Phytochemistry, 1971, Vol. 10, pp. 2323 to 2328. Pergamon Press. Printed in England.

THE INTRACELLULAR DISTRIBUTION OF TOCOPHEROLS IN PLANTS

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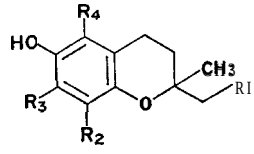
(Received 20 October 1970)

Abstract-The tocopherols of three plant tissues were examined and estimated colorimetrically after TLC. The plants were chosen on the basis of their high content of non-a tocopherols; *Fucus spiralis* thallus containing large amounts of δ-tocopherol and tangerine tomato fruit (*Lycopersicum esculentum*) and French bean leaves (*Phaseolus vulgaris*) having relatively large amounts of y-tocopherol. The various tissues were macerated and fractionated by centrifugation to yield a plastid fraction and a supernatant. a-Tocopherol was in each case found to be present in the plastid fraction, as was plastochromanol in the tomato, whereas the β-, γ- and δ-tocopherols were found in the supematant fraction. Differential centrifugation of the supernatant yielded a sediment at 30,000 *g* which contained β- and γ-tocopherols while sediments obtained at much higher *g* values contained some δ-tocopherol and some remained in the final supematant.

INTRODUCTION

EIGHT members of the vitamin E family are found in plants, a, β, γ and S-tocopherols and the four related tocotrienols (Fig. 1). The tocopherols, with the saturated side chain, are by far the most plentiful in plants, tocotrienols being found only in cereals, seed oils and the



Tocotrienols	Tocopherols
$R_1=[CH_2CH=C(CH_3)CH_2]_3H$	$R_1=[CH_2CH_2CH(CH_3)CH_2]_3H$
$R_2,R_3,R_4=CH_3,\alpha$	$R_2,R_3,R_4=CH_3,\alpha$
$R_2,R_4=CH_3;R_3=H,\beta$	$R_2,R_4=CH_3;R_3=H,\beta$
$R_2,R_3=CH_3;R_4=H,\gamma$	$R_2,R_3=CH_3;R_4=H,\gamma$
$R_2=CH_3;R_3,R_4=H,\delta$	$R_2=CH_3;R_3,R_4=H,\delta$

FIG. 1. THE NATURALLY OCCURRING TOCOPHEROLS AND TOCOTRIENOLS.

latex of *Hevea brasiliensis.*[1-3] In the tocopherol series a-, the trimethyl tocol is the most widespread and abundant especially in photosynthetic tissue.[4] Non a-tocopherols are found mainly in seed oils.[5] Many workers have shown that a-tocopherol is located in the chloroplasts[4,6-8] but little is known of the intracellular location of the non-a-tocopherols.

[1] D. MCHALE, J. GREEN, S. MARCINKIEWICZ, J. FEENEY and L. H. SUTCLIFFE, *J. Chem. Soc. 784 (1963).*
[2] J. F. PENNOCK, F. W. HEMMING and J. D. KERR, *Biochem. Biophys. Res. Commun.* 17,542 (1964).
[3] P. J. DUNPHY, K. J. WHITTLE, J. F. PENNOCK and R. A. MORTON, *Nature, Lond.* 207,521 (1965).
[4] V. H. BOOTH, *Phytochem.* 2,421 (1963).
[5] D. C. HERTING and E. E. DRURY, *J. Nutr.* 81, 4 (1963).
[6] C. BUCKE, *Phytochem. 7, 693 (1968).*
[7] R. A. DILLEY and F. L. CRANE, *Plant Physiology 38,452 (1963).*
[8] H. K. LICHTENTHALLER, *Ber. Deut. Botan. Ges. 79,* 111 (1966).

Booth[4] found that in yew and ivy leaves the ratio of γ- and δ-tocopherol to chlorophyll was lower in chloroplasts than whole tissue indicating that although there may have been γ- and δ-tocopherol in chloroplasts[7,9] the majority of these tocopherols were located elsewhere in the cell. However, the investigation was hampered by the low levels of these tocopherols, in particular δ-tocopherol.

It was decided to look at the intracellular location of tocopherols in tissues possessing large amounts of β-, γ-, or δ-tocopherols in addition to a-tocopherol. Suitable tissues were thalli of brown seaweed[10,11] which contains considerable amounts of δ-tocopherol, and tomato fruit and French bean leaves, which possess γ-tocopherol.

