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INCORPORATION OF SHIKIMIC ACID INTO ISOPRENOID QUINONES AND CHROMANOLS BY MAIZE SHOOTS

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Abstract-The patterns of incorporation of DL-[1,2-^{14}C]shikimic acid into plastoquinone-9, a-tocopherol, γ-tocopherol, a-tocopherolqumone and ubiquinone-9 by maize shoots have been investigated. The results showed that C-l, C-2 and C-6, C-3 and C-5 and C-4 of shikimic acid give rise to C-l, C-2 and C-6, C-3, and C-5 and C-4 in nlastoquinone-9 and a-toconherolauinone: C-4. C-3 and C-5. C-2 and C-6 and C-l in ubiquinone-9; and C-9, C-8 and C-10, C-5 and C-7 and C-6 in a-tbcopherol and γ-tocopherol.

INTRODUCTION

WHISTANCE, Threlfall and Goodwin[1,2] demonstrated that shikimic acid is a precursor of the p-benzoquinone nuclei of plastoquinone-9, a-tocopherolquinone and ubiquinone-9 and the aromatic nuclei of a-tocopherol and y-tocopherol in higher plants. Although the outlines of the pathways for the biosynthesis of plastoquinone-9, a-tocopherol, y-tocopherol, a-tocopherolquinone and ubiquinones from shikimic acid by higher plants have been elucidated (Scheme 1),[2–6] the exact orientations of the nuclear carbon skeleton of shikimic acid in the assembled quinone and chromanol molecules is not known.[3,4]

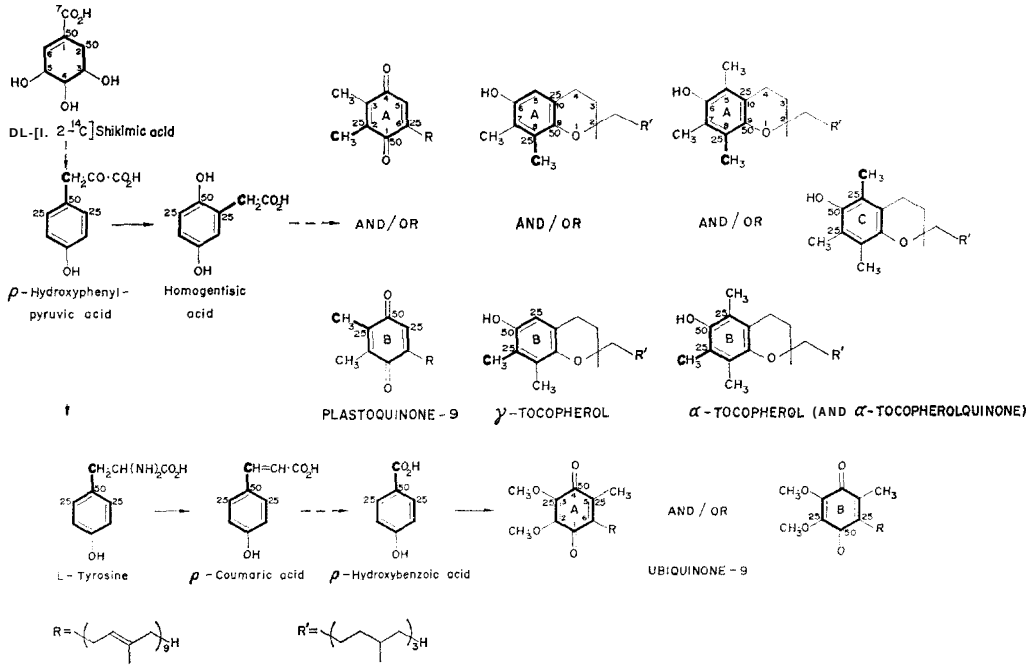
Reference to Scheme 1 shows that two possible orientations exist for each class of compound. Thus the four groups of carbon atoms of shikimate, C-l, C-2 and C-6, C-3 and C-5 and C-4 could give rise to C-l, C-2 and C-6, C-3 and C-5 and C-4 and/or C-4, C-3 and C-5, C-2 and C-6 and C-l of the p-benzoquinones and C-6, C-5 and C-7, C-8 and C-10 and C-9 and/or C-9, C-8 and C-10, C-5 and C-7 and C-6 of the benzochromanols.

