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# BIOSYNTHESIS OF TOCOPHEROLS: A RE-EXAMINATION OF THE BIOSYNTHESIS AND METABOLISM OF 2-METHYL-6-PHYTYL-1,4-BENZOQUINOL

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**Key Word Index**—*Lactuca sativa*; Compositae: *Spinacia oleracea*; Chenopodiaceae; biosynthesis; 2-methyl-6-phytyl-1,4-benzoquinone; 2,3-dimethyl-5-phytyl-1,4-benzoquinone; tocopherols.

**Abstract**—A number of previous studies of the involvement of 2-methyl-6-phytyl-1, 4-benzoquinol in the biosynthesis of α-tocopherol have failed to take account of the fact that this quinol and its quinone have very similar chromatographic properties to those of 2-methyl-3-phytyl-1,4-benzoquinol and 2-methyl-3-phytyl-1,4-benzoquinone respectively. It has now been shown that the two quinones can be separated from each other either by multidevelopment TLC or by HPLC and that the claims made earlier with regard to the biosynthesis and metabolism of 2-methyl-6-phytyl-1,4-benzoquinol in chloroplasts are correct. In particular, it has been established that this quinol is the only methyl phytylbenzoquinol formed from homogentisate and phytyl pyrophosphate in chloroplast preparations. It has also been shown for the first time that lettuce chloroplasts are able to synthesize $^3$H-labelled α- and γ-tocopherols from [*methylene*-$^3$H] homogentisate.

## INTRODUCTION

Schultz and his co-workers [1–4] have presented considerable experimental data which at first sight seem to show that, in spinach chloroplasts, the synthesis of α-tocopherol from homogentisate is carried out by the chloroplast envelope and proceeds by the pathway outlined in Scheme 1. It is now clear, however, that they failed to show unequivocally that the isomeric forms of the products of the first three reactions are indeed the ones depicted in the scheme. Their failure to characterize the products properly is entirely attributable to the fact that they believed that the three isomeric forms of phytyltoluquinone (**3**–**5**)† could be separated by adsorptive TLC [1], whereas, in practice, 2-methyl-6-phytyl-1,4-benzoquinone (**4**) cochromatographs with 2-methyl-3-phytyl-1,4-benzoquinone (**5**) in the systems quoted [5]. The use of the incorrect chromatographic data by the German group meant that in the characterizations of the quinols (as the corresponding quinones) and tocopherols, they failed to differentiate between 2-methyl-6-phytyl- and 2-methyl-3-phytyl-1,4-benzoquinone (**4** and **5**), between 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**6**) and its isomer 2,5-dimethyl-3-phytyl-1,4-benzoquinone (**8**) and between γ-tocopherol (γ-T, **20**) and 5,7-dimethyltocol (**21**) in their studies on the phytylation of homogentisate [3, 4] and the methylation of what, unbeknown to them, was a mixture of 2-methyl-6-phytyl- and 2-methyl-3-phytyl-1,4-benzoquinol [1, 2].

In our own investigations into the biosynthesis of tocopherols, we have shown that lettuce chloroplasts are able to synthesize 2,3-dimethyl-5-phytyl-1,4-benzoquinol (**2**) from homogentisate via the formation of 2-methyl-6-phytyl-1,4-benzoquinol (**1**) [6]. However, we obtained no evidence for the involvement of these compounds in the biosynthesis of lettuce tocopherols. The presence of the label in **1** (isolated as its quinone, **4**) was established by the demonstration that the δ-[$^3$H]tocopherol (δ-T, 18) obtained on chemical cyclization (Scheme 2) of a mixture of HPLC 'pure' $^3$H-labelled and unlabelled (carrier) **4** had the same specific radioactivity as the starting mixture. In retrospect, however, it appears that the unlabelled carrier quinone could have contained some 15% 2-methyl-3-phytyl-1,4-benzoquinone (**5**) and that the specific radioactivity of the δ-[$^3$H]T should have shown an 18% increase in specific radioactivity, unless, of course, the $^3$H-labelled **4** contained some 15% $^3$H-labelled **5**.

In this paper we report on (a) the spectroscopic properties and the HPLC and TLC separation of the three phytyltoluquinones (**3**–**5**) and (b) the re-examination of the products formed from homogentisate and phytyl pyrophosphate and from each of the isomers of phytyltoluquinol and *S*-adenosylmethionine (SAM) by chloroplasts of lettuce and spinach. It must be stressed that although labelled quinones were isolated in the biogenetic studies the true intermediates are the corresponding quinols from which the quinones are formed by oxidation in the course of the extraction procedures.