RESULTS AND DISCUSSIONS

Leaf tissue from French bean, thallus from *Fucus* or tomato fruits were fractionated to yield plastids and supernatant which were analysed for tocopherols. The results of the experiments are shown in Tables 1-3 and indicate that in each case a-tocopherol is found in the plastid fraction whilst β,-γ- or δ-tocopherol are found outside these particles.

It will be seen that the preparation of plastids was not as efficient as was hoped, as indicated by the relatively low recovery of chlorophyll in the fractionated tissue. With *Phaseolus* leaf tissue it was expected that there would be a low yield of chloroplasts, since

TABLE 1. TOCOPHEROLS PRESENT IN FRACTIONS OF *Phaseolus vulgaris* LEAF TISSUE

	mg Chl/g wet wt.	μg α-T/ mg Chl	μg γ-T/ mg Chl	μg γ-T/ μg α-T
Whole tissue	4·50	2·02	4.54	2·25
Chloroplast	0·34*	2·29	0.18	0·08
Supernatant	0·056*	2·30	28·3	12.16

* These values are calculated from the weight of tissue used for the separation and the chlorophyll content of the fractions, i.e. in this case 17.8 g of tissue was taken and 6·03 and 1·00 mg of chlorophyll estimated in chloroplast and supernatant, respectively.

the cells were ruptured only by the pressure of a spatula[12] thus leaving a residue with a large number of unbroken cells. Similarly with *Fucus*, maceration of the leathery tissue was not complete and considerable undamaged pieces were seen in the debris removed on filtration. Recovery of plastids in *Lycopersicum* (in terms of yield of a-tocopherol) was much better than in the other two tissues, the tomato fruit parenchyma being soft and easily disrupted.

Table 1 shows clearly the association of a-tocopherol with chlorophyll; the small amount of a-tocopherol in the supernatant fraction is presumably due to chloroplast contamination. γ-Tocopherol is present in the supernatant and the figures indicate that it is

[9] R. A. DILLEY Ph.D. Thesis, cited by F. L. CRANE, ***Biochemistry of Quinones*** (edited by R. A. MORTON), p. 183, Academic Press, London (1965).
[10] A. JENSEN, ***Proc. 5th Int. Seaweed Symposium,*** p. 281, Pergamon Press, Oxford (1966).
[11] F. BROWN, ***Chem. & Ind.*** 174 (1953).
[12] P. GHOOPRASERT, M.Sc. Dissertation, University of Liverpool (1967).

TABLE 2. TOCOPHEROLS PRESENTIN FRACTIONS OF *Fucus spiralis* THALLUS

	mg Chl/g wet wt.	μg α-T/ mg Chl	μg βT+γT/ mg Chl	μg δ-T/ mg Chl	μg δT/ μg α-T	μg β + γT/ μg α-T
Whole tissue	1.18	27.3	11·7	27.6	1.01	0·34
Chloroplast	0·062 *	23.8	3·7	5.2	0.22	0.16
Supematant	0·0057*	26.2	39.0	202	7·55	1.46

* These values are calculated from the weight of tissue used for the separation and the chlorophyll content of the fractions, i.e. in this case 112 g of tissue was taken and 7·04 and 064 mg of chlorophyll estimated in chloroplast and supernatant, respectively.

not associated with either cc-tocopherol or chlorophyll in the cell. Booth[4] compared tocopherol to chlorophyll ratios in whole leaf with those in chloroplasts and suggested that a-tocopherol was probably located in the chloroplast in ***Iris germanica*** and that y-tocopherol and possibly δ-tocopherol were not present in the chloroplast in yew ***(Taxus baccata)*** and ivy ***(Hederu helix).*** In the experiments of Booth the distribution of γ- and δ-tocopherols was not examined in detail, but the suggestion that these tocopherols were located in fractions other than the chloroplast is supported by our findings.

The work on ***Fucus*** spiralis (Table 2) shows conclusively that δ-tocopherol is located in the supernatant fraction and the presence of the small amount in the plastid fraction is probably due to incomplete separation of the fractions after centrifugation. As in ***Phaseolus,*** the association of y-tocopherol with chlorophyll indicated that a-tocopherol is sited within the chloroplast, the small amount of a-tocopherol in the supernatant again seeming to be due to chloroplast contamination. The β- and y-tocopherol also appear to be concentrated in the supernatant fraction but a rather higher proportion was found in the chloroplast than was the case with δ-tocopherol. As will be described later, δ-tocopherol is located in a different part of the cell to β- and y-tocopherol.

