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PHOTOSYNTHETIC APPARATUS OF CHILLING-SENSITIVE PLANTS

IX. THE INVOLVEMENT OF α-TOCOPHEROL IN THE ELECTRON TRANSPORT CHAIN AND THE ANTI-OXIDIZING SYSTEM IN CHLOROPLASTS OF TOMATO LEAVES *

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Summary

1. The role of tocopherols in tomato chloroplasts from fresh, cold and dark-stored as well as stored and illuminated leaves was studied.

2. The cold and dark storage of leaves results in a loss of chloroplast α- and γ-tocopherols of about 30—40% accompanied by an increase in chloroplast δ-tocopherol of about 40%. On illumination of stored leaves, an elevation of α- and γ-tocopherol level to about 110 and 95% of the control, respectively, occurs, whilst δ-tocopherol content is not affected.

3. Experiments performed with 2,2-diphenyl-1-picrylhydrazyl-treated chloroplasts show that only about 70% of total α-tocopherol is functionally active in the electron transport of Photosystem II between the diphenylcarbazide (DPC) donation site and the inhibition site of DBMIB.

4. A small amount of α-tocopherol quinone (about 10% of α-tocopherol content) is found in chloroplasts from fresh, fresh and illuminated as well as cold and dark-stored tomato leaves, whereas the illumination of the latter increases

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Abbreviations: PS I (II), Photosystem I (II); DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MDA, malondialdehyde; stored and illuminated leaves have been illuminated at 25°C (8000 lux) for 2 h following cold and dark storage for 3 days; cold and dark-stored leaves have been stored at 0°C in the dark for 3 days; Chl, chlorophyll.

the chloroplast α-tocopherol quinone content 3-fold. Moreover, following the illumination of chloroplasts from cold and dark-stored as well as stored and illuminated leaves, the oxidation of exogenous α-tocopherol to α-tocopherol quinone is 2-fold faster then in chloroplasts from fresh leaves.

5. The primary product ('α-tocopheroxide') formed during the α-tocopherol oxidation by illuminated chloroplasts was identified as 8a-hydroxy-α-tocopheron.

6. Exogenous α-tocopherol inhibits the lipid photoperoxidation by about 40—50% in chloroplasts from all three kinds of tomato leaf.

7. The results seem to suggest that chloroplast α-tocopherol is involved in both electron transport of PS II and antioxidizing system of chloroplasts.

Introduction

It was recently reported that tocopherols and/or their quinone forms may be involved in the chloroplast electron transport. The restoration of PS II activity by β- and γ-tocopherols, as measured in the presence of ferricyanide in acetone-extracted chloroplasts, was firstly proposed by Henninger et al. [1]. In contrast, Baszyński [2] and Baszyński and Tukendorf [3] pointed out that exogenous α-tocopherol restores completely both PS I activity and cyclic photophosphorylation in heptane-extracted spinach chloroplasts. The concentration of exogenous α-tocopherol required for the reconstitution of PS I suggests that α-tocopherol can play a structural role in restoration of thylakoid membranes. Moreover, as shown by Lichtenthaler [4], the level of α-tocopherol quinone (α-tocopherol oxidized form) in the photosynthetic membranes is equal to that of *P*-700 and cytochromes. In additions, Bishop [5] have reported that *Scenedesmus* mutant lacking α-tocopherol is unable to function normally. Recently, Barr and Crane [6] have proposed a new approach to the study of the role of α-tocopherol in spinach chloroplasts, using stable free radicals. They have postulated [6] that α-tocopherol is involved in the PS II electron transport chain between the DBMIB and DCMU inhibition sites. It is well known, however, that its quinone form (α-tocopherol quinone) rather than a chromanol (α-tocopherol) may be considered as a potential photosynthetic electron carrier [7].

It was also reported that in biological systems α-tocopherol may be an antioxidant which protects the membrane lipids against peroxidation (for reviews see Refs. 8 and 9). Singlet oxygen ($^1O_2$) initiates probably a photoperoxidation of chloroplast lipids, since illuminated chloroplasts produce superoxide radicals ($O_2^{\cdot-}$) [10] forming $^1O_2$ [11]. Furthermore, α-tocopherol can scavenge either $O_2^{\cdot-}$ [12,13] or $^1O_2$ [14]. In addition, α-tocopherol quinone was found among the oxidized products of scavenging [13,14].

