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STUDIES ON THE BIOSYNTHESIS OF THE TOCOPHEROLS IN HIGHER PLANTS

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Abstract—The incorporation of label from (methyl-^{14}C)L-methionine and either "A"-tritio NADPH (4R-diastereoisomer) or "B"-tritio NADPH (4S-diastereoisomer) into the tocopherols and tocotrienols formed when leaves and latex from *Ficus elasticus* were allowed to metabolize δ-tocotrienol was investigated. A stereospecific transfer of tritium label from "A"-tririo NADPH (4R-diastereoisomer) was detected but no one pathway of hydrogenation and methylation was shown to be paramount during the biosynthesis of α-tocopherol from δ-tocotrienol although methylation appears to precede hydrogenation.

INTRODUCTION

THE AROMATIC carbon atoms and the δ-methyl carbon atom of plastoquinone, α-tocopherol and α-tocopherylquinone have been demonstrated to be derived from exogenous tyrosine,[1] whilst the remaining nuclear methyl groups are formed by the transfer of methyl groups from L-methionine[2] in the form of *S*-adenosylmethionine. Whistance and Threlfall postulated these compounds might be synthesized by the pathway: prephenic acid → *p*-hydroxyphenylpyruvic acid → homogentisic acid (possibly as its β-glucoside) → homoarbutin → plastoquinones and tocopherols.[1] Supporting evidence for this theory has been presented to showing both *p*-hydroxyphenylpyruvic acid and homogentisic acid to be involved in plastoquinone biosynthesis.[3, 4]

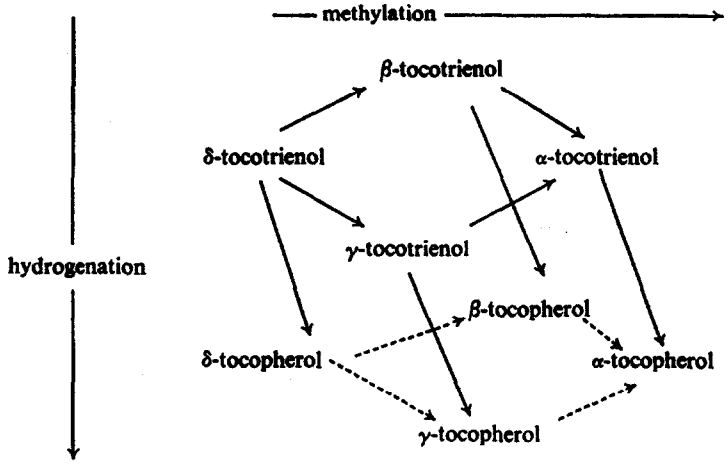
Pennock, Hemming and Kerr suggested the final stages of α-tocopherol biosynthesis involve the methylation of δ-tocotrienol to β- or γ-tocotrienol, a further methylation to α-tocotrienol, followed by a reduction to α-tocopherol; if other tocopherols occur then saturation occurs at the required step[5] (solid lines, Scheme I). A study of the incorporation of the label from (methyl-^{14}C)L-methionine by *Hevea brasiliensis* latex into the tocotrienols and α-tocopherol[6] failed to provide evidence that this possible route was the major pathway for α-tocopherol biosynthesis. Theoretically the members of the tocotrienols and tocopherols constitute a metabolic grid[7] providing six alternative pathways of hydrogenation and methylation for the biosynthesis of α-tocopherol from δ-tocotrienol (solid and dashed lines, Scheme I).