TABLE 3. FRACTIONATION OF THE PARENCHYMATOUS TISSUE OF THE FRUIT OF *Lycopersicum esculentum* (TANGERINE VARIETY)

	μg α-T/ g wet wt.	μg γ-T/ g wet wt.	μg PC/ g wet wt.	y-T/a-T	PC/a-T
Whole tissue	0.50	0·32	0.73	0·64	1·46
Plastid fraction*	0·19†	0	0.28	0	1·43
Supernatant	0·012†	0.18	0	148	0

* Plastid fraction is mainly chromoplasts but possibly some chloroplasts also.
† Values calculated from weight of tissue used for the fractionation, in this case 58·3 g was taken.

The recovery of β-, γ- and δ-tocopherol in ***Fucus*** and ***Phaseolus*** was lower than that of a-tocopherol. It has been reported[13] that y-tocopherol is destroyed enzymically in the damaged cell to a much lesser extent than is a-tocopherol and this might suggest that the recovery of a-tocopherol would be the lowest. However, extraction of the supernatant fraction, where the non-a-tocopherols were found, was tedious, involving large amounts of solvents and it is not unlikely that at this stage some loss in tocopherols occurred.

The tomato fruit, although green when young, contained no chlorophyll when used

[13] V. H. BOOTH, *Biochem. J. 84, 85 (1962).*

for these experiments. This tissue contains relatively low concentrations of tocopherol and plastochromanol but since the level of lipid is also low, these compounds are seen clearly on chromatograms and estimated easily. The data in Table 3 show that the plastid fraction contains a-tocopherol and plastochromanol while the supernatant once again contain y-tocopherol. Thus in a higher plant, a brown alga and the fruit of a higher plant, α-tocopherol has been found in the plastid fraction and the non-a-tocopherols in other parts of the cell. This pattern agrees with the generalization that a-tocopherol is the major tocopherol in chlorophyll-containing tissue and non-a-tocopherols, when found, are mainly in non-green tissues such as vegetable oils,[5] nuts,[14] fungi[15] and cereal seeds.[16]

A possible explanation for the observed distribution of tocopherols in bean leaf tissue fractions is that a-tocopherol is formed in the chloroplast as the shoot grows and γ-tocopherol is supplied from a storage reserve in the bean seed. However, this seems unlikely since examination of 20-day-old bean plants indicated there was much more y-tocopherol present in each plant than that present in the original bean seeds.

TABLE 4. DIFFERENTIAL CENTRIFUGATION OF *Fucus spiralis* THALLUS

Centrifuged fraction *g*	% of individual tocopherols a	β + γ	δ
5000	72	—	6
30,000	24	50*	—
100,000	—	so*	—
200,000	—	—	37
Supernatant	4	—	57

* The amounts of β and y-tocopherol were very small and these tocopherols were assessed to be present equally in the 30,000 and 100,000 *g* fractions by visual detection,

Some experiments were carried out to attempt to localize the non-a-tocopherols and the results for *Fucus* thallus are shown in Table 4. S-Tocopherol is predominantly in the 'soluble' part of the cell although some is sedimented at 200,000 g. Preliminary experiments have shown that part of the S-tocopherol can be precipitated by ammonium sulphate. Only small amounts of β- and y-tocopherol were found in *Fucus* and these were located in the fractions sedimented at 30,000 and 100,000 g. To check the intracellular location of y-tocopherol similar fractionations were carried out with tangerine tomato and *Phaseolus* which contain large amounts of this tocopherol. y-Tocopherol was present predominantly in the 30,000 g fraction of *Phaseolus* with smaller amounts in the plastids and supernatant. In tomato, over 95 per cent was found in the 30,000 g sediment. In *Phaseolus* a small amount of S-tocopherol was found and was located in the supernatant.