In previous communications from our laboratory [15,16] it was reported that the cold and dark storage of detached tomato leaves results in an irreversible inactivation of chloroplast cyanide-sensitive superoxide dismutase accompanied by an increase of chloroplast lipid photoperoxidation intensity. The aim of the present paper was to study the bifunctional role of α-tocopherol (in lipid antioxidation and electron transport) in the superoxide dismutase depleted

chloroplasts following the cold and dark storage of tomato leaves. Preliminary results of these studies have been reported [15].

Material and Methods

Plant material

Leaves of tomato (*Lycopersicon esculentum* Mill. var. Eurocross and Revermoon) were harvested from the plants grown for commercial purpose under green-house conditions (relative humidity, 85%).

Cold and dark storage, reactivation with light, isolation of chloroplasts and determination of photochemical activities

Procedures for all treatments are described in the previous paper [17] (see also Abbreviations). PS II activities were measured in the presence of 12 μg DBMIB per mg chlorophyll (cf. Ref. 18).

Picrylhydrazyl treatment of chloroplasts

The effect of stable radical (2,2-diphenyl-1-picrylhydrazyl) on the tocopherol level in tomato leaf chloroplasts as well as the restoration of photochemical activities were assayed in two ways according to Barr and Crane [6]. Method A involved the direct addition of various amounts of picrylhydrazyl to the reaction medium containing chloroplasts. After a 5 min dark preincubation period at room temperature the electron acceptor (DCIP) reduction was measured in light. Method B involved incubation of chloroplast preparations with various amounts of picrylhydrazyl for 15 min at 4°C. After the incubation chloroplasts were washed with 50 ml solution containing 0.4 M sucrose, 20 mM NaCl and 50 mM Hepes buffer (pH 6.1) and precipitated by centrifugation at $1000 \times g$ for 10 min. Such chloroplasts are referred to as picryl-treated chloroplasts.

Separation and determination of tocopherol content

Both chloroplast tocopherols and α-tocopherol quinone were extracted and determined by the modified method of Newton and Pennock [19].

Extraction. Chloroplast preparations were macerated in acetone using a Potter-Elvehjem homogenizer with glass pestle and the residue was discarded after filtration. The extracts were shaken with an equal volume of diethyl ether. Two layers were then produced by the addition of distilled water. The ether fraction was washed with water and dried with 'water-free' sodium sulphate. The diethyl ether was evaporated under N_2 and the resulting lipid was stored under hexane.

Chromatography. Lipid samples (in hexane) were partially fractionated by the column chromatography with neutral alumina, Brockman grade III [20] to separate the different polyprenyl quinones and tocopherols from β-carotene as well as the more polar xanthophylls and chlorophylls. The fraction containing tocopherols was then examined by two-dimensional thin-layer chromatography on silica plates in chloroform followed by 18% diethyl ether in hexane. The individual compounds could be then identified by both position on the plates and using spray reagents: 0.01% Rhodamine 6G in ethanol; equal volume of

0.2% ferric chloride and 0.5% 2,2′-bipyridyl in ethanol (Emmerie-Engel reagent); leucomethylene blue in acetone. The individual tocopherols were estimated quantitatively with Emmerie-Engel reagent as described by Newton and Pennock [19]. α-Tocopherol quinone was estimated in ethanol by measuring the difference in absorbance (ΔA) at 261 nm before and after reduction with 0.1% aqueous KBH_4 solution. A millimolar extinction coefficient of 17.02 $mM^{-1} \cdot cm^{-1}$ was applied. A standard α-tocopherol quinone was obtained by the oxidation of α-tocopherol with a 3% ferric chloride solution in 90% ethanol [21].

Determination of chloroplast lipid photoperoxidation

The peroxidation of lipids was followed by the thiobarbituric acid method in which thiobarbituric acid reacts with malondialdehyde, a decomposition product of the oxidation of polyunsaturated fatty acids [22]. For determination of lipid photoperoxidation the method of Heath and Packer [23] was used applying an extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$ at 532 nm, corrected for nonspecific absorbance at 600 nm. The malondialdehyde level was determined in chloroplasts following their illumination for 5, 10, 15 and 20 min. Illumination (20 000 lux) was performed at 25°C in a reaction medium containing 0.4 M sucrose, 20 mM NaCl and 50 mM Hepes buffer (pH 7.0). The rate of photoperoxidation measured by the rate of malondialdehyde formation is expressed as nmol/min per mg of chlorophyll.

