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α-TOCOPHEROL, ITS CO-OCCURRENCE WITH CHLOROPHYLL IN CHLOROPLASTS

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Abstract—α-Tocopherol has been found in leaves of 100 species of plants from widely separated families. Other plant parts that contained both chlorophyll and α-tocopherol included stems and fruit cases; roots contained little or no tocopherol. Potato tubers exposed to sunlight synthesized both chlorophyll and α-tocopherol. There was usually more tocopherol in deep green than in pale green leaves, and the green parts of variegated leaves were richer in α-tocopherol than yellow parts of the same leaves. Etiolated leaves had low concentrations of α-tocopherol. No α-tocopherol was found in cell sap from leaves; and the ratio of α-tocopherol to chlorophyll-*a* in extracts of leaves of iris was the same as in extracts of chloroplasts. For yew and for ivy, the ratio of γ-tocopherol to chlorophyll was much higher in extracts of leaf than in extracts of their chloroplasts.

This evidence suggests that in plants chlorophyll is always accompanied by α-tocopherol, which is probably sited inside chloroplasts, but that γ-tocopherol (and possibly δ-tocopherol) is mainly outside chloroplasts.

CERTAIN lipids, including β-carotene and luteol, are known invariably to accompany chlorophylls in leaves. Several others—including violaxanthin and other carotenoids, plastoquinones, vitamin K_1 and certain fluorescing lipids[1]—probably also invariably accompany chlorophylls. For a long time it has been presumed, though never proved, that α-tocopherol also occurs in all green leaves and therefore belongs to the group of essential phyllolipids. Evidence in support of the hypothesis is presented in this paper; indeed the evidence supports a wider hypothesis, namely that chlorophyll in any plant tissue is always accompanied by α-tocopherol.

Chlorophylls, carotenoids, and in fact most of a leaf's lipids are known to be in the chloroplasts. Hence tocopherols, being lipids, would be expected also to occur inside chloroplasts. Evidence here offered suggests that α-tocopherol is inside chloroplasts, although part of the γ-tocopherol appears to be outside.

RESULTS

Survey of Species

α-Tocopherol was found in leaves of all the plants analysed previously[2,3] and has now been found in a total of over 100 species of wild and cultivated plants, including evergreens, annuals, grasses, legumes, trees, shrubs, water plants, bracken, cacti and mosses. Reports on the tocopherol content of a few other species of plants are found in the literature.[4]

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1 V. H. BOOTH, *Biochem. J.* **84**, 444 (1962).
2 V. H. BOOTH and A. HOBSON-FROHOCK, *J. Sci. Food Agr.* **12**, 251 (1961).
3 V. H. BOOTH and M. P. BRADFORD, *Brit. J. Nutrit.* In press
4 D. C. HERTING, W. F. KUJAWSKI, M. L. LUDWIG and P. L. HARRIS, *Annotated Bibliography of Vitamin E*, Vol. V. Distillation Products Industries, Rochester N.Y. (1958).

Occasionally—once per 100 analyses—no tocopherol was seen on a chromatogram, but the absence was not confirmed when the analysis was repeated next day.

Relationship with Chlorophyll

In general, dark green leaves were found to be richer than light green leaves. For example seven varieties of lettuce having light green leaves had α-tocopherol contents of only 80 μg/g. On the other hand large dark green leaves of *Urtica dioica* (nettle) had 1000 μg/g in July. Intermediate in colour were *Crocus flavus* with 130 μg/g in spring and *Chenopodium alba* (fat hen) with 160 μg/g in summer. Leaves of *Seseli bocconei* (Sicilian parsley) were divided into two categories, whose α-tocopherol contents were: dark green, 700; light green, 180 μg/g respectively. Exceptions were encountered, but they were uncommon. For example, dark green and light green leaves of similar size taken from one *Sorbus aucuparia* (rowan) tree in September had 210 and 840 μg/g respectively. In December light green leaves of *Ilex aquifolium* (holly) had 1460 while dark green had 640 μg/g.

Etiolated Leaves

Green leaves of *Fragaria ananassa* (strawberry cv.) had a tocopherol content in April of 150, while achloric leaves had only 85 ug/g. Green leaves of chitted *Sinapis alba* (mustard) grown indoors in January had 210, while etiolated leaves of similar plants grown in the dark had no detectable tocopherol. The almost white heart of *Brassica oleracea* (cabbage) had 15 while the deep green outer leaves had 500 μg/g. In *Allium porrum* (leek) in December the almost white parts of leaves near the base had 55 μg/g, the upper, green, parts had 270, and intermediate parts had intermediate values.

