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THE DISTRIBUTION AND STABILITY OF α-TOCOPHEROL IN SUBCELLULAR FRACTIONS OF BROAD BEAN LEAVES

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Abstract—A method is described which enables the preparation of chloroplasts with the minimum enzymic breakdown of α-tocopherol. Using this method, it was shown that α-tocopherol is confined to the chloroplasts of young broad bean leaves. Fifty per cent of this α-tocopherol is removed from the chloroplast lamella by treatment with digitonin. This α-tocopherol is not located in plastoglobuli and is not removed from the lamellae by sonication.

INTRODUCTION

α-TOCOPHEROL (α-T) and α-tocopherolquinone (α-TQ) are present in all photosynthetic tissues except the photosynthetic bacteria and the blue-green alga *Anacystis nidulans*[1–6] but little is known of the function of either compound. It has been suggested that α-T may be an anti-oxidant,[7] an intermediate in photosynthetic electron transport[8] and a possible growth substance,[9] but it has not been proved that it performs any of these functions in plants. α-T is readily converted to α-TQ by gentle oxidation and this may be a functionally important reaction but there is no evidence that this conversion occurs *in vivo*. Before considering the function of a compound in a biological system it is important that its intracellular location be established; α-TQ is localized exclusively in the chloroplasts of young broad bean leaves[10] but there are conflicting reports about the intracellular location of α-T. Dilley and Crane[11] fractionated spinach leaves and from their results concluded that α-T was present in both chloroplasts and mitochondria. Booth[12] selected *Iris* leaves for study as this species lacked an enzyme which consumed α-T during the disruption of more commonly encountered tissues and showed that α-T is confined to the chloroplasts. Lichtenthaler[13] also noted that 90 per cent of the α-T in *Beta vulgaris* leaves is localized in the chloroplasts but unfortunately gave no details of the media used. The discovery of α-T in plastoglobuli,[14] which may be released from broken chloroplasts during isolation procedures, further confuses the issue.

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This paper describes a method for preparing chloroplasts from broad bean leaves without losing α-T by enzyme action and reports the results of experiments determining the intracellular and intrachloroplastic location of α-T.

RESULTS

Chloroplasts were isolated in a medium containing 0·33 M sucrose buffered with phosphate buffer, pH 6·8. These chloroplasts, from both broad bean and pea leaves, had a lower level of α-T (relative to chlorophyll) than acetone extracts of leaves harvested at the same time from plants of the same age grown in similar conditions (Table 1). This suggested that

TABLE 1. α-T AND CHLOROPHYLL LEVELS IN TOTAL LIPID EXTRACTS AND CHLOROPLASTS ISOLATED AT pH 6·8

Species	Sample	α-T (μmoles)	Chlorophyll (μmoles)	α-T:chl (μmoles/mmole)
Broad bean	Total lipid extract	0·72	10·5	69·0
	Chloroplasts	0·15	6·6	22·0
Pea	Total lipid extract	0·75	30·4	24·6
	Chloroplasts	0·063	6·15	10·2

Total lipid extracts prepared by grinding 12-g broad bean leaves or 25-g pea leaves in acetone. Chloroplasts were isolated from 50-g leaves in 0·33 M sucrose buffered with 0·1 M KH_2PO_4–NaH_2PO_4 at pH 6·8. For methods of extraction and estimation of α-T (see text).

α-T was lost during the isolation procedure, either as a result of enzyme action or as a result of physical breakdown of the chloroplast. Booth[12] noted that α-T was broken down by enzyme action in chloroplasts isolated at pH 6·8, but did not consider the effect of altering the pH of the grinding medium. Table 2 shows the effect of pH on the recovery of the endo-

TABLE 2. EFFECT OF pH ON THE RECOVERY OF α-TOCOPHEROL FROM HOMOGENATES OF BROAD BEAN LEAVES

Grinding medium	Chlorophyll (μmoles)	α-T	α-T:chl (μmoles/mmole)
Acetone	18·63	0·605	24·75
pH 5·6	7·61	0·014	1·84
pH 6·0	6·46	0·004	0·62
pH 6·4	6·20	0·033	5·32
pH 6·8	7·72	0·072	9·33
pH 7·2	6·34	0·146	30·28

15-g samples of leaves from plants aged 26 days were homogenized for 5 sec with 100 ml of medium containing 0·3 M sucrose buffered at the appropriate pH with 0·05 M ADA–NaOH, filtered through four layers of organdie and stirred at 20° for 15 min. Extraction as described in the text.

genous α-T from homogenates of broad bean leaves stirred for 15 min at room temperature: clearly there is considerable breakdown of α-T at pH 6·8 and below, but at pH 7·2 more α-T is recovered from the homogenate than from a control homogenized in acetone. The absence of enzymic breakdown of α-T at pH 7·3 was confirmed by incubating a homogenate of broad bean leaves at 25° for 90 min and examining aliquots at intervals (Table 3). It is clear that very little, if any, α-T is broken down and that the α-T and α-TQ levels vary together and not at one another's expense.