Other methods

The content of chlorophylls *a* and *b* was assayed by the method of Arnon [24]. Concentration of 2,2-diphenyl-1-picrylhydrazyl was determined spectrophotometrically at 520 nm using an extinction coefficient of 5.45 $mM^{-1} \cdot cm^{-1}$.

Reagents

2.2-Diphenyl-1-picrylhydrazyl was obtained from Aldrich Chemical Company. DPC was purchased from Eastman and was recrystallized from a water/methanol mixture. Neutral alumina (Al_2O_3) and silica plates were from Merck, F.R.G. Rhodamine 6G was obtained from BDH Chemicals while thiobarbituric acid was from Koch-Light, Ltd. DBMIB was kindly provided by Professor A. Trebst.

Results

Changes in chloroplast tocopherol content following the cold and dark storage of tomato leaves and their illumination

As is shown in Table I there occur four compounds of the tocopherol (vitamin E) family in tomato leaf chloroplasts. The α-tocopherol was found to be about 5- and 8-times more plentiful than the δ- and γ-tocopherols, respectively, whereas the α-tocopherol quinone content in these chloroplasts accounts for about 10% of α-tocopherol. The storage at 0°C in the dark of tomato leaves resulted in marked decrease of the γ- and α-tocopherol content (35 and 25% of the control, respectively), as well as an increase of about 40% in δ-tocopherol level, while it does not affect the chloroplast α-tocopherol quinone content.

TABLE I

EFFECTS OF STORAGE AND ILLUMINATION OF TOMATO LEAVES ON THE EXTENT OF CHANGES IN CHLOROPLAST TOCOPHEROLS, α-TOCOPHEROL QUINONE CONTENTS AND PHOTOSYSTEM II ACTIVITIES

Values of the representative experiments as well as the extremal values found in the indicated number of experiments (in parentheses) performed are included. Photochemical activities measured in the presence of DBMIB (12 μg per mg of chlorophyll) are expressed as μmol DCIP reduced per h per mg of chlorophyll. δ-, γ-, α-T, tocopherols containing one, two or three methyl groups substituted to the chroman ring, respectively. α-TQ, α-tocopherol quinone.

Source of chloroplasts	Photochemical activities		Tocopherol content (nmol per mg of chlorophyll)				
	$H_2O \rightarrow$ DCIP	DPC → DCIP	δ-T	γ-T	α-T (n = 5)	α-TQ (n = 5)	α-T + α-TQ
Fresh leaves	107.0	114.0	10.7 (5) 10.0—12.3	7.4 (5) 7.0—8.1	57.3 55.1—62.0	5.6 5.5— 7.2	62.9
Fresh and illuminated leaves *	99.0	108.5	12.7 (4) 11.9—15.0	9.1 (4) 7.8—9.2	60.5 59.4—65.7	6.5 6.5— 7.4	67.0
Stored leaves	6.0	106.0	15.3 (5) 14.5—17.5	4.9 (5) 4.5—5.8	44.1 44.0—46.6	5.9 5.7— 6.9	50.0
Stored and illuminated leaves	97.0	110.5	14.7 (5) 14.7—15.8	7.1 (4) 5.8—7.2	62.8 61.0—66.9	15.6 12.9—16.3	78.4

* For the procedure of the leaves treatment see Abbreviations and symbols.

Upon illumination of the cold and dark-stored leaves the chloroplast levels of the γ- and α-tocopherols increase by 95 and 110% over the control, respectively, while the δ-tocopherol content remains practically the same as in chloroplasts from cold and dark-stored leaves. In chloroplasts from stored and illuminated leaves, the α-tocopherol quinone level is 3-fold higher than that in chloroplasts from fresh leaves. The illumination of fresh leaves results in a small increase of α-tocopherol and the other studied tocopherols by 5 and 20% of the control, respectively.

The data of Table I indicate also that changes in tocopherol contents following the cold and dark treatment of leaves are accompanied by the inactivation of $H_2O \rightarrow$ DCIP reaction activity. This is due mainly to a depletion up to 50% of chloroplast manganese [25]. Illumination of cold and dark-stored leaves restores both $H_2O \rightarrow$ DCIP reaction activity as well as the manganese level. However, when DCIP reduction was measured in the presence of DPC, which donates electrons beyond the site damaged by the cold and dark storage of leaves [17] DCIP reduction is not affected by storage of leaves in the cold and dark (Table I). Therefore the photochemical activity of chloroplasts was measured with DPC as an electron donor.