## RESULTS

While studying the biosynthesis of a newly reported putative intermediate in the formation of 2-methyl-6-phytyl-1,4-benzoquinol (**1**) from homogentisate and phytyl pyrophosphate [7], we observed that on HPLC of a synthetic phytyltoluquinone mixture on a regenerated,

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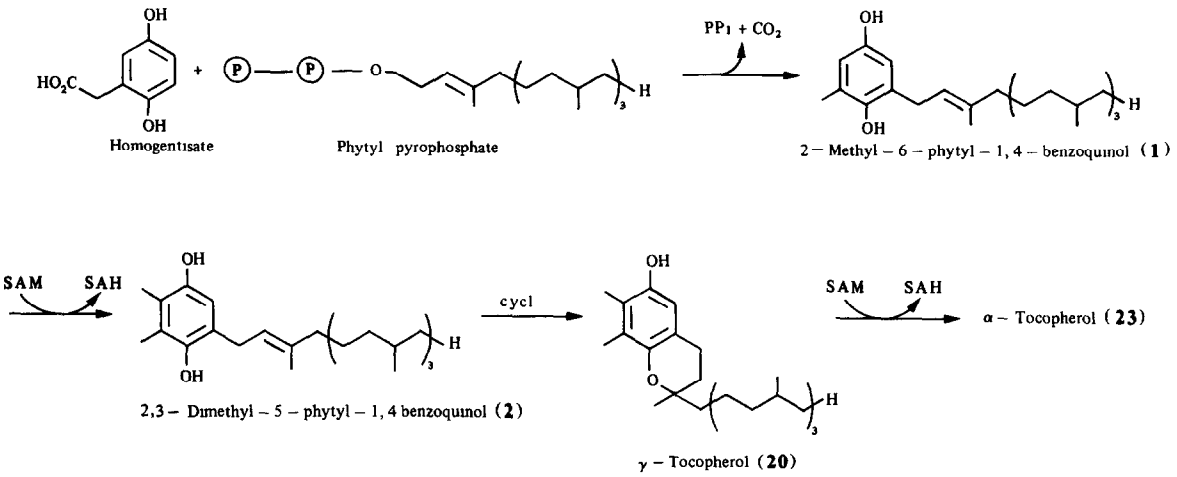
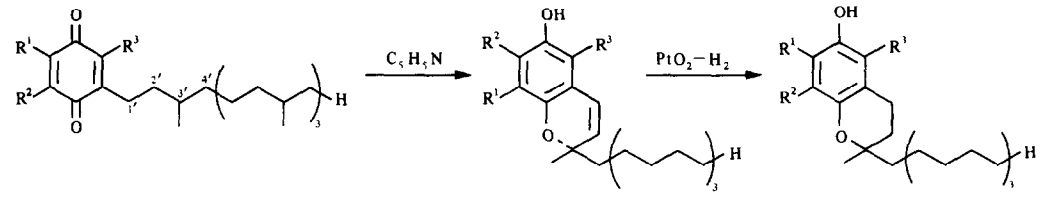
†Unless stated otherwise the phytyl side chain is in the *trans* configuration.




Scheme 1. Proposed pathway for the biosynthesis of α-tocopherol from homogentisate in spinach chloroplasts [1].




| R¹ | R² | R³ | | Quinone | Chromenol | Chromanol |
|---|---|---|---|---|---|---|
| Me | H | H | 3 | 2 – Methyl – 5 – phytyl – 1, 4 – benzoquinone | 10 | 17 7 – Methyltocol |
| H | Me | H | 4 | 2 – Methyl – 6 – phytyl – 1, 4 – benzoquinone | 11 | 18 8 – Methyltocol (δ – tocopherol) δ–T |
| H | H | Me | 5 | 2 – Methyl – 3 – phytyl – 1, 4 – benzoquinone | 12 | 19 5 – Methyltocol |
| Me | Me | H | 6 | 2, 3 – Dimethyl – 5 – phytyl – 1, 4 – benzoquinone | 13 | 20 7, 8 – Dimethyltocol (γ – tocopherol) γ–T |
| Me | H | Me | 7 | 2, 6 – Dimethyl – 3 – phytyl – 1, 4 – benzoquinone | 14 | 21 5, 7 – Dimethyltocol |
| H | Me | Me | 8 | 2, 5 – Dimethyl – 3 – phytyl – 1, 4 – benzoquinone | 15 | 22 5, 8 – Dimethyltocol (β – tocopherol) β–T |
| Me | Me | Me | 9 | 2, 3, 5 – Trimethyl – 6 – phytyl benzoquinone | 16 | 23 5, 7, 8 – Trimethyltocol (α – tocopherol) α–T |

Scheme 2. Structures, nomenclature and abbreviations.

prepacked column of LiChrosorb Si 60 5 μm (Merck) 2-methyl-6-phytyl-1,4-benzoquinone (**4**) was well resolved from 2-methyl-3-phytyl-1,4-benzoquinone (**5**) (e.g. Fig. 1). In addition, it was found that the two isomers were easily separated from each other by triple development TLC on silica gel developed with either diisopropyl ether–petrol (40–60°, 1:9) or dibutyl ether–petrol (40–60°, 1:9) (Table 1). The percentage composition of the phytyltoluquinone mixture was **3**, 36; *cis*-**3**, 12; **4**, 33; *cis*-**4**, 12; **5**, 6; *cis*-**5**, 2.