TABLE 3. EFFECT OF INCUBATION AT pH 7·3 ON α-T AND α-TQ LEVELS IN A HOMOGENATE OF BROAD BEAN LEAVES

Time (min)	Chlorophyll	α-T (μmoles)	α-TQ	α-T:chl (μmole/mmole)	α-TQ:chl	α-TQ:α-T (mole/mole)
0	10·41	0·47	0·11	45·5	10·4	0·23
10	10·34	0·62	0·16	60·3	15·2	0·25
20	10·90	0·64	0·14	59·0	13·1	0·22
30	10·94	0·67	0·16	60·9	14·5	0·24
50	11·14	0·58	0·13	52·2	12·0	0·23
90	10·20	0·61	0·15	59·4	14·3	0·24

100-g leaves from plants aged 35 days were ground for 1 min in a Townson and Mercer macerater with 400 ml 0·3 M sucrose buffered at pH 7·3 with 0·1 M tricine–NaOH. The homogenate was stirred at 25° and 50 ml aliquots were removed at the times indicated and extracted as described in the text.

Booth[15] also noted that enzymic breakdown of α-T occurs during even the brief period of comminution necessary to extract the plant lipids into a lipid solvent: such a breakdown may occur during homogenation in aqueous media. The results in Table 4 demonstrate that

TABLE 4. RECOVERY OF α-T FROM BEAN LEAF HOMOGENATES PREPARED IN DIFFERENT MEDIA

Experiment	Medium	α-T	Chlorophyll (μmoles)	α-T:chl (μmoles/mmole)
1	Acetone	0·14	14·54	9·63
	Sucrose–tricine	0·23	10·02	22·95
	NaCl–phosphate	0·17	9·71	17·51
2	Acetone	0·12	13·59	8·83
	Sucrose–tricine	0·22	9·80	22·45
	NaCl–phosphate	0·15	8·36	17·94

60-g leaves in each experiment were divided into three portions, which were each homogenized with 100 ml of the medium indicated for 60 sec in the Townson–Mercer macerater, filtered through four layers of organdie into 400 ml of acetone and extracted as described in the text.
"Sucrose–tricine" = 0·3 M sucrose in 0·067 M tricine–NaOH, pH 7·3;
"NaCl–phosphate" = 0·35 M NaCl in 0·1 M Na phosphate, pH 7·3.

[15] V. H. BOOTH, *J. Sci. Food Agri.* **11**, 8 (1960).

more α-T is recovered from homogenates prepared in 0·3 M sucrose buffered with 0·067 M tricine, pH 7·3, than from 0·35 M NaCl–0·1 M phosphate, pH 7·3 (similar to the medium used by Booth[12] at pH 6·8), or from an acetone extract. Thus comminution in a sucrose–tricine medium at pH 7·3 seems the most likely method of obtaining an indication of the true intracellular level of α-T.

Table 5 shows the distribution of α-T relative to chlorophyll in subcellular fractions of broad bean leaves obtained in three separate experiments. In each case the α-T:chlorophyll ratios in preparations of chloroplasts and chloroplast fragments and mitochondria (15,000 × *g* fraction) are similar and neither chlorophyll nor α-T could be detected in the supernatant after sedimentation of the 15,000 × *g* fractions. There is no evidence from these results for a site of α-T outside the chloroplast.

TABLE 5. α-T LEVELS RELATIVE TO CHLOROPHYLL IN THREE SUBCELLULAR FRACTIONS OF BROAD BEAN LEAVES

Experiment	α-T:chl (μmoles/mmole) Chloroplasts	(15,000 × *g*)	Supernatant
1	33·40	37·60	Neither compound detected
2	40·81	38·07	
3	54·26	47·81	

Age of beans: experiment 1, 21 days; 2, 28 days; 3, 31 days. Fractions prepared in 0·3 M sucrose buffered at pH 7·3 with 0·067 M tricine–NaOH and extracted as described in the text.