The investigations reported in the present paper were carried out to determine which of these orientations are found in the quinones from maize. The approach employed was to examine the patterns of incorporations of DL-[1,2-^{14}C]—shikimic acid into plasto-quinone-9, a-tocopherol, y-tocopherol, a-tocopherolquinone and ubiquinone-9 by excised, greening, etiolated maize shoots. This involved Kuhn-Roth oxidation of the ^{14}C labelled compounds after isolation.

RESULTS AND DISCUSSION

Six-hundred 7-day-old etiolated maize shoots were incubated for 24 hr under continuous illumination with 50 μc of DL-[1,2-^{14}C]shikimic acid. At the end of the incubation period the lipids were extracted from the shoots and fractionated by column and thin-layer chromatography (Table 1).

[1] G. R. WHISTANCE, D. R. THRELFALL and T. W. GOODWIN, *Biochem. Biophys. Res. Commun. 23,849 (1966).*
[2] *G.* R. WHISTANCE, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* 105, 145 (1967).
[3] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. Biophys. Res. Commun. 28,295 (1967).*
[4] *G.* R. WHISTANCE and D. R. THRELFALL. *Biochem. J.* 109, 577 (1968).
[5] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. J.* 109, 482 (1968).
[6] *G.* R. WHISTANCE and D. R. THRELFALL, *Biochem. J. 117, 593 (1970).*



SCHEME 1. POSSIBLE PATTERNS OF INCORPORATION OF DL-[1,2-^{14}C]SHIKIMIC ACID INTO PLASTOQUINONE-9, α-TOCOPHEROL, γ-TOCOPHEROL, α-TOCOPHEROLQUINONE AND UBIQUINONES IN HIGHER PLANTS.

The bold lines and carbon atoms represent the parts of the moleculesderived from the nuclear precursors.A,B and C represent the three possible incorporation patterns. The distribution of radioactivity (percentage of total in molecule) in individual carbon atoms is given on the formulae.

TABLE 1. INCORPORATION OF DL-[1,2-^{14}C]SHIKIMIC ACID INTO ISOPRENOID QUINONES AND CHROMANOLS &CAROTENE AND 4-DEMETHYLSTEROLS BY MAIZE SHOOTS

Column fraction (% E/P)*	^{14}C Radioactivity (dis/min)	Terpenoids examined	Amount (μmoles)	Specific radioactivity (dis/min/μmole)
0.25	8600	β-Carotene	2.86	30
		Phylloquinone	1·09	4840
1	31,080	Plastoquinone-9†	5.61	4860
3	33,666	a-Tocopherol	2.96	1783
5	14,600	Plastochromanol-8	0·33	50
		γ-Tocopherol	1.97	5500
		Ubiquinone-9‡	1.5	1457
12	4920	4-Demethylsterols	136	3
20	1885	a-Tocopherolquinone	1	1600

* Percentage of diethyl ether in light petroleum (b.p. 40–60°).

† Plastoquinone-8 (0·03 μmole) was also present.

‡ Ubiquinone-12 (trace), -11 (0.14 μmole), -10 (0.14 μmole), -8 (0·06 μmole) and -7 (trace) were also present.

The radioactivity present in the column chromatographic fractions was low, being only 0·85 per cent of the dose administered. However, as in a previous experiment with D-[G-^{14}C]-shikimic acid,[2] much of it was associated with plastoquinone-9, a-tocopherol, y-tocopherol, cc-tocopherolquinone and ubiquinone-9 (Table 1). A combination of ozonolytic, Kuhn-Roth and Schmidt degradations of plastoquinone-9 and comparisons[2,4] of the specific radioactivities of plastoquinone-9, a-tocopheroi, y-tocopherol, a-tocopherolquinone and ubiquinone-9 with those of p-carotene and 4-demethylsterols established that in all cases it was present almost entirely (> 98 per cent) in the nuclear carbon skeleton.