Effect of 2,2-diphenyl-1-picrylhydrazyl treatment of chloroplasts on PS II activity and on α-tocopherol content

Barr and Crane [6] reported that a stable nitrogen free radical, 2,2-diphenyl-1-picrylhydrazyl reacts with α-tocopherol in spinach chloroplast membrane

resulting in an inhibition of PS II activity. As is shown in Fig. 1 the treatment (the method A, see Methods) of chloroplasts from fresh, cold and dark-stored as well as stored and illuminated tomato leaves with an increasing amount of picrylhydrazyl results in an inhibition of DPC → DCIP activity up to 50% of the control at 0.6 μmol of picrylhydrazyl per mg of chlorophyll. The marked inhibition of DPC → DCIP activity (by about 30% of the control) is observed in chloroplasts isolated from leaves stored at 0°C in the dark, in the presence of picrylhydrazyl concentration as low as 0.3 μmol per mg of chlorophyll. In contrast, in chloroplasts from fresh as well as stored and then illuminated leaves, this activity is not affected by this concentration of picrylhydrazyl.

Picrylhydrazyl is known to react specifically with tocopherol in lipid mixture [6,26]. Thus, when α-tocopherol is added to the picryl-treated chloroplasts a partial reversal of inhibition of DCIP reduction by PS II in chloroplasts from cold and dark-stored tomato leaves is observed (Fig. 1). The addition of α-tocopherol results also in a partial restoration of DCIP reduction activity in all kinds of chloroplasts treated with higher concentration of picrylhydrazyl (up to 0.6 μmol per mg of chlorophyll).

When the changes in both DCIP reduction by PS II and α-tocopherol content of picryl-treated chloroplasts from fresh, cold and dark-stored as well as stored and illuminated leaves were studied by the method B (see Methods) the same pattern of the inhibition of DPC → DCIP activity in chloroplasts as that obtained by the method A is observed (Fig. 2).

A reversal by α-tocopherol of inhibitory effect of picrylhydrazyl on DCIP reduction activity is probably due to a restoration of picrylhydrazyl damaged

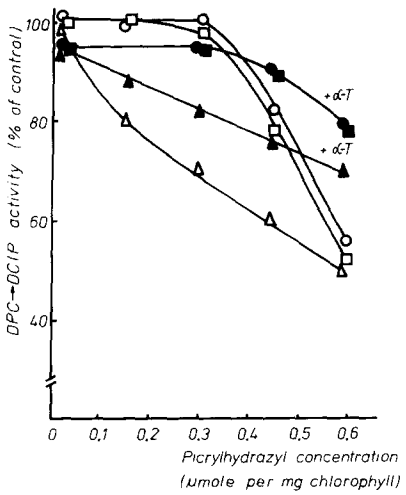

Fig. 1. Inhibition by stable radical 2,2-diphenyl-1-picrylhydrazyl of DCIP reduction in chloroplasts and restoration this activity upon the addition of α-tocopherol (α-T). The reaction medium (3 ml) contained 0.4 M sucrose, 20 mM NaCl, 50 mM Hepes buffer (pH 7.0), chloroplast preparation equivalent to 50 μg of chlorophyll and 300 μM DPC as an electron donor. Radical was added to the reaction medium in ethanol at the indicating amounts according to method A (see Methods). The PS II activity was measured before and after 2 min preincubation of chloroplasts with 200 μM α-tocopherol (empty and black symbols, respectively). Photochemical activity of control chloroplasts from fresh (○ and ●), cold and dark-stored (△ and ▲), as well as stored and illuminated (□ and ■) tomato leaves was 85.0, 80.1 and 84.5 μmol DCIP reduced per h per mg of chlorophyll, respectively.

