Update on Chlorophyll Breakdown

Chlorophyll Breakdown in Senescent Leaves

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The biosynthetic pathway of Chl, probably the most important pigment in the biosphere, has been studied in considerable detail and is mentioned in plant physiology textbooks. Oddly enough, however, the breakdown of Chl is barely mentioned. Yet, as pointed out by Hendry et al. (1987), an estimated one billion tons of Chl are broken down every year, seemingly without leaving a trace. The only enzyme that is taught is chlorophyllase, discovered by Arthur Stoll in 1912, which cleaves Chl into phytol and Chlide, the Mg-porphyrin moiety of Chl. For most of the subsequent 80 years, chlorophyllase has been the only known part of the catabolic system. Until quite recently all the subsequent steps of Chl breakdown remained undiscovered because no one was able to identify the breakdown products. We now know that the reason these products remained undetected is quite trivial: they are colorless. In mammals, the bile pigments biliverdin and bilirubin are colored breakdown products of heme; by analogy, plant biochemists may have expected the breakdown products of Chl to be colored as well, but this is not the case, which is probably why they were overlooked for so long. The identification of the structures of these colorless catabolites has, in the last few years, led to the elucidation of the pathway of Chl catabolism in the senescing leaves of higher plants.

CHL CATABOLITES

Nongreen catabolites of the porphyrin moiety were first discovered when extracts from senescent leaves of a non-yellowing genotype of Festuca pratensis were separated by TLC and compared with those from a normally yellowing wild type. In the presence of acidic solvents, pink and rust-colored artifacts of the colorless catabolites appeared on the silica-gel layers, but only in the case of senescent leaves (Matile et al., 1987, 1989). Subsequently, similar-colored compounds were found to be present in yellowing leaves of barley (Bortilik et al., 1990) and a number of other species. Unambiguous identification of catabolites was achieved through 14C-labeling of Chl in the pyrrole units during greening and tracing of breakdown products during subsequent senescence of barley primary leaves (Peisker et al., 1990) and cotyledons of oilseed rape (Ginsburg and Matile, 1993). HPLC resolved a large number of water-soluble catabolites from barley, whereas only three compounds accumulated progressively in degreening rape cotyledons; these three account for practically all of the Chl broken down. Radiolabeling in barley also revealed that carbon from the pyrroles was neither lost as CO2 nor exported from senescent leaves to other parts of the plant (Peisker, 1991). We may conclude that plants catabolize Chl into water-soluble porphyrin derivatives that merely accumulate in the mesophyll during foliar senescence.

In addition to such apparently final products of Chl breakdown, another kind of catabolite was identified by a distinctive blue fluorescence. These compounds occur in minute quantities and only when rates of Chl loss in the leaves are high. They were, therefore, thought of as possible primary or intermediary breakdown products that are converted into nonfluorescent final products in the course of senescence (Düggelin et al., 1988a, 1988b; Ginsburg and Matile, 1993). Nongreen catabolites thus fall into two categories on the basis of fluorescence, NCCs and FCCs. With the exception of one FCC, all catabolites so far identified in a number of species are different with regard to structure or retention time on reverse-phase HPLC, i.e. polarity. Therefore, it was necessary to introduce a convenient nomenclature (Ginsburg and Matile, 1993): a prefix indicates the plant species (e.g. Hv = Hordeum vulgare) and the individual compounds (NCC or FCC) are numbered according to decreasing polarity as judged by retention times on reverse-phase HPLC. Thus, Hv-NCC-1 represents the most polar of the three nonfluorescent catabolites accumulating in senescent oilseed rape (Brassica napus) cotyledons.

All NCCs from plants that have been analyzed so far exhibit the same basic structure of a 19-formyl-1-0xo-bilane, as first found in Hv-NCC-1 (Kräutler et al., 1991, 1992). Accordingly, the three NCCs from rape, also depicted in Figure 1 (Mühlecker et al., 1993; Mühlecker and Kräutler, 1996), as well as NCCs from autumn leaves of two species of Liquidambar (Iturraspe and Moyano, 1995) and Cercidiphyllum japonicum (Curty and Engel, 1996), appear to descend from Chl a or Pheide a, respectively. The structural comparison of Hv-NCC-1 to its putative Chl

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; FCC, fluorescent chlorophyll catabolite; NCC, nonfluorescent chlorophyll catabolite; Pheide, phophorbide.

Figure 1. Structure of Pheophorbide a compared with the structures of NCCs isolated from senescent leaves of several plant species. The references are given in the text.

