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Catabolites of chlorophyll in senescing barley leaves are localized in the vacuoles of mesophyll cells

(*Hordeum vulgare*/leaf senescence/pigments)

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ABSTRACT **Senescing barley leaves accumulate a series of pink pigments with the chemical properties of catabolites derived from chlorophyll. Levels of the major component of this group of pigments were quantified by HPLC and shown to be maximal in tissues exhibiting maximal rates of chlorophyll degradation. Protoplasts were isolated from senescent leaf tissue and fractionated to yield intact vacuoles and plastids. Although small but significant proportions both of total catabolites and of the dominant component of the series were recovered from the plastid fraction, the vast bulk of these compounds could be assigned to the vacuole. These observations suggest a role for the vacuole in the later stages of chlorophyll breakdown during senescence.**

Senescence has an important function in plant development, as senescent organs are sources of nutrients that are recycled to meet the requirement for growth or storage in other parts of the plant. For instance, a substantial proportion of the nitrogen that is accumulated in the form of reserve proteins in the grains of cereal plants originates as leaf protein that was mobilized from senescent foliage during the grain-filling period. It is unknown whether the four nitrogen atoms of chlorophyll molecules are also recycled; according to present ignorance, the chlorophyll simply disappears. The final products of degradation are not known, nor has the mechanism by which the Mg-porphyrin is catabolized been elucidated. Previously only derivatives of chlorophyll having an intact tetrapyrrole ring structure—namely, chlorophyll a-1 (1), pheophytin (2–4), pyropheophytin (4), chlorophyllide, and pheophorbide (2, 4)—had been detected in senescent leaves and other chlorophyll-degrading systems. During a recent study of senescence in leaf tissues of *Festuca pratensis*, a group of nongreen pigments was identified (5). That they might be products of chlorophyll breakdown *in vivo* was immediately suggested by their absence from tissue of a nonyellowing senescence mutant of the same species (6). Similar pigments were subsequently discovered in senescing primary leaves of barley and in yellowing leaves of a large number of other species, including several deciduous trees. In fact, we believe the phenomenon to be typical for all angiosperms in which chlorophyll is degraded during foliar senescence. Here it is demonstrated that almost all of the pink catabolites of chlorophyll are located not in the degenerate chloroplasts but in the vacuoles of senescing barley mesophyll cells.

MATERIALS AND METHODS

Plant Material. Primary leaves of *Hordeum vulgare* cv. Gerbel were excised 10 days after sowing, and segments of laminae excised between 10 and 50 mm from leaf tip were induced to senesce by incubation on distilled water in Petri dishes at 25°C (5).

Extraction and Analysis of Pigments. Leaf segments were homogenized with MeOH containing 5% (vol/vol) formic acid (100 μl/100 mg fresh weight). The homogenate was extracted three times with 1 ml of chloroform, and the combined extracts were passed through cotton wool and a small column (bed volumn *ca.* 0.5 ml) of silica gel beads (Merck 60, 70–230 mesh). The pink pigments were adsorbed at the top of the column, while pheophytins and most of the carotenoids were eluted. After the column was washed with chloroform, the residual pigments were eluted with MeOH/5% formic acid. The solvent was evaporated and the residue was dissolved in a defined volume of MeOH and used for HPLC analysis. HPLC was carried out on a reversed phase C_{18} column (Hypersil 5 ODS, 25 cm) with a solvent system of 10% MeOH/8.2% acetonitrile/5.8% tetrahydrofuran/1% H_3PO_4/75% water (by volume) at a flow rate of 1.0 ml·min^{-1}. A Kratos spectroflow 757 monitor recorded absorbance at 520 nm; peak areas integratedwith HP 3390 A integrator were used for quantifying pink pigments.

Pink pigments were subjected to degradation with chromic acid followed by TLC analysis of the maleimides produced (7).