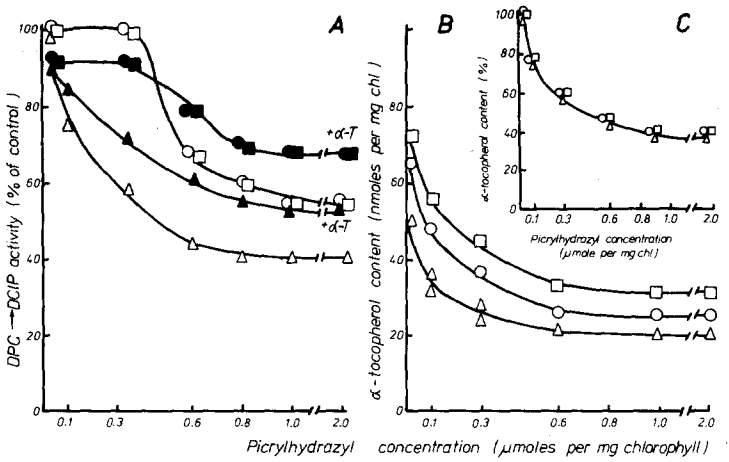

Fig. 2. Effects of picrylhydrazyl treatment of chloroplasts on PS II activity (A) and chloroplast α-tocopherol content (B,C). Treatment of chloroplasts with radical was performed according to method (B) (see Methods). α-Tocopherol (α-T) was added to picryl-treated chloroplast preparations to final concentration of 200 μM. The PS II activity in the presence of 300 μM DPC was measured in the reaction medium specified in legend to Fig. 1 before and after 2-min preincubation with α-tocopherol (empty and black symbols, respectively). The photochemical activity of control chloroplasts from fresh (○ and ●), cold and dark-stored (△ and ▲) as well as stored and illuminated (□ and ■) tomato leaves was 110.5, 105.3 and 108.4 μmol DCIP reduced per h per mg of chlorophyll, respectively. α-Tocopherol content was estimated in picryl-treated chloroplasts as described in Methods.

α-tocopherol site(s) in the membrane, although the reaction of α-tocopherol added with the residual amount of picrylhydrazyl adsorbed to the chloroplast membrane should be also considered. Treatment of chloroplasts with picrylhydrazyl results in the α-tocopherol extraction (Fig. 2B and cf. Ref. 6) by about 50—60% of the control. It is striking that the extent of α-tocopherol removal (Fig. 2C) is similar in chloroplasts of all kinds of leaf studied. These findings indicate that (i) only part (about 60%) of endogenous α-tocopherol content is accessible to picrylhydrazyl action, probably due to its location near the thylakoid membrane surface, and (ii) the picrylhydrazyl affects the membrane rather specifically since up to 40% of the photochemical activity remains following the treatment of chloroplasts with high (up to 2.0 μmol per mg of chlorophyll) concentrations of the radical (Fig. 2A). All these results suggest that only that part of α-tocopherol which remains following both cold and dark storage of leaves (Table I) and treatment of chloroplasts with 0.3 μmol of picrylhydrazyl per mg chlorophyll (Fig. 2B and C) (i.e. about 60—70% of the total) seems to play a structural role in the photosynthetic membranes.

Photooxidation of α-tocopherol in chloroplast preparations

Yagi and coworkers [12,13] reported that α-tocopherol dispersed in micelles of deoxycholate reacts with superoxide (O_2^-) generated in solution and results in spontaneous formation of α-tocopherol quinone [13].

On the other hand, it was also found [16] that (i) chloroplast cyanide-sensitive superoxide dismutase, a primary defence against O_2^-, is completely inactivated following the cold and dark storage of tomato leaves and (ii) the illumina-

tion of the stored leaves does not reactivate this activity. Thus, by cold and dark treatment of tomato leaves one can obtain chloroplasts suitable for studies of α-tocopherol as superoxide scavenger in the membrane. Moreover, a 3-fold higher α-tocopherol quinone content found in chloroplasts isolated from cold and dark-stored and then illuminated leaves depleted of superoxide dismutase as compared with that in chloroplasts from fresh leaves (Table I) suggests an active oxidation of α-tocopherol.

