrier at the cell wall similar to the complete barrier the cell wall of *Euglena gracilis* presents to mevalonic acid (Threlfall & Goodwin, 1967). In all cases except bean cotyledons the ¹⁴C radioactivity ran with ubiquinone until reversed-phase chromatography, when radioactivity was observed in ubiquinone and a zone running just ahead of ubiquinone corresponding to compound W. This pattern was observed both in bean leaves and *O. danica*, where the ubiquinone homologue is ubiquinone-10, and in maize roots where ubiquinone-9 is the homologue present, and suggests that two homologues of compound W exist, compound W-10 in the first case and compound W-9 in the second. This observation also offers support for the view that compound W is a precursor of ubiquinone. In bean cotyledons ¹⁴C radioactivity was present mainly in ubiquinone, but on radioautography of the

reversed-phase system a series of bands ahead of ubiquinone was observed, the natures of which are unknown.

The high incorporation of ^{14}C radioactivity into ubiquinone and the absence of radioactivity from other quinones, chromanols and phytosterols again leads to the conclusion that the nucleus of *p*-hydroxybenzoic acid has been incorporated specifically into ubiquinone (and compound W) in all tissues examined.

Experiments with [G- ^{14}C]shikimic acid, L-[U- ^{14}C]tyrosine and L-[U- ^{14}C]phenylalanine

It has been shown in micro-organisms that *p*-hydroxybenzoic acid can be formed by the shikimic acid pathway, probably arising at the level of chorismic acid (Cox & Gibson, 1966). In higher plants *p*-hydroxybenzoic acid can be formed from *p*-coumaric acid, which may be derived, depending on the species of plant, from either phenylalanine (via cinnamic acid) or tyrosine. The aromatic amino acids are of course themselves formed from shikimic acid. It was decided therefore to investigate the incorporation of shikimic acid, L-tyrosine and L-phenylalanine into ubiquinone and other terpenoids by illuminated etiolated maize shoots. The results of a preliminary experiment involving [G- ^{14}C]shikimic acid have already been reported (Whistance, Threlfall & Goodwin, 1966b). In the present experiment the components examined were subjected to more rigorous purification.

Etiolated excised maize shoots were dipped into

solutions of the ^{14}C -labelled substrates and illuminated for 24 hr. At the end of this period the lipids were extracted and examined by our routine procedures. The results of these experiments are given in Table 5.

The presence of some ^{14}C radioactivity in squalene, β -carotene and 3β -hydroxy sterols indicates that on metabolism shikimic acid, tyrosine and phenylalanine provide metabolites that can enter the terpenoid biosynthetic sequences. However, it is apparent from the extremely low specific activities of the 3β -hydroxy sterols and β -carotene compared with those of the quinones and chromanols that the nuclear portions of administered compounds are incorporated *in toto* into the terpenoid quinones and chromanols. To check this the plastoquinone and ubiquinone samples were all subjected to ozonolytic degradation to determine the ^{14}C radioactivity in the side chain (Table 6). In no case was significant ^{14}C radioactivity detected in the bis-2,4-dinitrophenylhydrazine derivative of laevulic aldehyde, which arises from the terpenoid portion of the molecule.

DISCUSSION

In agreement with work on other organisms (see the introduction) *p*-hydroxybenzoic acid was found to be readily incorporated into the nucleus of ubiquinone in a variety of plant tissues. The most striking result was the extremely effective incorporation (ranging from 0.4% in *O. danica* to 3.0% in bean leaves) of this compound into ubiquinone over short

Table 5. *Incorporation by maize shoots of [G- ^{14}C]shikimic acid, L-[U- ^{14}C]tyrosine and L-[U- ^{14}C]phenylalanine into terpenoid quinones and chromanols*

Etiolated 7-day-old maize shoots were excised and exposed with continuous illumination to the appropriate ^{14}C -labelled substrate for 24 hr. N.D., Not determined

Terpenoid	Sp. activity (counts/min./ μmole)		
	[G- ^{14}C]Shikimic acid (3.36 mc/m-mole): 500 shoots incubated with 10 μC	L-[U- ^{14}C]Tyrosine (5.5 mc/m-mole): 200 shoots incubated with 20 μC	L-[U- ^{14}C]Phenylalanine (15 mc/m-mole): 200 shoots incubated with 20 μC
Plastidic			
β -Carotene	0	0	13
Phylloquinone	3935	N.D.	0
Plastoquinone	2828	3855	814
α -Tocopherol	1365	Absent	448
γ -Tocopherol	3460	9720	1128
α -Tocopherolquinone	1874	4420	491
Extraplastidic			
Squalene	0	Trace	Trace
3β -Hydroxy sterols	6	63	30
Ubiquinone	1322	7440	4510

Table 6. *Ozonolytic degradation of plastoquinone and ubiquinone samples isolated from maize shoots exposed to [G-¹⁴C]shikimic acid, L-[U-¹⁴C]tyrosine or L-[U-¹⁴C]phenylalanine*

Purified samples of plastoquinone and ubiquinone were reductively acetylated and subjected to a scheme of ozonolytic degradation (see the Experimental section for details). The laevulic aldehyde arising from degradation of the prenyl side chain was isolated and purified as its bis-2,4-dinitrophenylhydrazine derivative and assayed for ¹⁴C radioactivity.

