Communication

Accumulation of \( \alpha \)-Tocopherol in Senescing Organs as Related to Chlorophyll Degradation

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ABSTRACT

\( \alpha \)-Tocopherol (\( \alpha \)-T) has been identified, using gas chromatography-mass spectroscopy and \( ^1 \)H- and \( ^13 \)C-nuclear magnetic resonance, in senescing leaves of \textit{Melia azedarach} L. The content of \( \alpha \)-T increased concomitantly with the breakdown of chlorophyll in senescing \textit{Vinca} and \textit{Melia} leaves. An increase in \( \alpha \)-T was found also in detached \textit{Melia} leaves, senescing in either light or darkness and in senescing, ethylene-treated orange leaves and fruit. The possibility that phytol, which is released from chlorophyll by chlorophyllase is utilized for the biosynthesis of \( \alpha \)-T is discussed. Senescing leaves of the low chlorophyll plants, parsley and tobacco, did not contain \( \alpha \)-T in measurable amounts.

Chl degradation occurs widely in nature as part of the senescence and ripening of green plant organs. The exact fate of the Chl molecule is not known, however (see Refs. 10 and 20 for recent reviews). Removal of the phytol by chlorophyllase has been suggested to be one of the earliest degradative steps, at least in certain plant species (2, 14).

Notwithstanding the progress made in recent years (13, 16) further identification of breakdown intermediates seems to be the most important task for the elucidation of the pathway of Chl catabolism.

In search for compounds which accumulate during the course of the disappearance of Chl we were intrigued by a peak in our HPLC system which eluted between Chl \( a \) and \( b \) and which increased markedly during senescence.

In the present paper we report the identification of this peak as \( \alpha \)-T\(^1\) and the accumulation of this compound during senescence in several plant species.

MATERIALS AND METHODS

Plant Material

Leaves of \textit{Melia azedarach} L., China tree, and \textit{Vinca major} representing different degrees of senescence were collected from plants growing on campus during autumn. Leaves at different degrees of senescence were picked also from tobacco (\textit{Nicotiana tabacum} L.) growing in pots in the greenhouse.

Parsley (\textit{Petroselinum sativum} L.) and celery (\textit{Apium graveolens} L.) leaves were purchased fresh in the market. Leaves were allowed to senesce at 25°C either in light or in darkness.

\(^1\) Abbreviation: \( \alpha \)-T, \( \alpha \)-tocopherol.

in Petri dishes containing moist filter paper covered with a Saran net, on which the leaves were laid to avoid direct contact with water. Dark green \textit{Melia} leaflets, picked by late summer (September), were also allowed to senesce by the same method.

Orange (\textit{Citrus sinensis} L. Osbeck cv. Shamouti) leaves and mature green fruit were harvested from the orchard and allowed to senesce in glass cylinders under a humid stream of air containing 20 \( \mu \)L \( \cdot \) \( 1 \)\(^{-1} \) of ethylene/L at 25°C in the dark.

Extraction

Plant materials were homogenized with a Polytron homogenizer in \( \sim 15°C \) acetone (0.2 g leaf tissue/5 mL; 0.5 g \textit{Citrus} fruit peel/5 mL) and left in the cold for 10 min. The homogenate was centrifuged to remove the debris and the supernatant filtered through a 0.45 \( \mu \)m pore size Millipore filter prior to analysis by HPLC.

HPLC Analysis

An LKB HPLC system equipped with a UV detector (Micromeritics 788) and HP3390 integrator was used. A 250 \( \times \) 4 mm (i.d.) Lichrosphere 100 RP-18 5\( \mu \)m column by Merck was used. All solvents were HPLC grade. Mixtures of solvents were used on a stepwise elution program. Solvent A consisted of 75:25 (v/v) methanol-water. Solvent B was ethyl acetate. The chromatogram was developed with a linear gradient from 15% to 50% B (made up with A) for 14 min, followed by additional 10 min of 50% B. Then a linear gradient of 50% to 75% B was run for 10 min, followed by additional 4 min of 75% B. Total development time was 38 min.

Twenty-\( \mu \)L samples were injected. The flow rate was 0.6 mL/min and the system was operated at room temperature (ca. 25°C). Principal absorbance detector wavelengths were 430 nm for Chl and 292 nm for \( \alpha \)-T.

For collection of fractions enriched with \( \alpha \)-T, extracts were prepared at 3 g tissue/8 mL of \( \sim -15°C \) acetone. A semipreparative 250 \( \times \) 7 mm (i.d.) Lichrosorb RP-18 7\( \mu \)m column by Merck was utilized. One-mL samples were injected each time and the flow rate was 1.5 mL/min.

Identification and Quantitative Determination

Chl \( a \) and \( b \) were tentatively identified by their retention time and by the absorbance spectra produced during the
HPLC run in comparison with markers, as previously described (2).

For identification of α-T, the fractions collected by semipreparative HPLC were dried under N₂ and dissolved in hexane. Identification of α-T was carried out using a Finniganmat-4600 GC-MS, and a Bruker AM-300 NMR spectrometer (300.1 MHz, FOR ¹H, 75.5 MHz FOR ¹³C); ALL NMR data are for a CDCl₃ solution and chemical shifts are reported in ppm from internal tetramethyl silane.