Whistance and Threlfall suggest tocopherol biosynthesis is complicated by precursor–product relationships as they find γ-tocopherol to have higher specific radioactivity than

[1] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. Biophys. Res. Commun.* **28**, 295 (1967).
[2] D. R. THRELFALL, G. R. WHISTANCE and T. W. GOODWIN, *Biochem. J.* **106**, 107 (1968).
[3] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. J.* **109**, 482 (1968).
[4] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. J.* **109**, 2577 (1968).
[5] J. F. PENNOCK, F. W. HEMMING and J. D. KERR, *Biochem. Biophys. Res. Commun.* **17**, 524 (1964).
[6] K. J. WHITTLE, B. G. AUDLEY and J. F. PENNOCK, *Biochem. J.* **103**, 21C (1967).
[7] J. D. BU'LOCK, *The Biosynthesis of Natural Products*, p. 88, McGraw-Hill, London (1965).

α-tocopherol during labelling experiments[2, 4, 8] and possibly some of the tocotrienols and tocopherols are not true intermediates but produced as a result of side reactions.[4] The same authors also point out that there are other possible biosynthetic sequences which could account for the biosynthesis of tocopherol. It is possible for the nucleus to acquire additional methyl substituents before the introduction of the isoprenoid side-chain. Alternatively, the tocotrienols and the tocopherols may be biogenetically distinct, the former derived from geranyl–geranyl pyrophosphate and the latter from phytyl pyrophosphate.

In a preliminary investigation, a stereospecific transfer of hydrogen from the nicotinamide ring of NADPH (tritium label from the 4R-diastereoisomer) to the isoprene residues of phytol and α-tocopherol was detected during biogenesis.[9] This paper describes the results of experiments with stereospecifically tritium labelled NADPH and (methyl-^{14}C)L-methionine in an attempt to elucidate the major pathway of biosynthesis of α-tocopherol in the leaves and latex of *Ficus elasticus* when allowed to metabolize δ-tocotrienol.



SCHEME I. (AFTER WHISTANCE AND THRELFALL[4].)

RESULTS AND DISCUSSION

To eliminate dilution errors arising from existing pools of tocopherols and tocotrienols, a parallel incubation of detached *Ficus* leaves without the addition of labelled compounds or δ-tocotrienol was carried out and the amounts of tocopherol and tocotrienol subtracted from those obtained from the radioactive incubations. The amounts of tocopherols and tocotrienols detected and the amounts of each due to the addition of δ-tocotrienol are shown in Table 1. The percentage recovery of tocopherol and tocotrienol due to the addition of δ-tocotrienol was approximately 60 per cent. No tocotrienols were detected under normal conditions of incubation.

It was not possible to distinguish between the amounts of tocopherols and tocotrienols in the leaves and the latex exudate in the incubation medium because of the necessity to allow for the pool of endogenous tocopherols and the variation in the flow of exudate. Consequently the site of biosynthesis in this instance remains obscure and it is not possible to state if the

[8] G. R. WHISTANCE, D. R. THRELFALL and T. W. GOODWIN, *Biochem. J.* **105**, 145 (1967).
[9] A. R. WELLBURN, *Phytochem.* **7**, 1523 (1968).

latex or other parts of the leaf are responsible for the observed metabolism of the δ-tocotrienol. It may well be that two or more possbile sites exist in higher plants and evidence has been

TABLE 1. AMOUNTS OF TOCOPHEROLS AND TOCOTRIENOLS DETECTED IN DETACHED (*Ficus elasticus*) LEAVES AND CORRESPONDING LATEX AFTER TRANSPIRATIONAL UPTAKE OF LABEL AND δ-TOCOTRIENOL (32·5 μmoles) FOR 24 hr

Compound	Amounts (μmoles)			Amounts due to addition of δ-tocotrienol (μmoles)	
	No label or δ-tocotrienol	Label (inc. "A"-tritio NADPH) and δ-tocotrienol	Label (inc. "B"-tritio NADPH) and δ-tocotrienol	Labelled using "A"-tritio NADPH	Labelled using "B"-tritio NADPH
α-Tocopherol	114·56	123·28	125·35	8·72	10·79
α-Tocotrienol	N.D.	1·35	1·82	1·35	1·82
β-Tocopherol	3·14	3·59	3·36	0·45	0·22
β-Tocotrienol	N.D.	1·19	0·86	1·19	0·86
γ-Tocopherol	4·39	5·24	5·82	0·85	1·43
γ-Tocotrienol	N.D.	0·81	0·75	0·81	0·75
δ-Tocopherol	1·51	2·11	2·62	0·60	1·11
δ-Tocotrienol	N.D.	2·81	2·57	2·81	2·57
Percentage recovery due to addition of δ-tocotrienol				62·9%	56·1%

N.D. = not detected.