### *Characterization of the three isomeric forms of phytyltoluquinone*

HPLC- and UV [log ε ($\lambda_{max}$, cyclohexane) *ca* 4.25]-pure samples of the *trans* isomers of 2-methyl-6-phytyl- and 2-methyl-5-phytyl-1,4-benzoquinone (**4** and **3**) and an 85% pure sample of the *trans* isomer of 2-methyl-3-phytyl-1,4-benzoquinone (**5**) were isolated as yellow oils from a crude mixture of *cis* and *trans* isomers of the three quinones (Experimental) by triple development TLC. The isolation of substantial amounts of good quality samples of the three quinones was aided greatly by the use of a TLC applicator. However, despite repeated TLC it was not possible to remove a UV-absorbing impurity ($R_f$ 41 min, system 5, Table 2) from the sample of 2-methyl-3-phytyl-1,4-benzoquinone (**5**).

The mass spectra of the three *trans* isomers were identical and contained the expected peaks at *m/z* 400 (11%) $[M]^+$ and 175 (46%) ($[C_{11}H_{11}O_2]^+$, pyrylium ion) [8, 9]. The UV spectra, however, showed small but distinct differences with regard to the shapes and $\lambda_{max}$ values of the absorption bands (Table 1). The $^1$H NMR spectra (Table 2) contained the expected signals for compounds of this type. The spectra of **3** and **4**, the two isomers which each have a ring proton *ortho* to the phytyl side chain, were very similar. In both spectra, each of the two ring protons and the nuclear *C*-methyl group gave rise to a multiplet signal (Table 2). The spectrum of **5**, however, differed markedly from those of its two isomers (Table 2). Thus the two ring protons, which are now *ortho* to each other, gave rise to a single singlet signal, as did the nuclear *C*-methyl group which is now *ortho* to the side chain. In addition, the signals due to H-4′ and H-2′ were shifted

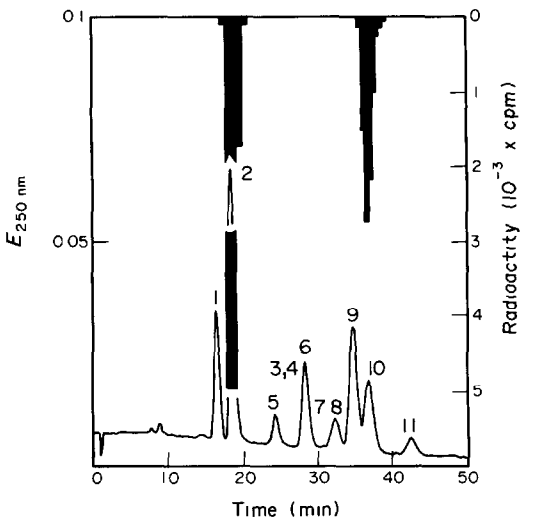



Fig. 1. Radio-HPLC of $^3$H-labelled phytyl-substituted quinones. A portion ($23 \times 10^3$ dpm) of the $^3$H-labelled quinone fraction recovered from TLC of the lipid extract of the incubaton mixture containing spinach chloroplasts, [$^3$H]homogentisate and phytyl pyrophosphate (Table 4) was taken up in 30 $\mu$l of cyclohexane containing synthetic **6** and a mixture of phytyltoluquinones supplemented with **5** and 20 $\mu$l subjected to radio-HPLC (system 5, Table 3). In the example chosen some 95% of the radioactivity injected onto the column was recovered in 2-methyl-6-phytyl- and 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**4** and **6**). 1, *cis*-**6**; 2, **6**; 3, 7; 4, **8**; 5, *cis*-**3**; 6, **3**; 7, *cis*-**5**; 8, *cis*-**4**; 9, **5**; 10, **4**; 11, contaminant in **5**

Table 1 UV spectral properties and TLC separation of phytyltoluquinones

| | UV $\lambda_{max}^{cyclohexane}$ nm | | | $R_f$† | |
|---|---|---|---|---|---|
| Quinone | max | shoulder | P* | Silica gel triple developed with dibutyl ether–petrol (40–60°) (1.9) | |
| **3** | 253 | 261 (sh/peak) | 5.3 | *cis* | 0.76 |
| | | | | *trans* | 0.71 |
| **4** | 254 | 260 | 5.5 | *cis* | 0.68 |
| | | | | *trans* | 0.63 |
| **5**‡ | 249 | 259 | 4.8 | *cis* | 0.62 |
| | | | | *trans* | 0.58 |

*P, Persistence ($\lambda_{max}/\lambda_{222\,nm}$)

†Although not quite as good, a similar separation of the *cis*/*trans* isomers was obtained with diisopropyl ether–petrol (40–60°) (1·9). Compound **4** was not separated from **5** on silica gel triple developed with $C_6H_6$, $C_6H_6$–petrol (40–60°) (2:3), $C_6H_6$–$CHCl_3$ (1 1) or $Et_2O$–petrol (40–60°) (1:9)

‡UV data obtained on an HPLC purified sample.

upfield whilst those due to $H_2$-1′ and the 3′-methyl group were shifted downfield.