As is shown in Table II the dark incubation for 40 min of isolated chloroplasts of all kinds of leaf with exogenous α-tocopherol results in only small increase of α-tocopherol quinone content. In contrast, the illumination of such chloroplast preparations for the same time markedly stimulate accumulation of α-tocopherol quinone in the reaction medium. Furthermore, the highest stimulation by light (about 3-fold) is observed in chloroplasts isolated from cold and dark-stored as well as stored and illuminated leaves as compared with chloroplasts from both fresh and fresh-illuminated leaves (about 2-fold stimulation). These results indicate that α-tocopherol quinone formation in this system is dependent upon the illumination of chloroplasts when there is no superoxide

TABLE II

FORMATION OF α-TOCOPHEROL QUINONE IN THE PRESENCE OF EXOGENOUS α-TOCOPHEROL FOLLOWING THE DARK AND LIGHT INCUBATION OF CHLOROPLASTS FROM FRESH, FRESH-ILLUMINATED, COLD-DARK STORED AND STORED-ILLUMINATED TOMATO LEAVES

Incubation was performed for 40 min at 25° C either in dark or in light (20 000 lux) in a reaction medium (10 ml) containing 0.4 M sucrose, 20 mM NaCl, 50 mM Hepes buffer (pH 7.0), 60 nmol of α-tocopherol per mg of chlorophyll and chloroplasts corresponding to 200 μg of chlorophyll per ml. After the incubation period, the reaction medium was extracted with acetone and diethyl ether. The ether fraction was evaporated, the residue was dissolved in hexane and then subjected to further purification as described in Methods. α-TQ formation does not proceed in the light in control medium without chloroplasts.

Source of chloroplasts	Hill reaction activity (μmol DCIP reduced per h per mg Chl)	α-Tocopherol quinone content (nmol per mg Chl)					
		Control	Incubation				
			In dark		In light		Stimulation factor
			Content	% of control	Content	% of control	
Experiment A							
Fresh leaves	91.0	5.6	8.0	143	16.1	294	2.0
Fresh-illuminated leaves	103.0	6.9	8.4	124	18.1	262	2.2
Stored leaves	9.0	5.8	8.3	143	23.4	403	2.7
Stored-illuminated leaves	88.0	15.3	18.4	120	55.4	361	3.0
Experiment B							
Fresh leaves	78.0	4.2	6.2	149	10.0	240	1.6
Fresh-illuminated leaves	75.5	5.9	8.0	135	14.1	238	1.8
Stored leaves	6.5	4.8	7.1	146	20.0	416	2.9
Stored-illuminated leaves	69.5	13.0	18.0	138	48.4	372	2.7

dismutase activity. Thus, it seems that α-tocopherol quinone can be a product of α-tocopherol oxidation by superoxide radical produced in chloroplasts in light [10,11].

When the chloroplast preparations from both kinds of leaves were illuminated with exogenous α-tocopherol another product of reaction was found beside the α-tocopherol quinone. This compound 'α-tocopheroxide' (R_F 0.40, compound A) migrates on thin-layer silica chromatograms developed in the hexane/diethyl ether (4 : 1, v/v) system ahead of α-tocopherol (R_F 0.33, compound B) and does not react with Emmerie-Engel reagent. 'α-Tocopheroxide' disappears following the prolonged illumination of chloroplasts and is probably converted to α-tocopherol quinone (R_F 0.09, compound C). It is interesting that this compound (A) is already formed in chloroplasts from cold and dark-stored as well as stored and illuminated leaves after 10 min of illumination and is completely converted to α-tocopherol quinone after a further 10 min, whereas in the case of chloroplasts from fresh leaves 'α-tocopheroxide' was not found before 30 min of illumination. Prolonged exposition of chloroplasts from fresh leaves to light caused also conversion of the primary oxidation product to α-tocopherol quinone.

The ultraviolet spectra of both α-tocopherol (B) and products (A, C) of its oxidation are presented in Fig. 3. The maximum absorption of the primary product (A) in ethanol was found at 238—240 nm (Fig. 3A). When this compound was acidified by HCl (600 μM final concentration) it was converted to α-toco-