TABLE 2. SPECIFIC ACTIVITIES OF TOCOPHEROLS AND TOCOTRIENOLS FROM DETACHED *Ficus elasticus* LEAVES AND CORRESPONDING LATEX, FORMED DURING TRANSPIRATIONAL UPTAKE OF δ-TOCOTRIENOL (32·5 μmoles), (METHYL-^{14}C)L-METHIONINE (0·1 mmole, 25 μc) AND EITHER "A"-TRITIO NADPH OR "B"-TRITIO NADPH (30 μmoles, 2 μc) FOR 24 hr

Compound	Disintegrations sec^{-1} μmole^{-1} (4R-diastereoisomer of NADPH used)*		Disintegrations sec^{-1} μmole^{-1} (4S-diastereoisomer of NADPH used)*	
	^{14}C	^{3}H	^{14}C	^{3}H
α-Tocopherol	4·42	84·89	6·66	10·27
α-Tocotrienol	13·36	8·55	10·72	6·12
β-Tocopherol	11·24	582·62	7·27	39·60
β-Tocotrienol	0·45	2·48	0·33	0·31
γ-Tocopherol	4·88	245·57	7·21	23·42
γ-Tocotrienol	33·84	3·49	28·77	1·20
δ-Tocopherol	0·90	472·73	1·17	21·97
δ-Tocotrienol	0·11	0·79	0·20	1·11

* Specific activities were calculated using the equation: total radioactivity in chromanol ÷ μmoles of newly synthesized chromanol.

presented that both cell-free latex[6] and developing chloroplasts[4] are capable of tocopherol biosynthesis.

The specific activities of the tocopherols and tocotrienols produced during incubation of *Ficus elasticus* leaves and latex with δ-tocotrienol are shown in Table 2. The results for the

incorporation of tritium label from stereospecifically labelled NADPH are in agreement with those reported earlier for α-tocopherol in seedlings of *Phaseolus vulgaris* and *Avena sativa*.[9] The side-chain of the tocopherols derive the hydrogen atoms for saturation stereospecifically from NADPH; that is the hydrogen atom H_A because the label is incorporated from the 4R-diastereoisomer.

Plastoquinone was used as an internal standard as reported earlier[9] to minimize errors arising from randomization of tritium label during biogenesis or from earlier incorporation of tritium from labelled NADPH during the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate. The specific activities for tritium of the tocopherols and tocotrienols shown in Table 2 have been corrected by subtracting, in proportion to the isoprene units they contain, an amount (four-ninths) related to the observed incorporation into plastoquinone (less than 30 disintegrations min^{-1} $\mu mole^{-1}$) assuming plastoquinone (PQ-9) to have zero incorporation of tritium label during isoprenoid saturation.

The tocotrienols are more susceptible to decomposition than the tocopherols and extensive purification via such procedures as acetylation was not possible. A portion of α-tocopherol was converted to the quinone.[10, 11] The material co-chromatographed with an authentic sample of α-tocopherolquinone and retained 93 per cent of both ^{14}C and ^{3}H counts of the starting material. This finding would rule out the possibility of the major portion of the tritium label appearing in the hydroxyl group of the chromanol moiety.