Final confirmation of the substitution patterns of the quinones was provided by the demonstrations that on cyclization followed by catalytic hydrogenation each quinone gave the expected chromanol (UV, TLC and colour reactions, and HPLC).

Table 2. $^1$H NMR data for the phytyltoluquinones (250 MHz, $CDCl_3$, TMS as int. standard)

| H* | Integral value† | **3** | **4** | **5** |
|---|---|---|---|---|
| R,R = H | 2 | 6.58 *m* | 6.54 *m* | 6.71 *s* |
| | | 6 51 *m* | 6.48 *m* | |
| 2′ | 1 | 5.14 *t* | 5.14 *t* | 4.93 *t* |
| 1′ | 2 | 3 11 *d* | 3 13 *d* | 3.21 *d* |
| R = Me | 3 | 2 03 *d* | 2.06 *d* | 2 04 *s* |
| 4′ | 2 | 2 01 *t* | 2.01 *t* | 1.96 *t* |
| 3′-Me | 3 | 1 54 *s* | 1.54 *s* | 1.73 *s* |
| Rest of side chain | | | | |
| >CH– | 3 | 1.43 *s* | 1.43 *s* | 1.54 *s* |
| $-CH_2-$ | 16 | 1 25 *m* | 1.25 *m* | 1 26 *m* |
| –Me | 12 | 0.86 *d* | 0.86 *d* | 0.87 *d* |

*The numbering of the side chain is given in Scheme 2.

†The integral values for **3** and **4** were as expected, as were those for **5** after allowance was made for the $-CH_2-$ and Me absorptions of the impurity in the sample.

*Chromatographic properties of quinones and chromanols*

Apart from the data given in our previous publication [6], there has been no comprehensive summary of the HPLC and TLC properties of these compounds, other than the brief and, in parts, inaccurate summary of the relative mobilities on TLC of the marker compounds used for the purification and identification of labelled intermediates in their incubation mixtures [1]. This deficiency in the literature is here rectified in Table 3.

*Biosynthetic studies*

*Experiments with* [methylene-$^3$H]*homogentisate.* A series of incubations were carried out in which chloroplast preparations of lettuce or spinach were incubated with [$^3$H]homogentisate in the presence or absence of either isopentenyl pyrophosphate (IPP) or phytyl pyrophosphate (Table 4). The lipid extracts from the various incubation mixtures were examined by radio-TLC (system 1, Table 3) and by radio-HPLC (systems 4–6, Table 3) of either the quinone ($R_f$ 0.35–0.60) and chromanol ($R_f$ 0.10–0.35) zones obtained from TLC (system 1, Table 3) of the lipid extracts or the 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone, plastoquinone-9, 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**6**), 2-demethylplastoquinone-9 to 2-methyl-3-phytyl-1,4-benzoquinone (**5**), α-T and γ-T zones from TLC. To facilitate the analysis of TLC fractions containing both nonaprenyl- and phytyl-substituted quinones, the fractions were subjected to radio-HPLC in system 4 (Table 3) and the 2-methyl-6-phytyl- and 2-methyl-3-phytyl-1,4-benzoquinones (**4** and **5**), which were not well resolved from each other, collected as a single fraction and subjected to radio-HPLC in system 5 (Table 3). In agreement with a previous study [6] most of the radioactivity in the lipid extracts was distributed between compounds which on TLC co-chromatographed with plastoquinone-9, 2-