Sample	Substrate	Sp. activity (counts/ min./mg.)	Details of the degradation			
			Amount degraded		¹⁴ C radioactivity in prenyl side chain*	
			Wt. (mg.)	¹⁴ C radioactivity (counts/min.)	(counts/min.)	(% of total radioactivity in molecule)
Plastoquinone	[G- ¹⁴ C]Shikimic acid	3780	2.1	7938	27	0.3
	L-[U- ¹⁴ C]Tyrosine	5150	1.2	6180	36	0.6
	L-[U- ¹⁴ C]Phenylalanine	1088	1.0	1088	24	2.0
Ubiquinone	[G- ¹⁴ C]Shikimic acid	1660	0.42	697	11	1.6
	L-[U- ¹⁴ C]Tyrosine	9340	0.18	1681	53	3.2
	L-[U- ¹⁴ C]Phenylalanine	5660	0.21	1188	42	3.5

* Calculated by using the equation: ¹⁴C radioactivity in prenyl side chain (counts/min.) = $9 \times$ specific activity (counts/min./μmole) of laevulic aldehyde derivative $\times$ μmoles of quinone degraded. The yield of purified laevulic aldehyde derivative in all cases fell within the range 60–75% of the theoretical.

periods of time (3–6 hr.) and the complete absence of ¹⁴C radioactivity from all other terpenoid quinones and chromanols examined. The results throw no light on the orientation of *p*-hydroxybenzoic acid with respect to the ubiquinone nucleus, although the knowledge that in maize tissues the side chain and nuclear *C*-methyl group are derived from mevalonic acid (Threlfall *et al.* 1967b) and methionine (Threlfall *et al.* 1967a) respectively leads to the conclusion that the carboxyl group is lost during the synthesis. The failure to find radioactivity in other terpenoid quinones and chromanols is, in the light of recent work with [3-¹⁴C]tyrosine (G. R. Whistance & D. R. Threlfall, unpublished work), not unexpected and probably reflects a specific requirement of *p*-hydroxybenzoic acid for ubiquinone biosynthesis (Scheme 1), rather than the failure of this compound to penetrate the chloroplast membrane and reach the site of synthesis of plastidic terpenoid quinones. This also explains why, on incubation of *p*-hydroxy[U-¹⁴C]benzoic acid with bean chloroplasts isolated by non-aqueous procedures, no metabolic transformation that might be associated with plastidic quinone biosynthesis can be demonstrated (G. R. Whistance, unpublished work).

Despite an intensive search no evidence could be found for the presence of 2-polyprenylphenols or 6-methoxy-2-polyprenylphenols, the decaprenyl forms of which are known ubiquinone-10 precursors in *R. rubrum* (Olson *et al.* 1965, 1966), in any tissue examined, although the possibility exists that these

compounds could occur as their glycosides, in which case our experimental techniques would not reveal them. However, in all tissues examined a compound with phenolic properties and possibly a prenyl side chain was detected, which from its relatively high specific activity and behaviour in a pulse-labelling experiment (Table 3) could be a ubiquinone precursor.

In maize tissues [G-¹⁴C]shikimic acid, L-[U-¹⁴C]tyrosine and L-[U-¹⁴C]phenylalanine were incorporated into all terpenoid quinones and chromanols examined. The incorporation into the fractions investigated was low compared with that from *p*-hydroxybenzoic acid, i.e. 0.25%, 0.15% and 0.06% for shikimic acid, tyrosine and phenylalanine respectively. However, this could be due to a combination of factors, e.g. number of plants taken in each experiment, cell-wall permeability and chloroplast permeability. Degradation studies on plastoquinone and ubiquinone and comparative studies with other terpenoids, e.g. 3β-hydroxy sterols, led to the conclusion that the nuclear portions of these substrates had been incorporated into the nuclear portions of the quinones and chromanols.

Thus shikimic acid can be regarded as the specific distal precursor of the nuclei of phyloquinone, plastoquinone, α-tocopherol, γ-tocopherol, α-tocopherolquinone and ubiquinone. Which part of the naphthaquinone ring of phyloquinone was labelled was not investigated.

The results with [U-¹⁴C]tyrosine and [U-¹⁴C]-

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