The results shown in Table 2 show methylation and hydrogenation occur upon the addition of δ-tocotrienol. Four preliminary experiments had already shown no incorporation of either label in the absence of exogenous δ-tocotrienol. Even upon the addition of δ-tocotrienol during three previous attempts to demonstrate the biogenseis of α-tocopherol little incorporation was observed. The intermediate situation of methylation or hydrogenation alone was not observed. The physiological conditions for biosynthesis to take place must obviously be correct and a control mechanism for appearance of biosynthetic capability may be involved. This induction or de-repression mechanism may be light dependent. On the day of successful incubation, natural daylight conditions were bright (max. about 50,000 lx) when incubation commenced (11.30 a.m.) but much duller (max. about 20,000 lx) the following day of harvest (11.30 a.m.). This finding is in good agreement with those of Booth who detected a rise in the levels of tocopherols in detached leaves on storage and under conditions of lowered illumination.[12] Little is known of the nature of the function of the tocopherols and their related compounds in higher plants. Whatever role is postulated it must inevitably explain the rise in level of tocopherols following the cessation of illumination.

The results shown in Table 2 would support the original theory of Pennock *et al.*[5] (solid lines, Scheme I) for biosynthesis of the tocopherols from their appropriate tocotrienols rather than the metabolic grid concept (dashed and solid lines) which would not be expected to show the higher specific activities for β-, γ- and δ-tocopherols than α-tocopherol observed. This elevated level of incorporation of label into the other tocopherols is in agreement with the findings of Threlfall and co-workers.[2, 4, 8] The incubation was allowed to continue over a period of 24 hr and in this time it is assumed a steady-state condition of some kind must be established for a period after the appearance of biosynthetic ability. How long this would take and last and for what period in the 24-hr period, is unknown and a subject of further investigation. With a decreasing supply of δ-tocotrienol to the system it is not possible to

[10] D. E. DUGGAN, *Arch. Biochem. Biophys.* **84**, 116 (1959).
[11] C. K. CHOW, H. H. DRAPER, A. S. CSALLANY and MEICHIU, *Lipids* **2**, 390 (1967).
[12] V. H. BOOTH, *Phytochem.* **3**, 273 (1964).

assume steady-state conditions prevailed throughout the whole period, although it is difficult to ascribe the elevated specific activities of β-, γ- and δ-tocopherol to anything other than being at the end of a "metabolic cul-de-sac". It is therefore not possible from this series of experiments to state β-, γ- and δ-tocopherol are definitely not the main precursors of α-tocopherol but indicate the most likely precursors of α-tocopherol are the tocotrienols.

The patterns of tocotrienols and tocopherols show a species variance. The balance between methylation and hydrogenation must be under genetic regulation although no genetic analysis of tocopherol patterns has been presented. The relative importance of each may be important taxonomically. It is unfortunate that, whilst having fewer ramifications, the alternative routes of biosynthesis of tocopherols, like those of the flavonoids which have been extensively studied, pose a difficult metabolic problem. This is yet another example of the difficulty of relating biosynthesis to genetic regulation in higher plants.

EXPERIMENTAL

(*A*) *Sources of Chemicals*

The modified method of San Pietro[13] to prepare (4-^{3}H)-NADP$^+$ employed by Cornforth *et al.*[14] was followed exactly. (Methyl-^{14}C)L-methionine (50 mc/mM) was obtained from the Radiochemical Centre, Amersham.

δ-Tocotrienol (19·5 mg) was isolated from commercial latex stabilized by ammonia (250 ml) using the method of Whittle *et al.*[15] The second method of extraction they describe was employed. The bulk of the total lipid after ether extraction was chromatographed on 200-g columns of alumina (Woelm, weakened to Brockmahn Grade III) and the 6–20 per cent (v/v) ether in light petroleum (E/P 60–40°b.p.) fractions containing δ-tocotrienol were collected and dried over anhydrous Na_2SO_4. The bulk of the solvent was removed under vacuum distillation and the remainder blown off under N_2 (O_2-free). Sterol was removed by crystallization from methanol at −30°. Final purification to remove δ-tocopherol and other material was carried out by reversed phase chromatography on paraffin-impregnated kieselguhr thin-layer (250 μ thick) plates developed with 75% (v/v) acetone in water, saturated with paraffin. All preparative operations were carried out in a darkened room.