Variegated Leaves

Ligularia (sometimes called *Farfugium*) has leaves with distinctly separated green and ivory parts. The former had an α-tocopherol content of 25, while the latter had only 9 μg/g based on fresh weight. The outer ivory-coloured parts of variegated *I. aquifolium* in winter had 30, while the inner deep green parts of the same leaves had 560 μg/g. The edges of the leaves of *Elaeagnus pungens maculata* in March were deep green with 700, the middles were yellow with 18, and intermediate light green parts had 230 μg/g. In variegated *Ligustrum vulgare* (privet) the yellow parts had 340, while the green parts of the same leaves had 900 μg/g in July. The α-tocopherol contents of leaves of variegated Irish yew were: entirely green 500; mainly green with small area yellow 320; mainly yellow with small area green 240 μg/g.

Green Parts other than Leaves

Most plant parts, other than flowers, that are exposed to sunlight contain chlorophyll, and some are known to contain α-tocopherol also. For example Brown[5] found α-tocopherol in the stem of kale and in the petiole of sugar beet. I have found α-tocopherol in green stems of *Petroselinum crispum* (parsley), *Nasturtium officinale* (watercress), and *Iris germanica*; also in other green but non-leaf tissues (Table 1).

Solanum tuberosum (potato) tubers growing below ground are usually nearly white, whereas those that are exposed to sunlight become green externally. The former contained negligible tocopherol, but the green outer layers of the latter contained a discernible amount of α-tocopherol.

[5] F. Brown, *J. Sci. Food Agr.* **4**, 161 (1953).

Roots

No tocopherol was detectable in the roots of *Brassica rapa* (turnip), *Beta vulgaris* (beetroot) or *Raphanis sativus* (radish).[3]

Chloroplasts

The co-occurrence of chlorophyll and α-tocopherol suggests that the latter is situated inside chloroplasts. Dam, Glavind and Nielsen[6] briefly stated that vitamin E occurred within

TABLE 1. α-TOCOPHEROL IN GREEN PARTS OTHER THAN LEAVES

Plant	Part	α-Tocopherol, μg/g
Apium dulce (celery)	Stem	65
Bryonia dioica (bryony)	Fruit case	110
Cucumis sativus (cucumber)	Skin	130
Datura stramonium (thornapple)	Seed case	65
Medicago sativa (lucerne, alfalfa)	Stem	45
Pisum sativum (pea)	Seed case	5*
Ribes uva-crispa (gooseberry)	Fruit	17
Rosa cv. (rose)	Seed case	20*

* Fresh weight.

the chloroplasts; no experimental details were given, and the analytical techniques available in 1940 were far from specific for α-tocopherol. Therefore it was desirable to re-investigate the problem.

All the chlorophyll is known to be in the chloroplasts. If the tocopherol also occurs there, the ratio of the two lipids should be the same in extracts of the whole leaf as in extracts of the

TABLE 2. RATIO OF α-TOCOPHEROL TO CHLOROPHYLL IN LEAVES AND CHLOROPLASTS FROM NARCISSUS (VARIETY FORTUNE)

Material	μg/g fresh weight		Ratio
	α-Tocopherol	Chlorophyll-*a*	T/C
Leaf	215	720	0·30
Chloroplast	610	3250	0·19

chloroplasts. Chloroplasts were isolated from *Narcissus* leaves, and both these and a corresponding batch of leaves were extracted with 80% acetone. The α-tocopherol and chlorophyll-*a* contents of the leaf and chloroplast extracts are given in Table 2. The comparison of the pair of tocopherol:chlorophyll ratios is 1·6. Similar results were obtained with leaves of *U. dioica*, *Hedera helix* (ivy), *Taxus baccata* (yew), *Sambucus nigra* (elder), *Phaseolus coccineus* (runner bean) and *Spinacea oleracea* (spinach), the average of the comparisons of the ratios in all the experiments being 1·7. The higher tocopherol:chlorophyll ratio for leaf than

[6] H. DAM, J. GLAVIND and N. NIELSEN, *Z. physiol. Chem. Hoppe-Seyler's* **265**, 80 (1940).

for chloroplast might mean that some tocopherol is located outside chloroplasts, or alternatively that some tocopherol was lost during the isolation of chloroplasts.