The labelling patterns in the nuclear carbon skeletons were determined by subjecting samples of plastoquinone-9, 5-methylperhydroplastoquinone-9 (synthesized chemically from [^{14}C]plastoquinone-9), a-tocopherol (natural), a-tocopherol (synthesized chemically from γ-[^{14}C]tocopherol), y-tocopherol, a-tocopherolquinone and ubiquinone-9 to Kuhn–Roth oxidation. Reference to Table 2 shows that approximately 25 per cent of the radioactivity in ubiquinone-9 was in C-5, approximately 25 per cent of that in plastoquinone-9 and y-tocopherol was in C-2 or C-3 and C-7 or C-8 respectively, and that approximately 25 per cent of that in a-tocopherol and a-tocopherolquinone was in C-5 or C-7 or C-8 and C-2 or C-3 or C-5 respectively.

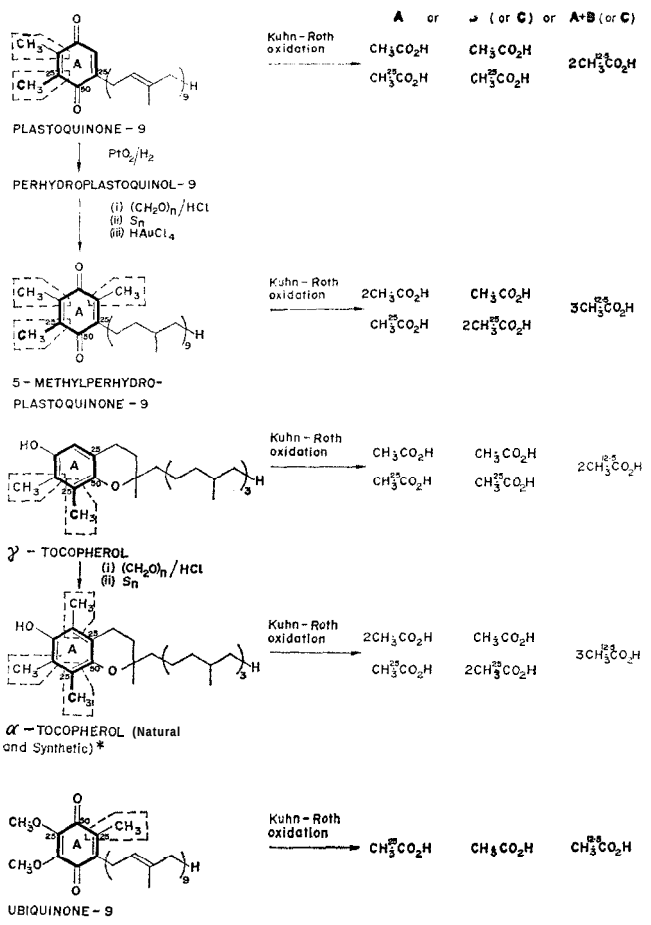
When considered in conjunction with Schemes 1 and 2 these findings establish that C-l, C-2 and C-6, C-3 and C-5 and C-4 of shikimic acid give rise to C-l, C-2 and C-6, C-3 and C-5 and C-4 in plastoquinone-9 and a-tocopherolquinone, C-4, C-3 and C-5, C-2 and C-6 and C-l in ubiquinone-9 and C-9, C-8 and C-10, C-5 and C-7 and C-6 in a-tocopherol and