Table 3. TLC and HPLC properties of quinones, quinols and tocopherols

| Compound | $R_f$ | | | $R_t$ (min) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Silica gel | | Paraffin-impregnated silica gel G [8] | LiChrosorb Si60 5 μm (125 × 4 mm, Merck)§ | | | ODS Hypersil 5 μm (150 × 4.6 mm)¶ | |
| | | | | % Dioxane in isooctane | | | | |
| | $C_6H_6$* (System 1) | $Et_2O$–petrol (1.9)† (System 2) | $Me_2CO$–$H_2O$ (9:1)‡ (System 3) | 0.06 (System 4) | 0.025‖ (System 5) | 1 (System 6) | MeCN–$H_2O$** (System 7) | MeOH–$H_2O$ (4:1) (System 8) |
| PQ-9 | 0.68 | 0.44 | 0.02 | 21 | 71 | — | 29.5 | — |
| Trimethylphytyl-benzoquinone | 0.64 | 0.48 | 0.12 | — | 14 | — | — | — |
| **6** | 0.63 | 0.42 | 0.20 | 9 | 19 | — | 15.5 | 11.2 |
| 2′,3′-dihydro-**6** | 0.63 | — | — | Runs with *c*-**6** | (Fig. 1) | — | — | — |
| **7** | 0.56 | 0.43 | 0.25 | 12 | 25 | — | 15.5 | 11.2 |
| 2-DPQ-9 | 0.56 | 0.35 | 0.04 | 41 | — | — | — | — |
| **8** | 0.55 | 0.41 | 0.26 | 13 | 27 | — | 15.5 | 11.2 |
| **3** | 0.55 | 0.40 | 0.33 | 18 | 28 | — | 12 | 7.6 |
| **4** | 0.50 | 0.37 | 0.35 | 14 | 36 | — | 12 | 7.6 |
| **5** | 0.49 | 0.35 | 0.38 | 14 | 34 | — | 12 | 7.6 |
| α-T | 0.39 | 0.15 | 0.56 | — | — | 12 | 14 | 8.8 |
| 5,7-Dimethyltocol | 0.32 | — | 0.62 | — | — | 14.5 | 12 | 7.6 |
| Trimethylphytyl-benzoquinol | 0.32 | 0.10 | 0.89 | — | — | 12 | — | |
| $PQH_2$-9 | 0.32 | 0.10 | 0.79 | — | — | 12 | 22.5 | — |
| γ-T | 0.25 | 0.10 | 0.72 | — | — | 24.5 | 12 | 7.6 |
| β-T | 0.25 | — | 0.72 | — | — | 22.5 | 12 | 7.6 |
| Quinol of **6** | 0.24 | 0.10 | 0.93 | — | — | 24.5 | 6.5 | 3.5 |
| δ-T | 0.17 | 0.07 | 0.83 | — | — | 40.5 | — | — |
| $DPQH_2$-9 | 0.17 | 0.07 | 0.89 | — | — | — | — | — |
| Quinols of: **7** | 0.16 | — | 0.93 | — | — | — | — | — |
| **8** | 0.15 | — | 0.93 | — | — | — | — | — |
| **3** | 0.14 | — | 0.95 | — | — | — | — | — |
| **4** | 0.13 | 0.06 | 0.95 | — | — | — | — | — |
| **5** | 0.13 | — | 0.95 | — | — | — | — | — |

*Rhodamine 6G-impregnated silica gel H (Merck) [8]

†Silica gel HF, TLC-ready-foil (Schleicher and Schüll). The order of migration of some of the compounds differs markedly from those reported in ref. [1].

‡Compounds visualized by spraying with rhodamine 6G in $Me_2CO$.

§1.1 ml/min.

‖Regenerated column.

¶2 ml/min.

**MeCN–$H_2O$: 4:1, 5 min; 4:1 → 9:1, 5 min; 9:1, 5 min; 9:1 → MeCN, 5 min; MeCN, to end.

Table 4. Incorporation of radioactivity from [$^3$H]homogentisate into quinones and tocopherols in chloroplast suspensions

| Supplements | Radioactivity ($10^{-3}$ × dpm)* | | | | | |
|---|---|---|---|---|---|---|
| | **4** | **6** | γ-T | α-T | 2-DPQ-9† | PQ-9† |
| Experiment 1. Lettuce chloroplasts | | | | | | |
| None | 4.3 | 11.2 | 0 | 0 | 0 | 0.4 |
| IPP | 10.1 | 15.5 | Tr(?) | 0 | 10.5 | 5.6 |
| Phytyl pyrophosphate | 21.2 | 42.8 | Tr(?) | 0 | 0 | 0.6 |
| Experiment 2. Spinach chloroplasts | | | | | | |
| None | 3 3 | 1.2 | 0 | 0 | 0 | 0.3 |
| IPP | 23 4 | 34.1 | Tr(?) | 0 | 6.2 | 11.4 |
| Phytyl pyrophosphate | 61 6 | 47.9 | Tr(?) | 0 | 0 | 0.9 |
| Experiment 3. Lettuce chloroplasts | | | | | | |
| None | 7.0 | 2 0 | 0 | 0 | 0.1 | 0 |
| SAM | 0 | 21 2 | 8.3 | 0.8 | 0 | 0 3 |
| SAM, IPP | 0 | 37 1 | 13 2 | 3.3 | 0 | 10.2 |

In experiments 1 and 2 each incubation mixture (total vol. 1 ml) contained: suspension buffer, pH 7.6, chloroplast preparation (0.5 mg chlorophyll), 10 μmol $MgCl_2$, [$^3$H]homogentisate (2 μCi) and either 0.5 μmol $Li_3IPP$ or 0.1 μmol $(NH_4)_3$ phytyl pyrophosphate. The mixtures were incubated in 10 ml test tubes with constant shaking and illumination (1350 μmol/m$^2$/sec) at 20° for 30 min. In experiment 3 each incubation mixture (total vol. 3 ml) contained: suspension buffer, pH 7.6, chloroplast preparation (0.3 mg chlorophyll), 10 μmol $MgCl_2$, [$^3$H]homogentisate (2 μCi), ± 1 μmol $Li_3IPP$ and 0 5 μmol SAM. The mixtures were incubated in 25 ml conical flasks under the conditions just described. To provide enough $^3$H-labelled material for characterization studies, each incubation was performed in duplicate or triplicate. The results obtained for the replicates which were analysed were essentially the same as those recorded in the table.