When leaves are damaged they lose tocopherol by enzymic oxidation,[7] and since isolation of chloroplasts involves crushing of the leaf cells it might be expected that the ratio of tocopherol to chlorophyll would fall during manipulation. That this happens was confirmed by determining the concentrations of α-tocopherol and chlorophyll-*a* in leaf of *Populus nigra* before and after maceration for 4 min in a blendor. The values for tocopherol and chlorophyll-*a* in July were 225 and 2340 μg/g dry weight, changing to 130 and 2330 after maceration. Hence the tocopherol:chlorophyll ratio fell from 0·096 to 0·056.

Two schemes offered a chance of avoiding the enzymic destruction of tocopherol: (a) the use of inhibitors,[7] and (b) the use of leaves devoid of the tocopherol-destroying enzyme. When leaves and chloroplasts from *U. dioica* were extracted without cyanide, the tocopherol: chlorophyll ratio was four times as high for the leaf as for the chloroplasts. In the presence

TABLE 3. RATIO OF α-TOCOPHEROL TO CHLOROPHYLL IN LEAVES AND CHLOROPLASTS FROM *Iris germanica*

Material	μg/g fresh weight		Ratio T/C	Comparison of ratios, leaf/chloroplast
	α-Tocopherol	Chlorophyll-*a*		
Leaf	73	520	0·14	
Chloroplast	600	3960	0·152	0·92
Leaf	65	480	0·135	
Chloroplast	570	3900	0·146	0·92
Leaf	70	550	0·13	
Chloroplast	56	515	0·109	1·19
Leaf	80	420	0·19	
Chloroplast	115	612	0·188	1·01
			Mean comparison	1·01

of 0·5 mM sodium cyanide the leaf ratio was only twice as high as the chloroplast ratio. It is now known that cyanide does not completely inhibit the enzyme: hence it remains unknown whether the leaf and chloroplast ratios would be the same if the enzyme were inhibited completely.

The alternative scheme was therefore examined. The activity of the tocopherol-oxidizing system in leaf of *I. germanica* is very low:[7] at pH 4·3 only 10 per cent of the tocopherol was lost from leaf macerate in an hour at room temperature. At lower temperatures, and at pH 6·8 the loss is negligible. Iris leaves were therefore used for determining ratios. The effect of the tocopherol gradient along iris leaf[2] was avoided by taking similar samples for both leaf extracts and chloroplast preparations. Four experiments were done on different occasions, and with minor modifications of the procedure for the preparation of the chloroplasts, particularly in the speed used for centrifuging. The α-tocopherol and chlorophyll-*a* contents respectively of leaf and chloroplasts are summarized in Table 3. The average of the four leaf:chloroplast comparisons was 1·01, which provides evidence that in iris tocopherol accompanies chlorophyll inside the chloroplasts.

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

Gamma-tocopherol

Although α-tocopherol is the principal isomer in leaves, both γ- and δ-tocopherols may also occur in some species but usually only in concentrations below 20 μg/g of the dry weight. *T. baccata* and *H. helix*, however, stand out from 100 species in having considerable concentrations of what are believed* to be γ- and δ-tocopherols.[1] Chloroplasts were separated from both species, and γ-tocopherol:chlorophyll-*a* ratios were calculated as in the experiments with α-tocopherol (Table 4). Since enzymic loss of γ-tocopherol is only small,[7] the high leaf: chloroplast comparisons suggest that most of the γ-tocopherol is outside the chloroplasts.

There is evidence from one experiment that the same reasoning applies to δ-tocopherol. The δ-tocopherol contents of the yew leaf and chloroplasts in the third experiment of Table 4

TABLE 4. RATIO OF γ-TOCOPHEROL TO CHLOROPHYLL IN CHLOROPLASTS

Material	μg/g fresh weight		Ratio T/C	Comparison of ratios
	γ-Tocopherol	Chlorophyll-*a*		
H. helix, leaf	15	1200	0·0125	
chloroplast	6	4500	0·00133	9·4
T. baccata, young leaf	70	780	0·09	
chloroplast	20	1160	0·017	5·3
T. baccata, old leaf	38	1320	0·029	
chloroplast	19	2400	0·008	3·6

were 6 and 4 μg/g respectively. The δ-tocopherol:chlorophyll ratios were therefore 0·0046 and 0·0017; and the comparison of these ratios was 2·7.