Subcellular Localization of Chlorophyll Catabolites. Excised presenescent primary leaves were allowed to senesce in permanent darkness for 4 days. Protoplasts and vacuoles were prepared by established procedures (8, 9). The separation of unlysed protoplasts from intact vacuoles required adjustment of the densities of media used for the flotation of vacuoles. Mixtures of 0.6 M sorbitol and 0.6 M betaine were used for step gradients layered on top of the product of protoplast lysis. The sediment of chloroplasts produced upon flotation of vacuoles was resuspended in 0.6 M sorbitol/25 mM Tricine buffer, pH 8.0/1 mM EDTA/0.1% bovine serum albumin), and the chloroplasts were purified by centrifugation for 10 min at 1000 × *g* through a layer of the above sorbitol medium containing 20% Percoll. Based on contents in protoplasts of corresponding markers, the yields were 25% for vacuoles and 44% for chloroplasts. All fractions obtained were made up to defined volumes. Protoplasts, vacuoles, and chloroplasts in aliquots of corresponding suspensions were sedimented, and the pellets either were used for the extraction of chlorophyll catabolites or were resuspended in sorbitol medium for analysis of enzyme activities and marker compounds by standard procedures. Data of a single experiment are given. In three independent additional experiments, the choice of markers was different and/or the isolation of vacuoles was associated with lower yields or higher contaminations, or both. The demonstration of the vacuolar location of chlorophyll catabolites was unambiguous in all experiments.

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RESULTS AND DISCUSSION

Chromatographic and Spectroscopic Characteristics of Chlorophyll Catabolites. The absorption spectrum of a prominent representative of the pink series of catabolites (Fig. 1) exhibits a maximum around 520 nm with a shoulder towards smaller wavelengths and a second maximum at 315 nm. As judged from the spectra as well as from other observations, members of the group of catabolites composed of one major and several minor pink compounds as shown in the HPLC chromatogram of Fig. 1 are closely related with each other. The compounds are sufficiently polar to be water soluble, yet they can be extracted from leaves with chloroform when acidified with formic acid. They have no affinity with a C_{18} reversed-phase HPLC column when neutral solvents are used. The chromatographic separation shown in Fig. 1 required strongly acidic solvents, suggesting that the pink pigments are acid in nature. This also holds true with regard to another group of putative chlorophyll catabolites of lower polarity and yellow appearance. The most prominent of the pink compounds, marked with an asterisk on the chromatograms of Fig. 1, has been purified. It has a molecular mass of 718 (S. Brown, personal communication), but its detailed chemical structure is as yet unknown.

Evidence That Pink Pigments Are Derived from Chlorophyll. Several findings strongly support the conclusion that the pink pigments represent intermediary products of natural chlorophyll breakdown. First, their abundance in barley leaves induced to senesce in permanent darkness increased dramatically during the period of rapid decline of chlorophyll and decreased subsequently (Fig. 2). Treatments promoting the retention of chlorophyll, such as exposure of excised leaf segments to natural daylight (Fig. 2) or treatment with cytokinin in permanent darkness (5), resulted in low contents

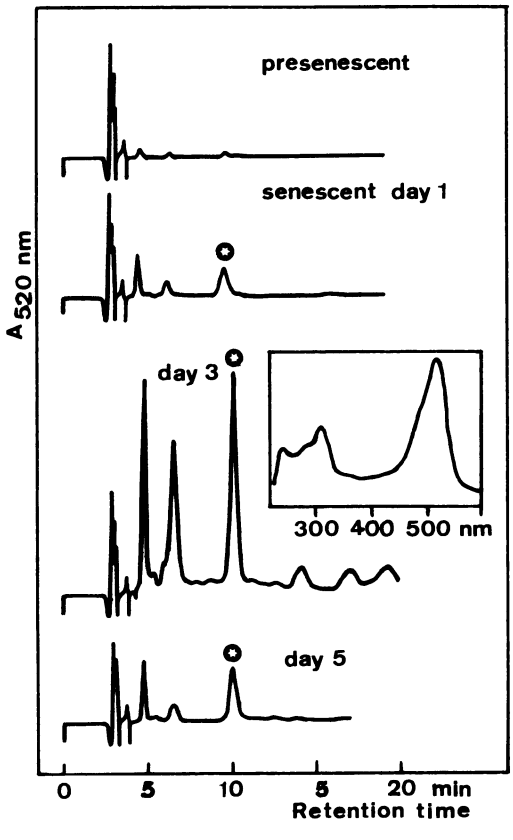

FIG. 1. HPLC of pink catabolites of chlorophyll extracted from barley primary leaf segments induced to senesce in the dark for 1, 3, and 5 days, respectively. (*Inset*) Spectrum of the purified compound marked with an asterisk on the chromatograms.