The location of α-T within the chloroplast lamella was studied by fragmenting chloroplasts with 0·5 per cent digitonin by a method similar to that of Boardman and Anderson,[16] but using a buffered 0·3 M sucrose solution so that the fragmentation was due only to the digitonin and not to osmotic shock. The results in Table 6 show that approximately half

TABLE 6. DISTRIBUTION OF CHLOROPHYLL AND α-T IN CHLOROPLAST FRAGMENTS OBTAINED BY DIGITONIN TREATMENT

Fraction	Chlorophyll (μmoles)	α-T	α-T:chl (μmoles/mmole)	% of total α-T
1,000 × *g* (10 min)	19·53	0·39	19·97	16·96
10,000 × *g* (30 min)	15·77	0·41	26·00	17·83
50,000 × *g* (30 min)	7·01	0·17	24·25	7·39
70,000 × *g* (60 min)	1·98	0·12	60·61	5·22
Supernatant	2·54	1·21	476·38	52·61
Untreated	5·72	0·24	41·96	

Beans aged 18 days. Chloroplasts isolated in 0·3 M sucrose–0·067 M KH_2PO_4–Na_2HPO_4, pH 7·3, as described in text, resuspended in the same medium + 0·5 per cent digitonin and incubated at 0° for 75 min. The digitonin solution was prepared without the use of ethanol.

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the chloroplast α-T is removed from the denser chloroplast particles by digitonin and is found in the 70,000 × *g* supernatant. The distribution of α-T between the fractions is similar to that found for the plastoquinones by Friend *et al.*,[17] who suggest that the high level of plastoquinones in the supernatant fraction may be due to the disruption of plastoglobuli by the digitonin. To test this theory in the case of α-T, broad bean chloroplasts were fragmented by sonication in a hypo-osmotic medium, a method which releases the plastoglobuli from chloroplasts.[18] Chloroplasts were treated with ultrasound for 15 and 45 sec and 3 min. Treatment for 15 sec disrupted all the chloroplasts, in 3 min there was slight fragmentation of lamellae. Table 7 summarizes the distribution of α-T and chlorophyll between the chloroplast fragments produced by sonication. No α-T was found in the final supernatant until

TABLE 7. DISTRIBUTION OF α-T AND CHLOROPHYLL IN FRACTIONS OF BROAD BEAN CHLOROPLASTS PRODUCED BY SONICATION

Duration of sonication	Fraction	Chlorophyll (μmoles)	α-T	α-T:chl (μmoles/mmole)
15 sec	Large fragments	10·83	0·41	37·49
	Small fragments	1·67	0·04	25·15
	Supernatant	0	0	0
45 sec	Large fragments	10·41	0·20	18·92
	Small fragments	1·97	0·05	27·91
	Supernatant	0	0	0
3 min	Large fragments	7·10	0·19	26·76
	Small fragments	6·74	0·05	7·72
	Supernatant	Trace	0·04	—

In each experiment chloroplasts were isolated from 55-g leaves of plants, aged 25 days, in 0·3 M sucrose buffered with 0·067 M tricine–NaOH, pH 7·3. The chloroplasts were resuspended in 0·067 M tricine–NaOH pH 7·3 and sonicated for the time stated using a Dawe Soniprobe generating 1·4 A at 20,000 kc/sec. "Large fragments" were precipitated in the same manner as the original chloroplasts, "small fragments" were sedimented at 171,000 × *g* for 18 hr. Light microscopy showed that all chloroplasts were broken after sonication for 15 sec.

the chloroplasts had been sonicated for 3 min. The α-T in the supernatant after digitonin fragmentation of broad bean chloroplasts did not, therefore, arise from the disruption of plastoglobuli. However, small particles, rich in α-T, can be removed from the lamellae by prolonged treatment with ultrasound. These results are consistent with the observations of Greenwood *et al.*[18] that chloroplasts of young broad bean leaves contained few plastoglobuli and, in fact, the final supernatants after sonication contained little lipid material. To demonstrate that plastoglobuli are released from chloroplasts by sonication for only 15 sec chloroplasts from *Pelargonium zonale* leaves were treated in this manner; the final supernatant after centrifugation at 171,000 × *g* included a visible layer of plastoglobuli and contained α-T.

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DISCUSSION

α-T and α-TQ are almost ubiquitous in aerobic photosynthetic organisms, are localized exclusively in the chloroplast and are largely confined to the chlorophyll-containing lamellae. The two compounds are so readily interconvertible that it is hard to believe that their functions *in vivo* are not closely related, yet no such close relationship has been demonstrated *in vivo*. The present work indicates that α-T and α-TQ concentrations vary together and not at one another's expense when bean leaf homogenates are incubated at pH 7·3, and Griffiths *et al.*[19] note that α-TQ is not formed at the immediate expense of α-T during the greening of etiolated maize seedlings. Similarly, Lichtenthaler[20] found that the ratio of α-T to α-TQ was similar in green and etiolated barley seedlings even though the level of each in the green seedlings was double that in the etiolated seedlings.