Figure 2. The first identifiable product, Bn-FCC-2, in the case of Brassica napus, as confronted with the substrate, Pheide a, of the ring-cleaving reaction catalyzed by Pheide a oxygenase in conjunction with a stroma protein.

for the metabolic (or artifactual) path to catabolites exhibiting structural characteristics of pyropheophorbides (see Engel et al., 1991; Gossauer, 1994). The apparently uniform, direct lineage from Chl a (Pheide a), but not from Chl b (Pheide b), of the NCCs from plants still lacks an experimentally verified explanation. It may be associated with the recently discovered existence of a metabolic reversal of the biosynthetic path from Chl a to Chl b (Ito et al., 1993, 1994; Ito and Tanaka, 1996).

Very recently, the constitution of Bn-FCC-2, the first identifiable Chl catabolite in senescent rape cotyledons, has been elucidated by modern spectroscopic means (Mühlecker et al., 1996). As expected, it is also a 19-formyl-1-oxo-bilane, i.e. a linear tetrapyrole derived from Pheide a (Fig. 2). In Bn-FCC-2, the chromophoric system responsible for \( A_{360} \) as well as for the characteristic fluorescence maximum at 450 nm, extends over the five-membered rings, joining the southern, unsaturated mesoposition.

These features of Chl catabolites from plants appear to have a structural parallel with some “red pigments” excreted by the green alga Chlorella protothecoides (Oshio and Hase, 1969) that have been structurally characterized in recent years (see Engel et al., 1991; Gossauer, 1994). These linear tetrapyroles have functional traits of Chl a or of Chl b, (Iturraspe et al., 1994) but they are thought to arise from these by an oxygenolytic cleavage of the porphinoid macrocycle at the same position found so far in the plant catabolites.

A third group of linear tetrapyroles that is likely to be derived from Chls has been discovered in some lumines-
cent compounds from the marine dinoflagellates *Pyrocystis lunula* and *Euphausia pacifica* (Nakamura et al., 1988, 1989). However, in these cases the presumed ring opening occurs at the western mesoposition, i.e. between C-20 and C-1.

For reasons of completeness, phytol and its fate during Chl breakdown is also considered briefly here. Phytol seems to be quite stable during leaf senescence and it persists largely in esterified form (Csupor, 1971; Park et al., 1973; Peisker et al., 1989); in barley the acetyl ester of phytol was found to accumulate in the plastoglobuli of gerontoplasts (Bortlik, 1990).

### PATHWAY OF CHL BREAKDOWN

The breakdown of Chl into phytol, Mg$^{2+}$, and a primary cleavage product of the porphyrin moiety occurs in three consecutive steps, catalyzed by chlorophyllase, Mg-dechelatase, and pheophorbide $a$ oxygenase (Fig. 3). The third step is the most significant for the yellowing of senescent leaves because the opening of the porphyrin macrocycle is associated with the loss of green color. The first identifiable product of Pheide $a$ oxygenase is an FCC, which has so far been observed to occur in several plant species. This FCC is the most apolar compound of the group of FCCs found in each species. Thus, the first identifiable product in rape cotyledons is Bn-FCC-2, whereas in barley the identical FCC is Hv-FCC-4, because in this species three additional, less apolar FCCs are present.

The apparent primary product of Chl breakdown that has a ring-opened structure is represented by Bn-FCC-2, newly characterized by Mühlecker et al. (1996). It has the molecular formula C$_{23}$H$_{30}$N$_{4}$O$_{2}$ (Pheide $a$, C$_{43}$H$_{36}$N$_{4}$O$_{2}$). Therefore, the first steps of the breakdown of Pheide $a$ in degreening cotyledons of rape can be deduced to formally comprise the addition of two atoms of oxygen and four atoms of hydrogen. From the available structural information on Bn-FCC-2, this can now be specified to occur by an oxygenolytic cleavage of the conjugated chromophoric system of Pheide $a$ between C-4 and C-5, followed by reductive saturation at the mesopositions C-10 and C-20. It remains to be seen whether Pheide $a$ oxygenase inserts both atoms of molecular oxygen in the decisive cleavage step. The analogous reaction in *C. protothecoides* appears to proceed via a monoxygenase-catalyzed epoxidation, followed by the hydrolysis of the epoxide (Curty et al., 1995).