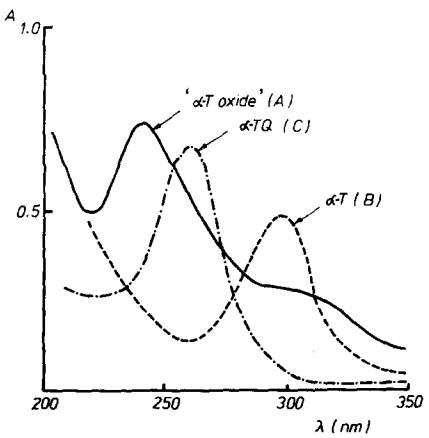

Fig. 3. Ultraviolet spectra of the oxidation products of α-tocopherol. The oxidation of α-tocopherol was performed by illumination of chloroplasts (20 000 lux at 25°C) isolated from fresh, cold and dark-stored as well as stored and illuminated tomato leaves. Chloroplasts from both stored as well as stored and illuminated tomato leaves (equivalent to 200 μg of chlorophyll per ml) were illuminated for 0, 10 and 20 min in 10 ml reaction medium containing 0.4 M sucrose, 30 mM NaCl, 50 mM Hepes buffer (pH 7.0), chloroplast equivalent to 200 μg of chlorophyll per ml and 60 nmol of α-tocopherol per mg of chlorophyll. The chloroplasts from fresh leaves were illuminated for 0, 30 and 40 min (see text). Immediately after illumination of chloroplasts the reaction medium was extracted as described in Methods. The ether fraction was evaporated and the residue dissolved in small volume of hexane was subjected to thin-layer chromatography on a silica plate (0.25 mm thick) uisng hexane/diethyl ether (4 : 1, v/v) as a solvent. Compounds were detected by spraying them with either 0.01% Rhodamine 6G in ethanol or with Emmerie-Engel reagent. A, B and C indicate the compounds eluted from the plate for spectrophotometric analysis (see text). The ultraviolet spectra of these compounds were recorded in ethanol with an Acta III Beckman spectrophotometer. 'α-T oxide', 'α-tocopheroxide'; α-TQ, α-tocopherol quinone.

TABLE III

INHIBITION BY α-TOCOPHEROL OF LIPID PHOTOPEROXIDATION IN CHLOROPLASTS FROM FRESH, COLD AND DARK-STORED AND STORED AND ILLUMINATED TOMATO LEAVES

Chloroplasts were preincubated for 5 min with α-tocopherol (α-T) (about 60 μmol per mg of chlorophyll) in the dark, at 25°C and then malondialdehyde (MDA) production was estimated following illumination of chloroplasts for 5, 10, 15 and 20 min. Illumination of chloroplasta (100 μg chlorophyll per ml) was performed as described in Methods. Hill reaction activity is expressed in μmol DCIP reduced per h per mg of chlorophyll. It was not affected by the preincubation of chloroplasts with α-tocopherol.

Source of chloroplasts	Hill reaction activity	Photoperoxidation (nmol MDA/min per mg Chl)		% of inhibition by α-T
		−(α-T)	+(α-T)	
Experiment A				
Fresh leaves	90.6	2.2	1.5	42
Stored leaves	9.4	4.3	1.9	56
Stored and illuminated leaves	84.0	4.1	2.3	44
Experiment B				
Fresh leaves	79.0	2.1	1.4	35
Stored leaves	5.1	4.9	2.5	49
Stored and illuminated leaves	74.5	5.0	3.2	36

pherol quinone. Although these findings substantiate the conclusion that the primary product consists of 8a-hydroxy-α-tocopheron [13,27], in our hands it was not completely reduced to α-tocopherol by the addition of ascorbic acid as would be expected for this compound [27].

Effect of exogenous α-tocopherol on lipid photoperoxidation

It was found previously [16] that the enhanced photoperoxidation of chloroplast lipids may result from the inactivation of cyanide-sensitive superoxide dismutase activity which may be considered as an endogenous effector controlling the rate of photoperoxidation. Since α-tocopherol is present in the chloroplast membranes [1—7] we used it as an anti-oxidant for the experiment presented in Table III. As can be seen, the exogenous α-tocopherol inhibits the light-induced malondialdehyde formation by about 35—55% of the control when added in high excess to chloroplast preparations isolated from fresh, cold and dark-stored, and stored and illuminated leaves. An excessive amount of α-tocopherol does not affect the Hill reaction activity. In order to check the specificity of α-tocopherol action we have added the same amount of monogalactosyl diacylglycerol, a neutral chloroplast lipid. However, it affected neither the Hill reaction nor the light-driven malondialdehyde formation.

Exogenous α-tocopherol may be easily oxidized following chloroplast illumination (Table II) and may inhibit superoxide-dependent photoperoxidation (Table III). Thus, it seems likely that this compound can react with the superoxide generated in chloroplasts.