TABLE 2. KUHN-ROTHOXIDATIONS: LABELLING PATTERNS A, B AND C ARE SHOWN IN SCHEMES 1 AND 2

	Kuhn-Roth oxidation				
		Radioactivity in acetic acid (percentage of total radioactivity in molecule)			
			Theory		
Sample	Amount degraded (dis/min)	Found	A	B (or C)	A + B (or C)
Plastoquinone-9*	10,223	23·5†	25	25	25
5-Methylperhydro-plastoquinone-9 (from plastoquinone-9)	5920	22.3	25	50	37·5
a-Tocopherol (natural)	4820	22.1	25	50	37·5
a-Tocopherol (from y-tocopherol)	3825	23	25	50	37.5
γ-Tocopherol	4200	21.5	25	25	25
a-Tocopherolquinone	1496	21.4	25	50	37.5
Ubiquinone-9	1660	21	25	0	12.5

* Ozonolytic degradation of a sample of plastoquinone-9 (5110 dis/min) showed that not more than 1·5% of the total radioactivity was in the isoprenoid side chain.

† Schmidt degradation of the recovered acetic acid showed that the radioactivity was present entirely in the carboxyl carbon atom.



SCHEME 2. KUHN–ROTH OXIDATIONS OF PLASTOQUINONE-9, 5-METHYLPERHYDROPLASTOQUINONE-9, a-TOCOPHEROL, γ-TOCOPHEROL, α-TOCOPHEROLQUINONE AND UBIQUINONE-9 FROM MAIZE SHOOTS ADMINISTERED DL-[1,2-^{14}C]SHIKIMIC ACID.

The labelling patterns A, B and C are given in Scheme 1. The distribution of radioactivity (percentage of total in molecule) in individual carbon atoms is given on the formulae.

γ-tocopherol. Further, they show that, as proposed by Whistance and Threlfall,[3–6] in plastoquinone-9, a-tocopherol, y-tocopherol and a-tocopherolquinone the a-carbon atom ofhomogentisic acid gives rise to the nuclear methyl group *meta* to the polyprenyl side chain.

EXPERIMENTAL

Radiochemicals

DL-[1,2-^{14}C]Shikimic acid (8.7 mc/m-mole) containing equal amounts of radioactivity in C-l and C-2 was purchased from Calbiochem, Los Angeles, California, U.S.A.

Incubation of Maize Shoots with DL-[1,2-^{14}C]*Shikimic Acid*

Shoots from 600 etiolated 7-day-old maize seedlings (*Zea mays* var. Rhodesian White Horse Tooth) were incubated (by dipping the cut ends into 300 ml of H_2O containing the radiosubstrate) with 50 μc of DL-[1,2-^{14}C]shikimic acid for 24 hr at 28" under continuous illumination (300 L/ft^2).[2,4]

Extraction and Fractionation of Lipids

The lipids were extracted by our routine procedure.' A preliminary fractionation of the extract into various isoprenoid quinone-and chromanolcontaining fractions was carried out bychromatographing it on a column of acid-washed alumina (Brockmann grade III, Woelm, anionotropic) developed by stepwise elution with Et_2O in light petroleum (see Table 1).[8] Phylloquinone, plastoquinone-9, plastochromanol-8, α-tocopherol, y-tocopherol, a-tocopherolquinone, ubiquinone-9, β-carotene and 4-demethylsterols were isolated from the appropriate column fractions by adsorptive and reversed-phase TLC.[2,6]

Chemical Conversion of γ-[^{14}C]Tocopherol to α-[^{14}C]Tocopherol

The procedure employed was a two step synthesis involving chloromethylation (Step 1) and reduction (Step 2).

Step 1. Chloromethylation of γ-[^{14}C]tocopherol. A mixture of 0·47 mg γ-[^{14}C]tocopherol (6000 dis/min), 10 mg unlabelled y-tocopherol and 50 mg paraformaldehyde was dissolved in 15 ml of dry Et_2O and gassed for 1·5 hr with dry HCl.

Step 2. Reduction of 5-chloromethyl-γ-[^{14}C]tocopherol. The reaction mixture from Step 1 was treated with granulated Sn and allowed to stand for 30 min at R.T. The resulting α-[^{14}C]tocopherol was extracted from the mixture as follows: The ethereal phase was diluted with light petroleum (2 vol.), washed (H_2O) until free of HCl, then evaporated to dryness under N_2. The residue was chromatographed on TLC on Silica gel G developed with benzene–$CHCl_3$ (1: 1, v/v). α-[^{14}C]Tocopherol (R_f 0·5) was recovered from the layers by eluting with Et_2O. The yield of purified product was 64%.