*Experiments 1 and 2: 488 × $10^3$ dpm ≡ synthesis of 1 pmol of product/hr/mg chlorophyll; Experiment 3: 293 × $10^3$ dpm ≡ 1 pmol of product/hr/mg chlorophyll.

†2-DPQ-9, 2-demethylplastoquinone-9; PQ-9, plastoquinone-9

demethylplastoquinone-9, 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**6**) and 2-methyl-6-phytyl-1,4-benzoquinone (**4**) (plus **5**). More importantly, however, radio-HPLC analysis supported the previous claims [3, 4, 6] that the only labelled phytylquinones produced in the various reaction mixtures were 2-methyl-6-phytyl- and 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**4** and **6**) (e.g. Fig. 1). HPLC-purified $^3$H-labelled **4** also cochromatographed with the single mass peak given by an isomeric mixture of phytylquinones (**3**–**5**) on reversed-phase HPLC (system 6, Table 3) and, after reduction (sodium borohydride), with 2-methyl-6-phytyl-1,4-benzoquinol (**1**) (system 7, Table 3). In addition, on TLC of the products obtained on cyclization (Scheme 2) of a mixture of the radioactive quinone and an isomeric mixture of synthetic phytyltoluquinones the radioactivity migrated with the chromenol of **4** (**11**). The identity of the radioactive **6** was confirmed by reversed-phase HPLC, by reduction to the corresponding quinol and by cyclization and reduction to form γ-T (**20**).

The addition of SAM, as expected [6], resulted in the accumulation of **6** at the expense of **4** (Table 4). It also led to the appearance of $^3$H-labelled γ- and α-T (**20** and **23**) in some experiments. The identities of γ- and α-T, chromanols which on TLC and adsorptive HPLC have the same chromatographic properties as 2,3-dimethyl-5-phytyl-1,4-benzoquinol (**2**) and plastoquinol-9 respectively (Table 3), were established by reversed-phase HPLC followed by ordinary phase HPLC of the tocopherol fractions recovered from TLC. In the reversed-phase system (system 7, Table 3) used the γ-T was well resolved from **2** and any **6** formed by oxidation of **2** on elution of the gel with ether and α-T was well resolved from plastoquinol-9 and plastoquinone-9. In fact very little radioactivity was associated with quinols showing that they had been oxidized almost completely to quinones in the course of the extraction of the lipids from the incubation medium. Adsorptive HPLC (system 6, Table 3) established that the dimethyltocol was γ-T and not β- (**22**) or 5,7-dimethyl tocol (**21**).

*Experiments with phytyltoluquinols.* Spinach chloroplasts were prepared and incubated with [Me-$^{14}$C]SAM and samples of each of the phytyltoluquinols (prepared from our purest samples of phytylquinones) and two analogues of 2-methyl-6-phytyl-1,4-benzoquinol under the same conditions as those employed by Soll and Schultz [1], except that the quinol-forming reaction mixtures were not purified before use. The incubation mixtures were examined by procedures similar to those just described under (a) (Table 5).

It was found that $^3$H-labelled 2,3-dimethyl-5-phytyl-1,4-benzoquinone (**6**), γ-T (**20**) and α-T (**23**) were accumulated in the incubation mixture supplemented with 2-methyl-6-phytyl-1,4-benzoquinol (**1**). Compound **6** was also accumulated in the reaction mixture supplemented with the quinol of **3** whilst small amounts of **7** were formed in the incubation supplemented with the quinol of **5**. The incubations containing *cis* and 2′,3′-dihydro forms of **1** produced *cis*- and 2′,3′-dihydro-**6** respectively.

All of the extracts contained $^{14}$C-labelled compounds

Table 5. Incorporation of radioactivity from [Me-$^{14}$C]SAM into dimethyl-substituted quinones and tocopherols in spinach chloroplast suspension supplemented with quinols

| Quinol (0.5 mM) | Radioactivity ($10^{-3}$ × dpm)* Unknown† | 6 | γ-T | α-T |
|---|---|---|---|---|
| Control | 4.9 | 0 | 0 | 0 |
| **1** | 5.9 | 27.9 | 7.1 | 0.9 |
| **3** | 6.1 | 3.9 | 0 | 0 |
| **4** | 8.7 | 1.8 (**7**) | 0 | 0 |
| *cis*-**1** | 8.6 | 3.8 (*cis*-**6**) | 0 | 0 |
| 2′,3′-dihydro-**1** | 10.8 | 7.5 (2′,3′-dihydro-**6**) | 0 | 0 |

Each incubation mixture (total vol. 1 ml) contained 0.91 ml of spinach chloroplast suspension (1.8 mg chlorophyll/ml), pH 7.6, 50 μl of [Me-$^{14}$C]SAM.$H_2SO_4$ (1 μCi), 50 μl 0.2 M $NaHCO_3$ and either 20 μl of EtOH containing a crystal of $NaBH_4$ or 20 μl of EtOH containing 0.5 μmol quinol plus reacted and unreacted $NaBH_4$. The additions were made in the order described. The mixtures were incubated in 10 ml test tubes with constant shaking and illumination (1350 μmol/m$^2$/sec) at 20° for 30 min.