Quantitative determinations were based on integrator data which were calibrated according to the spectrophotometric absorbance of pure standards. Chl standards were prepared in the laboratory and α-T standards were from Merck.

**RESULTS**

Scanning of acetone extracts from green and senescing leaf tissue at different wavelengths revealed the presence of a material which increased during the course of senescence. The material could not be seen in the 430 nm scan used to detect Chl and carotenoid pigments. It appeared, however, as a major peak eluting between Chl a and b when scanned at 292 nm, a wavelength which also reveals the Chl (Fig. 1). Melia azedarach L. leaves seemed to be particularly rich in this material and were used, therefore, for collection of large amounts for the purpose of identification. When collected by semipreparative HPLC and injected into a gas chromatograph, the material eluted as a single peak, as detected by the flame ionization detector. The material could be analyzed by GC-MS without further purification.

The dominant features of the mass spectrum were intense peaks at m/z 430(M⁺, 100%), 205 (10) and 165 (76), which matched the fragmentation ions of the α-T molecule.

The ¹H-NMR spectrum (0.85, 0.86, 0.87 and 0.87 [each d, 6.5, 3H, H₃-4a', H₃-8a', H₂-12a' and H₂-13'], 1.23 [s, 3H, H₃-2a], 1.75 and 1.80 [ABq of t, J₆₋₇ = 14Hz, J₇₋₆ = 7Hz, 2H, H₂-3] 2.11 [bs, 6H, H₃-5a and H₃-8b], 2.16 [bs, 3H, H₂-7a], 2.60 [bt, 7Hz, 2H, H₂-4], 4.20 [b, 1H, OH]) was in good agreement with the data reported in the literature for α-T (3, 4). Conclusive identification came from the ¹³C-NMR, in which the chemical shifts for all carbons in the material we isolated were within 0.3 ppm or less of those reported by Matsuo and Urano (12) (their carbon numbering was used in the assignment of the ¹H data, vide supra).

Determination at different stages of senescence in Vinca and Melia leaves showed a prominent increase of α-T concomitant with the decline in Chl (Fig. 2). Calculation of the amount of Chl and α-T (on a molar basis) showed that the total phytol moiety present in these compounds did not...
change during the course of senescence (Fig. 2), except for the 'Yellow' stage of *Vinca*, in which the α-T started to decline.

Detached *Melia* leaves, which already contained a considerable amount of α-T on the day of harvest, showed a moderate further increase in α-T (25–20%) during senescence (Table 1). *Melia* leaves were found to lose their Chl more rapidly in light than in darkness, but α-T accumulated to the same extent under light and dark conditions. Similar levels of α-T were obtained after 4 and 6 d of senescence, although the loss of Chl was more prominent at 6 d.

Accumulation of α-T was evident also in orange leaves and fruit upon exposure to ethylene (2.04 and 1.82 μmol α-T per g fresh weight, respectively).

A careful search in fresh and senescent leaves of parsley, celery (senescing in either darkness or light), and tobacco did not reveal the presence of α-T.

**DISCUSSION**

The results of the present study demonstrate a transient increase in α-T during the course of senescence in several plant species. The amounts of α-T accumulated in the senescing *Vinca* and *Melia* leaves (0.7 and 1.5 μmol g−1 fresh weight, respectively) are relatively high, compared with values reported in the literature (6–8). A marked increase in α-T was reported recently also by Sawamura et al. (15) for senescing citrus fruit peel.

Little is known about the turnover of α-T in leaves and our data are open to more than one line of interpretation. Because of its antioxidant character (17) and free radical scavenger activity (9), α-T may be turning over rapidly in young leaves, when photosynthetic activity is high, but accumulate during senescence, upon the decline in photosynthetic rates and genesis of radical species. However, if this is the case, one would expect at least some difference between the levels of α-T accumulated in light (where photosynthetic activity still takes place) and in darkness (where photosynthesis is completely abolished). Our data (Table 1) do not show such a difference. In addition, the accumulation of α-T in senescing plant organs is somewhat difficult to reconcile with the hypothesis which relates the advent of senescence to conditions which favor oxidative reactions in plant cells (16).

Another possibility is to link the build up of α-T during senescence with the degradation of Chl (Fig. 2), although our detached leaf senescence data (Table 1) do not suggest a strict correlation between these two processes. Both α-T and Chl contain a phytol moiety as part of their molecular and both are biosynthesized within the chloroplast, although at different sites (19). The phytol utilized for their biosynthesis may be derived from a common pool—a *Scenedesmus* mutant which lacks the ability to reduce geranylgeraniol and form phytol did not contain α-T and did not have normal Chl (11). Administration of phytol has been shown to bring about a marked increased in the biosynthesis of α-T, in intact cells (5) as well as in *in vitro* systems (18) which seemed to have sufficient phytol kinase activity (18).

The phytol released by chlorophyllase during the initial stages of Chl breakdown (2, 14), may, therefore, be utilized for the biosynthesis of α-T during senescence. Leaves of the low chlorophyllase plants parsley and tobacco (1, 2) did not contain α-T in measurable amounts and did not accumulate it during senescence.

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**LITERATURE CITED**