(*B*) *Incubation Media Preparation*

The reduction of (4-^{3}H)-NADP$^+$ to the 4R- and 4S-tritium labelled diastereoisomers of NADPH described by Cornforth *et al.*[14] was carried out as described previously.[9] In addition a third incubation was carried out using unlabelled NADPH$^+$. To each of these incubations (total volume 10·4 ml) was added 1 ml of 0·1 M potassium phosphate buffer (pH 7·5) containing 20 μmoles of ATP, di-sodium salt (recorrected back to pH 7·5 with 6 N KOH), 0·1 mmole of (methyl-^{14}C)L-methionine (25 μc) and 32·5 μmoles of δ-tocotrienol. The δ-tocotrienol was brought into contact with the aqueous phase by first blending with 0·20 ml of freshly collected *Ficus elasticus* latex, adding this to 1 ml of phosphate buffer and finally to the NADPH incubate while vigorously agitating the tubes by means of a Whirlimixer (Fisons). In the case of the third incubation medium, the radioactive L-methionine was replaced by unlabelled L-methionine. The δ-tocotrienol, but not the additional latex, was omitted.

(*C*) *Conditions of Incubation*

Leaves (lamina length 25 ± 0·3 cm and width 14 ± 0·2 cm) from *F. elasticus* plants approximately 1·25 m high were used in all experiments. Using a new razor blade, the leaves were severed as close as possible to the main stem. The angle of cut was approximately 30° to the axis of the petiole. The cut petiole was immediately dipped under the appropriate incubation medium. During incubation a continuous exudation of latex from the cut petiole was allowed to fall to the base of the container. A rapid transfer of leaf to medium resulted in prolonged exudation with simultaneous transpirational uptake of the medium indicated by the slow fall in level of fluid. The liquid level was adjusted periodically by the addition of 0·075 M potassium phosphate buffer (pH 7·5) to prevent exposure of the cut ends of the petioles to the atmosphere resulting in coagulation of the latex and cessation of transpirational uptake. The angle of cut of the petiole was important as it allowed the exuded latex to fall clear from the conducting vessels of the petiole as incubation progressed.

[13] A. San Pietro, *J. Biol. Chem.* **217**, 579 (1955).
[14] J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják and G. J. Schroepfer, *Proc. R. Soc.* B **163**, 492 (1966).
[15] K. J. Whittle, P. J. Dunphy and J. F. Pennock, *Biochem. J.* **100**, 138 (1966).

Six leaves were placed in each of the three incubation media contained in small beakers, the sides of which were covered with black paper to exclude light, leaving only a slight aperture for use as a depth gauge. Cotton wool was used to hold the leaves tightly in the neck of the vessel to prevent undue evaporation and avoid photodecomposition. The incubation was allowed to continue for 24 hr under physiological conditions similar to those prior to excision.