These findings pose problems with regard to both function and biosynthesis of tocopherols. The non-a-tocopherols may be biosynthetic precursors of a-tocopherol and be

[14] G. LAMBERTSEN, H. MYKLESTEAD and 0. R. BRAEKHAN, *J. Sci. Food Agric. 13, 617 (1962).*
[15] H. KUBIN and H. FINK, *Fette Seifen Anstrichmittel63, 280* (1961).
[16] J. GREEN, *J. Sci. Food Agric. 9,* 801 (1958).

synthesized outside the chloroplast and transported there only for the final methylation step. On the other hand, all the tocopherols may be synthesized in one part of the cell and then transported to their separate sites of action. This would imply separate functions for a- and the non-a-tocopherols. Another possibility is that there are different sites for the biosynthesis of a-tocopherol and for the other tocopherols. a-Tocopherol may function in photosynthesis but little is known about the other members of the tocopherol family. A clearer knowledge of their intracellular location may promote some ideas.

EXPERIMENTAL

(i) *Solvents and Medium*

Et_2O, dried over Pb-Na alloy (B.D.H. Chemicals Ltd.) and distilled over reduced Fe immediately prior to use; light petroleum (b.p. 40–60°) dried over Pb-Na alloy and distilled immediately prior to use; acetone dried (K_2CO_3) and distilled immediately prior to use; absolute EtOH refluxed with 10 g/l. of Zn dust and 20 g/l. KOH for 6 hr and then distilled, the 78" distillate being collected for use; isopropanol, distilled immediately prior to use; $CHCl_3$ as supplied containing 1% EtOH as stabilizer; S.F.D.P. medium, 0·25 M sucrose, 1 mM Mg $(OAc)_2$, 2 mM EDTA and 0·02 mM Na_3 citrate in 0.025 M Tris buffer adjusted to pH 7·8 with 1 N HOAc and solute added to make it up to 5% dextran, 2·5% ficoll, 0·05% cysteine, 0·01% bovine serum albumin and 2 % polyvinyl pyrrolidone.

(ii) *Tissues*

French Dwarf Beans (*Phaseolus vulgaris*) were grown in damp vermiculite and the leaves removed for analysis at 20 days after planting. A tangerine mutant of tomato (*Lycopersicum esculentum*) was grown at the University Gardens, Ness, by Mr. J. K. Huhne and used when ripe, i.e. after all green tinges had disappeared. The brown seaweed, *Fucus spiralis* was collected locally.

(iii) *Extraction of Whole Tissue*

Small strips of bean leaves, *Fucus* thallus or mashed tomato pith were macerated in acetone with a Polytrons homogenizer, the residue discarded after filtration. The extracts were shaken with an equal volume of light petroleum, two layers then being produced by the addition of H_2O. The petrol fraction was washed (H_2O), dried ($NaSO_4$), evaporated under N_2, the resultant lipid being stored under cyclohexane.

(iv) *Fractionation of Tissues and Extraction of the Fractions*

(a) ***French dwarf bean leaves.*** Weighed samples of bean leaves, from plants left overnight in the dark (to obviate any complications arising during centrifugation due to the presence of starch granules), were taken, one sample being extracted as in (iii). The other sample was cut into thin strips which were placed in a chilled mortar with SFDP medium (100 ml per 20 g tissue) and squeezed against the mortar with a plastic spatula to eject the chloroplasts and other cell contents into the medium.'" The liquid was then filtered through double muslin and retained for centrifugation, the chloroplast fraction being sedimented at 2000 *g* (10 min at 0–5°). It was found that the isolation of intact plastids in all three tissues was more successful if the speed was increased slowly to maximum, thus avoiding the rupture of these particles. This sediment was extracted as in (ii). To the supernatant, including cell sap, 9 vol. of isopropanol was added and then the mixture was diluted with 8 vol. of H_2O. An equal volume of petrol was added, the whole shaken and the petrol fraction removed and evaporated down under N_2 to yield the final lipid extract.

(b) ***Fucus spiralis.*** Weighed samples of *F. spiralis* thallus were homogenized in 0·01 M KCl-0.5 M sucrose medium (60 g per 100 ml medium), the homogenate then being filtered through 6 layers of muslin. The filtration was slow and the muslin became quite clogged with debris and it was found more convenient to collect only part (usually about 70 per cent) of the filtrate. The filtrate was centrifuged at 3000 g for 20 min at O-5" to produce a sediment, the chloroplast fraction, which was extracted as in (iii) and a supernatant which was filtered through glass wool and treated as in (a).