*$109 \times 10^3$ dpm ≡ synthesis of 1 nmol of **6** or γ-T/hr/mg chlorophyll or 0.5 nmol of α-T/hr/mg chlorophyll.

†See text for TLC and HPLC properties.

which on TLC (system 1, Table 3) migrated with the same $R_f$ values as **7** and **8**. On radio-HPLC (system 5, Table 3), the $^{14}$C-activity was eluted as two major peaks ($R_t$ 10 and 12) just prior to the mass peak of *cis*-**4** and a minor peak ($R_t$ 20 min) just after the mass peak of *trans*-**4**. Soll and Schultz [1] made no reference to the presence of these compounds in their incubation mixtures, although Professor Schultz (personal communication) has reported that they were present.

## DISCUSSION

It has been shown that 2-methyl-6-phytyl-1,4-benzoquinone (**4**) can be separated from 2-methyl-3-phytyl-1,4-benzoquinone (**5**) either by multidevelopment TLC or by HPLC (Tables 1 and 3). The successful separation of these two quinones from each other by HPLC on a commercial column which for some two years from new had failed to resolve the two isomers from each other appears to be attributable to an improvement in the resolving power of the column brought about in the course of its regeneration.

The results of the two types of biogenetic experiment (Tables 4 and 5 and Fig. 1) reported in this paper have confirmed the claims of previous workers [1–4, 6]. In particular, they show that 2-methyl-6-phytyl-1,4-benzoquinol (**1**) is the only detectable product of the phytylation of homogentisate in chloroplast preparations of lettuce and spinach and that 2,3-dimethyl-5-phytyl-1,4-benzoquinol (**2**) is the first detectable methylation product of **1**. They also show that although **2** is the only possible product which can be formed by the sequential operation of the first two enzymes on the pathway outlined in Scheme 1, the *C*-methylase whilst most active with **1** can convert 2-methyl-5-phytyl-1,4-benzoquinol to **2**, 2-methyl-3-phytyl-1,4-benzoquinol to 2,6-dimethyl-3-phytyl-1,4-benzoquinol, *cis*-**1** to *cis*-**2** and 2′,3′-dihydro-**1** to 2′, 3′-dihydro-**2**. The formation of **2** from 2-methyl-5-phytyl-1,4-benzoquinol is perhaps somewhat surprising, since all of the other conversions involve *C*-methylation of the nuclear *C*-atom *para* to the side chain.

It was also shown for the first time that lettuce chloroplasts are able to form $^3$H-labelled α- and γ-T from [$^3$H]homogentisate and SAM. In support of a precursor–product relationship between **2** and γ- and α-T, it was confirmed that spinach chloroplasts supplemented with **1** and [Me-$^{14}$C] SAM are able to form γ- and α-T in addition to **2**. It must be stressed, however, that whilst the synthesis of **2** from homogentisate and phytyl pyrophosphate via **1** appears to be beyond doubt as a result of a pulse chase experiment [6] and the experiments reported in this paper, the formation of α- and γ-T from **2** still awaits unequivocal confirmation. On balance, however, all the available data are consistent with the pathway outlined in Scheme 1.

## EXPERIMENTAL

*Radiochemicals.* *S*-Adenosyl-L-[Me-$^{14}$C]methionine (60 μCi/μmol) and [*methylene*-$^3$H] homogentisate (0.88 Ci/μmol) were purchased from The Radiochemical Centre, Amersham, Bucks, UK. The [$^3$H]homogentisate was purified before use by TLC [6].

*Chemicals.* The quinones and chromanols used in this study were either synthesized [6] or obtained from the sources listed in ref. [6] $Li_3IPP$ and phytyl pyrophosphate $(NH_4)_3$ were synthesized by the methods described in refs. [6] and [10] respectively. All organic solvents were redistilled before use.