Chemical Conversion of Plastoquinone-9 to 5-*Methylperhydro*[^{14}C]*plastoquinone-9*

The procedure employed was a four step synthesis involving catalytic hydrogenation (Step 1), chloro methylation (Step 2), reduction (Step 3) and oxidation (Step 4).

Step 1. Catalytic hydrogenation of [^{14}C]Plastoquinone-9. A mixture of 1·58 mg [^{14}C]plastoquinone-9 (10,223 dis/min) and 10 mg unlabelled plastoquinone-9 was dissolved in 10 ml of cyclohexane–EtOH (1: 1, v/v) and shaken for 1 hr in the presence of H_2 and PtO_2 (5 mg). At the end of this time the mixture was filtered and the solvent evaporated under N_2.

Steps 2 *and 3.* Chloromethylation of Perhydro[^{14}C]plastoquinol-9 and Reduction of 5-Chloromethyl-perhydro[^{14}C]plastoquinol-9. These were carried out under conditions identical to those described above for the conversion of γ-[^{14}C]tocopherol to α-[^{14}C] tocopherol.

Step 4. Oxidation of 5-Methylperhydro[^{14}C]plastoquinol-9. This was carried out with chloroauric acid.[9] The product, 5-methylperhydro[^{14}C]-plastoquinone-9, was purified by TLC on Silica gel G developed with diisopropylether-light petroleum (b.p. 40–60°) (1:9, v/v) (R_f 0.8). The yield of purified 5-methylperhydro [^{14}C]plastoquinone-9 was 58 per cent.

Chemical Degradations

(a) *Kuhn-Roth oxidations of* [^{14}C]*-labelled* 5-*Methylperhydroplastoquinone-9, Plastoquinone-9, a-Tocopherol, y-Tocopherol, a-Tocopherolquinone and Ubiquinone-9.* The samples were each made up to 10 mg with the appropriate unlabelled material and then subjected to Kuhn-Roth oxidation under the conditions described by Threlfall *et al.*[10] On completion of the oxidation, [^{14}C]acetic acid was removed from the reaction mixture by steam distillation, purified and assayed for ^{14}C radioactivity.[10]

(b) *Schmidt Degradation of* [^{14}C]*Acetic Acid obtained by Kuhn-Roth oxidation of* [^{14}C]*Plastoquinone-9.* This was carried out in the manner described by Threlfall *et al.*[10]

(c) Ozonolytic Degradation of [^{14}C]*Plastoquinone-9.* The procedure used was that described by Whistance *et al.*[2]

Quantitative Assay

Phylloquinone, plastoquinone-9, a-tocopherol, y-tocopherol, a-tocopherolquinone, ubiquinone-9, β-carotene and 4-demethylsterols were assayed by our routine procedures.[6] Plastochromanol-8 was estimated from its extinction at 295 nm in cyclohexane by using an $E^{1\%}_{1\,cm}$ value of 56."

Radioassay

Details of scintillation counting and proportional counting were as described by Whistance *et al."* All counts were corrected for background and instrument efficiency.

[7] W. T. GRIFFITHS, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J. 103, 589* (1967).
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[10] D. R. THRELFALL, G. R. WHISTANCE and T. W. GOODWIN. *Biochem. J.* 106.107 (1968).
[11] K. J. WHITTLE, P. J. DUNPHY and J. F. PENNOCK, *Biochem. J. 96, 17C* (1965).

Solvents

Light petroleum (b.p. 40–60°) and Et_2O were dried (Na-Pb alloy) and redistilled; Et_2O was also distilled over reduced Fe immediately before use. Spectroscopic solvents were of the appropriate grade; all other solvents were of AnalaR or equivalent grade.

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