Discussion

It is commonly accepted that (i) α-tocopherol is a major tocopherol in photosynthetic tissues [2,3,19,28,29] and (ii) it is accumulated parallel to

chlorophyll during light-induced thylakoid formation [28]. The α-tocopherol content in tomato leaf chloroplasts (about 60 nmol per mg of chlorophyll) was found to be several times higher than that of other chloroplasts tocopherols (Table I). Its level is similar to this determined in spinach chloroplasts [30] but exceeds 3—5 times that measured in chloroplasts from both pea and bean leaves [19,31]. The discrepancies found by various authors with respect to α-tocopherol content in the same plants can be due to the different conditions of illumination and/or different age of tissues used for experiments [32].

The cold and dark storage of tomato leaves results in a decrease of γ- and α-tocopherol levels about 35% accompanied by an increase of the δ-tocopherol content by about 40%. The illumination of stored leaves for 2 h restores the γ- and α-tocopherol levels, whereas it does not effect δ-tocopherol amount. These phenomena can be explained on the basis of the location of tocopherol in plant cell. Firstly, γ- and α-tocopherols are synthesized in chloroplasts and their formation is stimulated by light [19,33,34], so their contents are changed following the cold and dark storage as well as illumination of leaves. Secondly, the monomethylated tocopherol precursors (including δ-tocopherol) are formed directly from homogentisic acid [35,36] probably in two independent pathways [33,37] operating on the chloroplast envelope [38]. Thus, the accumulation of δ-tocopherol may indicate that the final methylation steps do not accur during the cold and dark storage of leaves (Table I).

Using the free radical, 2,2-diphenyl-1-picrylhydrazyl, as both an electron transport inhibitor (Fig. 1 and 2A) and α-tocopherol extractor (Fig. 2B and C) it is shown in this work that α-tocopherol, a predominant chloroplast tocopherol, plays a functional role in the PS II dependent electron transport of tomato chloroplasts. This is in agreement with reports by Barr and Crane [6] on spinach chloroplasts. However, it is found that only about 70% of total chloroplast α-tocopherol content is involved in electron flow, i.e., that part of it which is not affected by the cold and dark treatment of tomato leaves (Table I and Figs. 1 and 2). These results seem to suggest that α-tocopherol molecules exist in the chloroplast membranes as a stoichiometrically excessive 'pool' similar to that of plastoquinones [18]. However, there is also a possibility that the part of α-tocopherol is located in chloroplast outside of the thylakoid membrane, e.g. in osmophilic plastoglobuli [4,39]. This hypothesis can explain the lack of the effect of treatment of chloroplasts with low concentration of picrylhydrazyl on photochemical activity (Fig. 1).

Both α-tocopherol and α-tocopherol quinone, which form a lipophilic redox system in chloroplasts, appear to excist in equilibrium [21]. In chloroplasts from fresh tomato leaves α-tocopherol quinone accounts only about 10% of α-tocopherol content. In contrast, following the cold and dark storage and then illumination of leaves there is the 3-fold increase of chloroplast α-tocopherol quinone content (Table I). Moreover, illumination in the presence of exogenous α-tocopherol of chloroplast preparations isolated from both cold and dark-stored as well as stored and illuminated leaves results in a faster production of α-tocopherol quinone than in those from fresh leaves (Table II). Thus, similarly to observations by Yagi and coworkers [12,13] the formation of α-tocopherol quinone appears to be facilitated by an increased level of superoxide anion due to an inactivation of chloroplast superoxide dismutase activity following cold and dark storage of tomato leaves [16].

The antioxidizing function of α-tocopherol is evident from the inhibition (by about 50% of the control) of superoxide dependent chloroplast lipid photoperoxidation (Table III), as it was also observed in the presence of exogenous superoxide dismutase [15].

Following the illumination of chloroplast preparations in the presence of exogenous α-tocopherol a primary product of its oxidation was detected. Based on the spectrophotometric and chromatographic analysis it is defined probably as 8a-hydroxy-α-tocopheron, recently found by Nishikimi et al. [13] in the reaction medium following oxidation of dispersed α-tocopherol with superoxide anion in vitro. This primary product was formed more quickly in chloroplast preparations from cold and dark-stored as well as stored and illuminated leaves (i.e. when superoxide dismutase is inactivated) than in those from fresh leaves.

All these findings suggest that in chloroplasts α-tocopherol can play a role as a lipophilic antioxidant and its presence in the membranes may provide also the basic environment for electron transport.

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