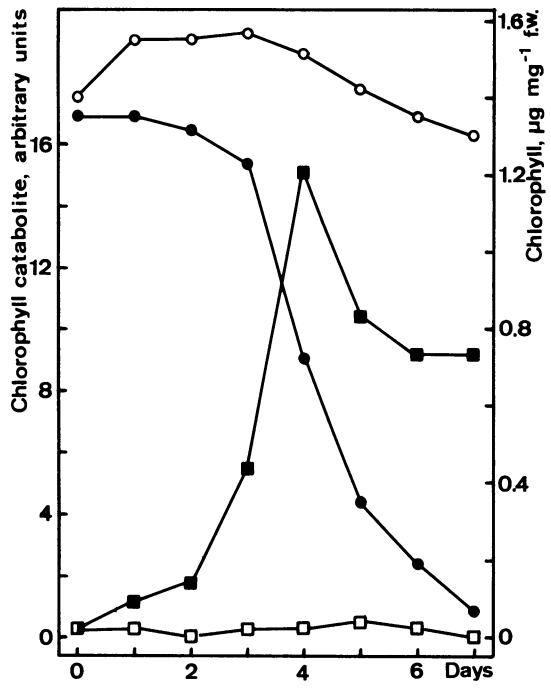

FIG. 2. Levels of a pink chlorophyll catabolite (□) and chlorophyll (○) in segments of barley primary leaves induced to senesce either in permanent darkness (black symbols) or under natural daylight conditions (open symbols). Segments of presenescent primary leaves, 10 days after sowing, were excised between 10 and 50 mm from the tip and placed on distilled water in Petri dishes. The prominent compound marked in Fig. 1 with an asterisk was measured and expressed as the integrated peak area per unit of fresh leaf weight. Chlorophyll was determined in separate segments.

of pink pigments. Conversely, the extremely rapid degradation of chlorophyll as a result of treatments with methyljasmonate (10) was associated with a transiently very high level of pink pigments, coinciding again with the main period of chlorophyll degradation. It should be mentioned that the photooxidation of chlorophyll in illuminated segments treated with methylviologen is not associated with the appearance of pink pigments (unpublished observations), suggesting that the mechanism of natural chlorophyll catabolism differs fundamentally from the nonenzymic photodynamic process by which such herbicides bleach leaf tissue.

Second, the pink putative chlorophyll catabolites are not identical with anthocyanins or anthocyanidins to judge from their optical properties (Fig. 1, *Inset*); moreover, senescent leaves of beet, a species which is not competent to synthesize anthocyanins, contained the same spectrum of pigments as in barley and other competent species.

Third, the pigments react with diazotized dyes such as 4-nitro-*o*-anisidine (5), as does bilirubin, the product of hem porphyrin breakdown. The term phytobilirubins may be justified for the whole group of chlorophyll catabolites.

Fourth, persuasive evidence for the porphyrin origin of pink pigments can be drawn from the result of oxidative degradation with chromic acid. Of the products obtained from the principal compound (Fig. 1, asterisk), methylvinyl maleimide derived from pyrrole ring I of chlorophyll could be identified.

As judged from the spectral properties, similar pigments have been observed during rapid degradation of bacteriochlorophyll in *Rhodopseudomonas sphaeroides* (11). Moreover, it is interesting to note that a compound with an absorption maximum at 520 nm has also been obtained *in vitro* through the interaction of chlorophyll with the anion radical of molecular oxygen (12).

Table 1. Localization of pink chlorophyll (Chl) catabolites in vacuoles and chloroplasts prepared from mesophyll protoplasts of senescent barley leaves

	Markers, Chl, and Chl catabolites, % of total							
						Pink Chl catabolites		
	Chl	α-Manno-sidase	Glyceraldehyde-3-phosphate dehydrogenase	Malate dehydro-genase ($NADP^+$)	Malate	Peak*	Total	Peroxidase
Chloroplasts†	100	0	64.4	2.1	0.81	3.6	9.2	0
Vacuoles‡	ND	100	13.6	5.6	97.9	108.6	103.1	31.7
% recovery§	89	93	106	91	136	—¶	—¶	106

ND, not done.
*Peak marked with an asterisk in Fig. 1.
†Assuming 100% of Chl is located in chloroplasts.
‡Assuming 100% of α-mannosidase activity is located in vacuoles (8).
§Sum of fractions prepared related to contents/activities in protoplast (= 100%).
¶Calculation impossible as the compounds cannot be extracted from supernatant fractions.