*Separation of* cis- *and* trans-*phytyltoluquinones.* A sample (140 mg) of a crude mixture of *cis*- and *trans*-phytyltoluquinones (**3**–**5**) prepared by condensation of phytol with toluquinol in dioxane in the presence of $BF_3$-etherate [6] was subjected to TLC on rhodamine 6G-impregnated 0.5mm silica gel H plates developed with $Et_2O$–petrol (2·23). To ensure that discrete and straight bands were obtained the samples were applied to the gel by means of a commercial TLC applicator in this and all subsequent TLC separations of the phytyltoluquinones After

development, the phytyltoluquinones (50 mg) were recovered from the gel with $Et_2O$ and rechromatographed on $Et_2O$-washed (by development) rhodamine 6G-impregnated silica gel H plates which were triple developed with either diisopropyl ether–petrol (40–60°) (2:23) or $Bu_2O$–petrol (40–60°) (2:23). After development ($R_f$ values, Table 1), **5**, **4** plus small amounts of *cis*-**5**, **3** plus some *cis*-**4** and *cis*-**3** were recovered from the plate with $Et_2O$ and rechromatographed in the same system to give samples of each of the three *trans* isomers (**3**, 7.5 mg; **4**, 4.7 mg; **5**, 3 mg) of phytyltoluquinone and *cis*-**3** (2 mg). In the case of **3**, the front of the band was taken in order to obtain a sample free from *cis*-**4**. The TLC, HPLC, UV, MS and $^1H$ NMR properties of the three *trans* isomers of phytyltoluquinone are given in the Results section and in Tables 1–3. *Cis*-**4** was obtained by TLC of an old sample of **4** in which some 12% of the *trans* isomer had isomerized to the *cis* isomer as a result of prolonged storage and periodic exposure to light.

*Preparation of 2′,3′-dihydro-***4**. This compound (UV $\lambda_{max}$ 255 nm (cyclohexane); MS *m/z* 402$[M]^+$) *was prepared by catalytic hydrogenation of* **4** under the same conditions as those used in the preparation of 2′,3′-dihydro-**6** [8].

*Conversion of quinones to chromenols and chromanols.* The quinone (or mixture of quinones) was converted into its chromenol by refluxing in $C_5H_5N$[9]. The chromenol (or mixture of chromenols) was then converted into its chromanol (monomethyltocol or dimethytocol) by catalytic hydrogenation ($PtO_2$–$H_2O$) [8]. The TLC properties ($R_f$ and colour reaction on spraying with 0.2% (w/v) Fast Blue in EtOH followed by aq. 5% (w/v) KOH of the three monomethyl chromenols and chromanols (**10–12**, **17–19**) on silica gel H developed with $Et_2O$–petrol (4:1) were as follows: **11**, 0.29, brown; **10**, 0.33, orange-brown; **12**, 0.38, red; **18**, (δ-tocopherol) 0.29, brown; **17**, 0.34, orange-brown; **19**, 0.39, red. The TLC and HPLC properties of the dimethyltocols (**20–22**) are given in Table 3.

*Biological material.* Lettuce (*Lactuca sativa* L. var Bizet or Ostinata) plants and spinach (*Spinacia oleracea* L. hybrid 102) seedlings were grown from seed at the Botanic Gardens, University of Hull.

*Isolation of chloroplasts.* Lettuce chloroplasts were prepared from lettuce plants by the method outlined in ref. [6], with the modification that the period of homogenization was reduced from 90 sec to 2 × 10 sec. Spinach chloroplasts were prepared from 7- to 10-day-old seedlings by the method of ref. [11] as modified by ref. [12].

*Incubation mixtures.* The various incubation mixtures used in these studies are given in Tables 4 and 5. In the incubations containing phytyltoluquinols, the phytyltoluquinol was formed by reduction of the appropriate amount of phytyltoluquinone in 20 μl of EtOH with a small crystal of $NaBH_4$. The reaction mixture was used without purification and was the last addition made to the incubation mixture

*Analysis of incubation mixtures.* The lipids were extracted from the incubation mixtures with $Me_2CO$ from which they were then partitioned into petrol [1]. In the cases of the incubations containing [Me-$^{14}$C]SAM 50 μg each of α- and γ-T and 4 μg of **6** were added to the $Me_2CO$ extract. The petrol soluble lipids along with the appropriate marker compounds were then subjected to TLC on either rhodamine 6G-impregnated silica gel H or silica gel $HF_{254+366}$ developed with $C_6H_6$. After development, the plates were examined under a UV (254 nm) lamp to locate the positions of the marker quinones and either the quinone ($R_f$ 0.30–0.60)- and tocopherol ($R_f$ 0.19–0.30)-containing areas or the individual quinone- and tocopherol-containing bands from the plate eluted with $Et_2O$. The $Et_2O$ extracts, after addition of appropriate amounts of authentic samples of quinones (1–4 μg) and, if appropriate, chromanols (10–20 μg), were then subjected to HPLC in one or more of the systems listed in Table 3. The effluent from the column was monitored at either 254 nm in the case of quinones or 280 nm in the case of tocopherols. The effluent from the monitor was collected at intervals of 1 min between mass peaks and 30 sec over the mass peaks and the individual fractions assayed for radioactivity in a liquid scintillation counter [6]. In the case of the fractions collected on reversed-phase HPLC the solvent was removed prior to radioassay.

The recovery of radioactivity from HPLC was usually in excess of 95% of the amount injected onto the column. The chemical conversions of mixtures of $^{14}$C- or $^3$H-labelled and unlabelled quinones to their corresponding chromenols and chromanols were carried out by the methods outlined above.

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