(c) ***Tangerine tomatoes.*** Weighed samples of parenchymatous tissue of the fruit were homogenized in 0·01 M KC1 0·5 M sucrose (40 g/100 ml medium), the homogenate then being filtered through 6 layers of muslin. The filtrate was centrifuged at 3000 *g* for 20 min at O-5". the sediment of orange-yellow plastids so produced being extracted as in (iii). The supematant was filtered and then extracted as in (a).

(v) *Differential Centrifugation*

Homogenates of *Phaseolus* and *Fucus* were prepared as in (iv(b)) and of *Lycopersicum as* in (iv(c)). As has been described in the text, the dimethyl tocopherols (β- and γ-tocopherols) could be found in sediments produced at about 30,000 *g* while δ-tocopherol, where present, remained in the supematant at this centrifugal force. It was not possible to use a standard centrifugation technique for all three tissues as the forces

and times required varied and those described below were established after preliminary experiments. ***Phaseolus*** homogenate was centrifuged at 1000 ***g*** for 20 min to yield plastids and then a sediment was obtained at 30,000 ***g*** for 45 min; ***Lycopersicum*** homogenate was centrifuged at 3000 ***g*** (20 min) and the supernatant recentrifuged at 30,000 ***g*** (90 rnin); ***Fucus*** homogenate sediments were collected at 5000 ***g*** (10 min), 30,000 ***g*** (30 min), 100,000 ***g*** (120 min) and 200,000 ***g*** (120 min). In each case the sediments and final supernatant were extracted as in (iv).

(vi) ***Chromatography***

Lipid samples were examined by 2-dimensional TLC on silica plates in $CHCl_3$ followed by 18% Et_2O in petrol. The various lipid components could then be identified by position and by using the following spray reagents: 0·01% fluorescein in EtOH UV-absorbing compounds showed up as dark spots on a yellow fluorescent background under UV illumination: equal volumes of 0·2% $FeCl_3 6H_2O$ and 0·5% 2-2′-bipyridyl in EtOH; reducing compounds giving a pink colour : equal volumes of 0·2% $FeCl_3 6H_2O$ and 0·4% 2,4,6-tri-(-2-pyridyl)-1,3,5-triazine in EtOH; reducing compounds produced a blue colour : 10% phosphomolybdic acid in EtOH produced a blue colour with reducing compounds in the cold, and with lipids generally, on heating: 0·5% aqueous Fast Blue salt B, tetrazotized di-o-anisidine Zn double salt (Fluka) followed by 0·1 N aqueous NaOH: this reagent coupled with phenols having a free *ortho* or *para* position, producing a characteristic slate grey colour with δ-tocopherol and a blue-grey colour with y-tocopherol and plastochromanol

(vii) ***Estimations***

Chlorophyll. The chlorophyll contents were estimated by measuring the absorptivity in Et_2O at 600 nm; chlorophyll conc. = 100·5 x Extinction 600 nm mg l^{-1}.[17]

Tocopherols and Plastochromanol. The tocopherols (and plastochromanol in the case of tomato extracts) were separated by TLC as in (v), then extracted from the silica gel with Et_2O. The Et_2O was evaporated under N_2 and the lipid dissolved in 0·8 ml EtOH: 0·1 ml of 0·625% ethanolic 2·2′ binyridvl was added. followed-by 0·25% ethanolic $FeCl_3$. The absorptivity at 520 nm was "measured, 3 rnin after the addition of $FeCl_3$ in the case of δ-tocopherol, and 2 min for the other reducing compounds. Tocopherol contents could then be calculated from: μg Tocopherol/spot = (Absorbance 520 nrn minus blank) x factor where factor = 24 for a-tocopherol (α-*T*); 23·5 for β-tocopherol (β-*T*); 22 for y-tocopherol (γ-*T*); 19 for δ-tocopherol (S-T); 40 for plastochromanol (PC). The amounts of β- and y-tocopherol in *Fucus* were quite small and separation of the two on TLC was not good. However, visually the amounts of each seemed constant so they were estimated together. These tocopherols were isolated from a sample of whole tissue and separated by preparing the nitroso-derivatives which can easily be separated. It was found that the ratio of β-*T*/γ-*T* was 1·7/l. and so a common factor of 23 was used to estimate both together in other experiments.

[17] J. H. C. SMITH and A. BENITEZ, ***Modern Methods of Plant Analysis*** (edited by K. PAECH and M. V. TRACEY), Vol. 4, p. 142, Springer-Verlag, Berlin (1955).