Alternative Sites

If part of the α-tocopherol were located outside chloroplasts, it might be possible to find it. A buffered (pH 6·8) macerate of 45 g iris leaf was centrifuged to remove chloroplasts, the brown supernatant solution was freeze dried, and the acetone extract of the residue was transferred to light petroleum and applied to filter paper. After development no tocopherol was found on the chromatograms although the cell sap preparation came from 100 times as much leaf as would normally be used. In another experiment the chloroplast-free supernatant solution from macerated leaf of *U. dioica* was vigorously shaken with acetone and light petroleum. After the phases separated, the petroleum was washed to remove acetone, concentrated and applied to paper. No tocopherol was seen on the chromatogram. Yet after an α-tocopherol concentrate was dispersed in another portion of the supernatant solution, it was recovered to the extent of 85 per cent showing that tocopherol, when present, is extractable from the diluted cell sap.

* The concentrations of γ- and δ-tocopherols varied widely from "not-certainly-detected" to ten times the concentration of α-tocopherol. The γ-isomer was identified by its position on the chromatograms supported by dianisidine and ferric chloride tests. The δ-isomer was identified by dianisidine and ferric chloride tests, and by mixed chromatography with an authentic specimen. Because both tocopherols have so seldom been found during studies in this laboratory, although reported elsewhere in several species of leaf, the problem of their occurrence is under investigation.

DISCUSSION

No chlorophyll-*a*-containing higher plant tissue has been proved to be devoid of tocopherol although tocopherols occur, for example in fruits and seeds,[3,8] without chlorophyll. Moreover, evidence is provided that the α-tocopherol in iris leaves is within chloroplasts. Therefore the hypothesis that chlorophyll-*a* is always accompanied by *a*-tocopherol, at least in higher plants, is supported. An apparent exception was provided by Green, Price and Gare[9] who found no tocopherol in two chlorophyll-containing bacteria, but these differ from higher plants in many ways including the nature of their chlorophyll.

The functions of α-tocopherol and chlorophyll may be associated. α-Tocopherol is an antioxidant, and the most obvious functional hypothesis would be based on this property. Griffiths *et al.*[10] showed that chlorophyll can be destroyed by the oxygen produced when chlorophyll-containing bacteria are illuminated, unless carotenoids are present to protect it. α-Tocopherol also may protect chlorophyll from oxidation inside chloroplasts of leaves. It would be used up during active photosynthesis, which is in keeping with the observation[2] that tocopherol concentration is lowest in fast-growing leaves.

METHODS

Tocopherols

For those experiments in which only the tocopherol content was to be measured, about 300 mg of leaf (or more of other tissues) were extracted with acetone followed by 30–40° light petroleum.[11] The extract was freed of acetone by washing with water,[12] and applied to filter paper treated with $ZnCO_3$.[13,14] Chromatograms were developed in two dimensions, then tocopherol spots were cut out and determined by the dipyridyl–$FeCl_3$ technique.

When the concentrations of both tocopherol and chlorophyll were required in order to calculate ratios, the procedure was slightly different. The chlorophyll was determined as described below; an aliquot of the acetone solution (for tocopherol determination) was poured into about the same volume of 30–40° light petroleum standing over half-saturated ammonium sulphate solution in a separating funnel,[11] and further manipulations then proceeded as described above.

Tocopherol concentrations are based on dry matter unless otherwise stated, as for example in the experiments with chloroplasts.

Chlorophyll

About 1 g of leaf or 50 mg of chloroplasts were ground with quartz under acetone–water (80% v/v) in small beakers.[11] The extract was decanted, and the extraction repeated until all pigment was removed. Aliquots of the total extract were diluted and briefly centrifuged. The extinction at 663 mμ was used to calculate the content of chlorophyll-*a*.[15] Chlorophyll was used only as a reference substance so that systematic errors—for example that due to chlorophyll-*b*—were unimportant.

[8] G. LAMBERTSEN, H. MYLKESTAD and O. R. BRAEKKAN, *J. Sci. Food Agr.* **13**, 617 (1962).
[9] J. GREEN, S. A. PRICE and L. GARE, *Nature* **184**, 1339 (1959).
[10] M. GRIFFITHS, W. R. SISTROM, G. COHEN-BAZIRE and R. Y. STANIER, *Nature* **176**, 1211 (1955).
[11] V. H. BOOTH, *Analyst* **84**, 464 (1959).
[12] V. H. BOOTH, *Carotene, its Determination in Biological Materials*. W. Heffer & Sons, Cambridge (1957).
[13] J. GREEN, S. MARCINKIEWICZ and P. R. WATT, *J. Sci. Food Agr.* **6**, 274 (1955).
[14] V. H. BOOTH, *Analyst* **88**, in press (1963).
[15] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).

Preparation of Chloroplasts

Chloroplasts were isolated from leaves by maceration under buffered sodium chloride solution followed by differential centrifugation, all manipulations being carried out at approximately 0°.[1]

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