It is conceivable that α-TQ might function in photosynthetic electron transfer reactions and *in vivo* as much as 60 per cent of the total chloroplast α-TQ is in the form of the quinol[10] but it has been demonstrated that α-TQ can restore only cytochrome *c* reduction of the photochemical activities removed by extraction of chloroplasts with organic solvents.[21–24] It is even less likely that α-T is concerned directly in photosynthetic electron transfer; indeed it is probable that in every published experiment in which plastoquinone A was removed from chloroplasts by solvent extraction[25–27] most, if not all, of the α-T was also removed and yet the photochemical activities were restored by adding only pure plastoquinone A to the extracted chloroplasts.

The level of α-T relative to chlorophyll in the tissues used in these experiments varied widely, even though the tissues were of similar ages and were grown in similar conditions in a constant environment cabinet. The temperature, day-length and humidity were constant throughout, the only factor varying from experiment to experiment was the condition of the soil. This could explain the variations in α-T levels, since Coic *et al.*[28] noted that in tobacco plants α-T was present in significant amounts only in plants lacking nitrogen, and Booth[29] showed that the α-T level rises as tissues wilt: none of the tissues used here was wilting but the soil-water and nitrogen levels may have varied.

Lichtenthaler and Peveling[30] suggest that the plastoglobuli may be reservoirs for lipoquinones. The low level of plastoglobuli in chloroplasts of young broad bean leaves and the absence of α-T from them does not disagree with this theory since a rapidly growing tissue would presumably incorporate all the available α-T into the lamellae.

The elucidation of the nature of the function of α-T is the aim of further studies.

MATERIALS

Plant Tissues

Broad beans (*Vicia faba* L. var. Giant Windsor) were grown in John Innes No. 1 compost at 21° in a growth chamber in a regime of 16 hr light and 8 hr dark. Peas (*Pisum sativum* L.

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var. Laxton's Superb) were grown in vermiculite in the same growth chamber. Plants of a red-flowered variety of *Pelargonium zonale* were grown in a growth chamber in 13 hr light and 11 hr dark at 25°.

Chemicals

Diethyl ether, light petroleum (b.p. 40–60°) and ethanol were purified as described previously.[10] Acetone was distilled from activated charcoal to remove an impurity which can interfere with the determination of α-T.[31]

Activated neutral alumina (Woelm) was deactivated with 6·0 per cent (w/w) water. Thin layers of silica gel G (Macherey Nagel) were prepared by the application of a 1:2 slurry to glass plates 20 cm square using a Shandon spreader set at 0·25 mm thickness. DL-α-Tocopherol was purchased from Sigma Chemical Co., London, and α-TQ was prepared from it by oxidation with $AgNO_3$.[32] Tricine (N-tris-(hydroxymethyl)methylglycine)[33] and ADA (N-(2-acetamido)iminodiacetic acid)[34] were purchased from British Drug Houses, Poole, Dorset.

METHODS

Extraction of Tocopherols

The tocopherols were extracted from whole leaves by macerating them thoroughly with acetone in a Townson and Mercer top-drive macerater. Subcellular fractions were extracted by stirring them with sufficient acetone to give a final concentration of 80 per cent acetone. Further extraction of the acetone extracts was as described previously.[10]

Chromatography

Occasionally the tocopherols were partially purified by alumina chromatography: acetone extracts were dissolved in light petroleum and applied to 10-g columns of alumina, β-carotene was removed by elution with 0·5 per cent diethyl ether in light petroleum, then the tocopherols and quinones were eluted with 20 per cent diethyl ether in light petroleum. Routinely, total lipid extracts were dissolved in cyclohexane and aliquots were applied as bands to thin layers of silica gel G together with separate spots of authentic α-T; after development with 20 per cent heptane in benzene the marker α-T was located by covering the rest of the plate and spraying with 0·2 per cent ethanolic $FeCl_3$ + 0·5 per cent ethanolic αα′-dipyridyl (50–50) (Emmerie–Engel reagent).[35] The band corresponding to the marker α-T was scraped off the plate, eluted with 5 ml of spectroscopically pure ethanol and the α-T estimated. The recovery of α-T from thin layers of silica gel G was 101 per cent ± 2.

α-T was estimated by reduction with $NaBH_4$,[36] α-T by the method of the Analytical Methods Committee[37] and chlorophyll by the method of Arnon.[38]

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Isolation of Subcellular Fractions

The method used for chloroplast isolation was based on that of Walker:[39] 50-g tissue was ground with 200 ml medium for 10 sec at the full speed of an M.S.E. Atomix, the brei was filtered through four layers of white permanent fine muslin (organdie) and the filtrate centrifuged by allowing the 8×50 ml high-speed angle head of an M.S.E. 6L centrifuge to accelerate to 6000 rev/min and then braking it rapidly. The supernatant was centrifuged at $15{,}000 \times g$ for 1 hr in the 6×90 ml angle head of a Griffin-Christ Omikron centrifuge to produce a final supernatant and a precipitate containing chloroplast fragments and mitochondria.

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