Sites of Catabolite Accumulation in Mesophyll Cells. The degradation of chlorophyll and proteins in senescent leaves are biochemical expressions of the differentiation of chloroplasts into gerontoplasts. Analysis of the fine structure of senescent mesophyll cells in various species suggests that plastids persist, although the constituents of the thylakoids and of the stroma are largely broken down (13). In fact, in barley primary leaves, the number of chloroplasts per mesophyll cell was found to be practically unchanged during the main period of degradation of chloroplastic proteins and chlorophyll (14). Corresponding findings have been reported for senescent *Festuca* and wheat leaves (15–17). A logical conclusion would be that chloroplast constituents, including the thylakoid pigments, are degraded *in situ*, within the senescent plastids. Hence, intermediary products of chlorophyll breakdown might be expected to accumulate in the chloroplasts. However, in plastids isolated from leaf cell fractionations, the pink pigments were hardly detectable and could be accounted for as impurities rather than true constituents of the organelle preparations. In conventional homogenates of senescent leaf tissue, the bulk of chlorophyll catabolites was recovered in the soluble fraction, indicating that they are either located in the cytosol or released from a fragile compartment such as the vacuole. Most surprisingly, analysis of vacuoles isolated from protoplasts of senescent mesophyll cells showed that a major proportion of the pink pigments is located in the sap of these organelles (Table 1). The result is unambiguous, although the isolation of vacuoles from senescent protoplasts is quite difficult and contamination of the preparations is comparatively high (6–14% as calculated from the content of the extravacuolar markers malate dehydrogenase ($NADP^+$) and glyceraldehyde-3-phosphate dehydrogenase). On the other hand contamination of the chloroplast fraction with vacuoles was low, judging by the absence of the vacuolar marker α-mannosidase. Whether or not the small percentage of pink pigments recovered in the plastids is truly representative of catabolites located in the organelle of origin was determined by comparison with an abundant vacuolar metabolite, malate. We found that contamination of chloroplasts with malate was lower by a factor of 4–10 than the abundance of pink pigments in this fraction; thus, these chlorophyll catabolites indeed appear to be present in the chloroplasts, as expected, albeit in very low concentrations.

The Pathway and Subcellular Organization of Chlorophyll Catabolism. The vacuolar location of chlorophyll catabolites opens new possibilities for approaching the problem of chlorophyll breakdown and certainly also poses a number of new problems. In the functional state, the photosynthetic pigments are complexed with the apoproteins of light-harvesting complexes and photosystems, and it must be assumed, therefore, that disassembly of thylakoids and the dissociation of protein pigment complexes represent initial steps of chlorophyll catabolism. Whether dephytylation by the action of chlorophyllase is necessary for further breakdown is unknown (4). Saponification of the pink pigments has revealed that phytol seems to have been cleaved off as shown by GLC analysis with chlorophyll a as the reference compound. As far as the chromophore is concerned, removal of Mg and modifications in the isocyclic ring seem to occur (1–4), but the reaction or reactions responsible for the decisive opening of the porphyrin ring, which must be associated with the loss of green or brownish colors, remain(s) obscure. In the analogous case of hem catabolism, there is a specific monooxygenase producing biliverdin and CO. CO was not detectable in the head space of senescent barley leaves enclosed in a small chamber, so it may be that the ring-opening enzyme in plants is different from that of animals.

The presence of nongreen catabolites in both chloroplasts and vacuoles suggests that the initial catabolic reactions occur in the chloroplasts. The products then appear to be exported to vacuoles and temporarily accumulate there, probably because further catabolism is comparatively slow. The vacuole had been proposed to be the "lytic compartment of plant cells"(18). Vacuolar hydrolases (19–21) seem unlikely to play a role in the destruction of pyrrole systems; but vacuoles also contain oxidases, including a substantial proportion of the total peroxidase activity present in presenescent (22–24) and senescent (Table 1) cells. A role for peroxidase in the breakdown of chlorophyll catabolites must be seriously considered because peroxidases are known to bleach Mg-porphyrins (25–28). It will be difficult to follow the catabolic sequence to the very end without the development of a method for specifically radiolabeling chlorophyll in presenescent leaves. Such an approach will greatly facilitate identification of the final products and establishment of whether the yellowing of leaves is associated with the recycling of the N atoms of chlorophyll as well as being an aesthetically significant phenomenon. Elucidation of initial reactions occurring in the chloroplasts and yielding the first nongreen breakdown products of Mg-porphyrins will be of great significance for our understanding of the principle of chloroplast demolition.

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