(D) Lipid Extraction and Purification of the Tocopherols and Tocotrienols

The incubated leaves were chopped into small pieces and macerated three times in 80% (v/v) acetone (approx. 2 ml/g tissue) using an Ultraturrax gun homogenizer in bursts of 20 sec duration and filtered under reduced pressure each time through a sintered-glass funnel. The combined acetone extracts, diluted with water (1:3), were extracted three times with freshly distilled ether. The incubation media containing exuded latex were extracted according to the second method of Whittle *et al.*[15] using $CHCl_3$–MeOH (2:1, v/v). After standing, the $CHCl_3$ layer was reduced in volume by distillation under vacuum and extracted with ether. The ethereal extracts from the leaves were each combined with their appropriate extracts from the incubation media, washed with water, dried (Na_2SO_4) and the solvent removed by vacuum distillation. The lipid extracts were chromatographed on 200-g columns of alumina and the 6–20% (v/v) E/P fractions were collected. Sterol was removed by crystallization from methanol at −30°. The tocotrienol and tocopherol mixture was chromatographed as lines using 22% (v/v) di-isopropyl ether in P/E (40/60° b.p.) as developing solvent. All 20 × 20 cm plates coated with silicagel G (250 μ thick) were freed from lipid by a preliminary development with freshly distilled di-ethyl ether. The major band, containing mainly α-tocopherol and the more polar minor bands were indicated by spraying with 0·01% (w/v) fluorescein in ethanol[16] and the two areas eluted with ether and blown to dryness under N_2. The material eluted from the main band containing α-tocopherol was chromatographed in like manner in $CHCl_3$. The band of material containing α-tocopherol and the more polar band containing other tocotrienols and tocopherols were eluted separately with ether and blown to dryness with N_2. The latter material was combined with the other polar material from the first series of chromatographic separations. The two-dimensional TLC method described by Pennock *et al.*[5] for the separation of tocotrienols and tocopherols was employed for the purification of (*a*) the material containing mainly α-tocopherol and (*b*) the combined polar material. The tocotrienols and tocopherols were visualized as before, and the silica gel removed from the plate with a suitably shortened razor blade, eluted with ether and blown to dryness with N_2. The initial separation of the two fractions was essential, as the major material (α-tocopherol) would otherwise affect the chromatographic separation of the minor constituents. In this system β-tocotrienol and γ-tocopherol have identical R_f. Separation of these compounds was achieved by reversed-phase chromatography on kieselguhr coated 20 × 20 cm plates impregnated previously with 3% (v/v) paraffin in P/E (60–80° b.p.) and developed with 90% (v/v) acetone in water. The bands of material, which had identical chromatographic properties as those of authentic β-tocotrienol (R_f 0·63) and γ-tocopherol (R_f 0·45), were visualized by the method of Dunphy *et al.*,[17] eluted with ether and blown to dryness under N_2. The contaminating paraffin was removed by TLC in $CHCl_3$, eluting the γ-tocopherol or β-tocotrienol as before. Plastoquinone was extracted and purified as described previously.[9] All extractions and purifications were carried out in a darkened room.

(E) Estimation to Tocopherols and Tocotrienols

The tocopherols and tocotrienols were estimated by the method described by the Analytical Methods Committee[18] and the conversion factor described by Whittle *et al.*[15] to take account of the slow colour production of Emmerie–Engel reagent with δ-tocotrienol was employed.

(F) Radioactivity Counting Procedure

All samples were assayed for ^3H and ^{14}C content with a Nuclear Enterprises Automatic Liquid Scintillation Spectrometer (Model NE 8303) with toluene containing 2,5-diphenyloxazole (0·5%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (0·03%) as scintillation fluid. Counting was by the screening method[19,20] and efficiencies were 19·8% for H^3 and 24·6% for ^{14}C; less than 0·1% of the ^3H counts appeared in the ^{14}C channel, but 13·4% of the ^{14}C counts were found in the ^3H channel. Counts were corrected for background, quenching and the efficiency of counting (allowing for the ^{14}C counts in the ^3H channel) and expressed as dis/sec. Where sufficient radioactivity permitted, a Panax two-dimensional thin-layer radioactivity scanner was employed to monitor the purification of radioactive compounds.

[16] P. J. DUNPHY, K. J. WHITTLE and J. F. PENNOCK, *Chem. Ind.* 1217 (1965).
[17] P. J. DUNPHY, J. D. KERR, J. F. PENNOCK and K. J. WHITTLE, *Chem. Ind.* 1549 (1966).
[18] Analytical Methods Committee, *Analyst* **84**, 356 (1959).
[19] Y. STEIN and O. STEIN, *Biochim. Biophys. Acta* **60**, 58 (1962).
[20] G. T. OKITA, J. J. KABARA, F. RICHARDSON and G. V. LEROY, *Nucleonics* **15**, 111 (1957).