The primary FCC can be detected in intact, isolated gerontoplasts (Ginsburg et al., 1994) and its generation from Chl $a$ and Pheide $a$, respectively, can be studied in vitro by incubating gerontoplast membranes or protein preparations thereof under appropriate conditions (Schellenberg et al., 1993; Ginsburg et al., 1994; Hörtensteiner et al., 1995). The reaction requires dioxygen and it was not surprising to find that the oxygenase contains Fe, but it was quite unexpected that the redox cycle turned out to be driven by reduced Fd (Schellenberg et al., 1993). Fd may even be newly synthesized during senescence for the purpose of Chl breakdown, since a gene with high homology to Fd I has been cloned from senescent maize leaves (Smart et al., 1995). Another unexpected property of the oxygenase is its specificity for Pheide $a$ as a substrate, Pheide $b$ being a competitive inhibitor of FCC production in vitro (Hörtensteiner et al., 1995). A typical assay mixture contains solubilized gerontoplast membranes (or a pigment-free protein preparation), Pheide $a$ as substrate, Fd, and cofactors (NADPH, Glc-6-P, and Glc-6-P dehydrogenase) required to keep Fd in the reduced state. The reaction can also be driven by light in the presence of Fd if unsolubilized membranes are employed as a source of enzyme (Fig. 4).

An important component of assay mixtures has so far not been mentioned: the production of primary FCC from Pheide $a$ requires the presence of a soluble stroma protein. Its function has not yet been firmly established, but it is almost certain that it represents an NADPH-dependent reductase that is responsible for the reduction of double bonds in the pyrrole system following (or preceding?) the action of the ring-opening oxygenase (Fig. 4).

Chlorophyllase (EC 3.1.1.14) is a classical enzyme that has been purified and characterized (e.g. Shioi and Sasa, 1986; Trebitsh et al., 1993), whereas the second enzyme of the pathway, Mg-dechelatase, has so far merely been demonstrated to exist in terms of enzymic conversion of Chlide into Pheide (e.g. Bazzaz and Rebeiz, 1978; Ziegler et al.,

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**Figure 3.** Chl breakdown in senescent leaves: putative sequence of enzymic reactions.

![Figure 3](https://www.plantphysiol.org/)

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Chlorophyll 
\[ \text{Chlide} \]
\[ \text{Pheide a} \]
\[ \text{NADPH I [Intermediary compound]} \]
\[ \text{Stroma protein (Reductase)} \]
\[ \text{NADP}^+ \]
\[ \text{NADPH} \]
\[ \text{Pentose-phosphate cycle} \]
\[ \text{Glucose-6-phosphate} \]
\[ \text{Primary FCC} = \text{Bn-FCC-2} \]
\[ \text{Modified FCCs} \]

Figure 4. The oxidative cleavage of the porphyrin macrocycle.

1988; Shioi et al., 1991; Langmeier et al., 1993). Enzymic dechelation of chlorophyllin has recently been employed for measuring dechelatase activity in solubilized chloroplast membranes (Vicentini et al., 1995b). Shioi et al. (1996) reported catalytic conversion of Chlide to Pheide by virtue of a low molecular weight compound present in extracts of Chenopodium album leaves. This heat-stable compound may represent the prosthetic group, which, upon tissue extraction, is easily dissociated from the dechelatase protein.

The activities of chlorophyllase and dechelatase can be demonstrated in presenescent as well as in senescent leaves. But the third activity of the pathway, Pheide a oxygenase, is detectable only in senescent leaves (Ginsburg et al., 1994; Hörtensteiner et al., 1995) and, therefore, may represent a key enzyme of Chl breakdown. A stay-green genotype of F. pratensis was found to be equipped with normal levels of chlorophyllase and dechelatase, but senescent leaves contained an extremely low oxygenase activity compared with the wild type (Vicentini et al., 1995a). Both mutant and wild type were competent regarding the stroma factor required for the ring-opening reaction. Students of the history of genetics may be interested to learn that Gregor Mendel’s green peas (mutants) differ from the yellow isoline by virtue of the same lesion in the oxygenase (Thomas et al., 1996).

At first glance, the apparent control of breakdown at the third step of the pathway rather than at an earlier step seems strange. There is likely to be another, undefined element of the catabolic system located upstream of chlorophyllase that is regulated in a senescence-specific manner.

COMPARTMENTATION OF BREAKDOWN IN THE CELL

An appreciation of the spatial organization within senescent mesophyll cells and gerontoplasts is crucial for understanding Chl breakdown. The present state of the art is summarized in the model depicted in Figure 5.

To start with the end of the process, the central vacuole of senescent mesophyll cells has been identified as the dumping ground of Chl catabolites (Matile et al., 1988; Düggelin et al., 1988b; Bortlik et al., 1990; Hinder et al., 1996). This is not particularly surprising, since structures of NCCs (Fig. 1) demonstrate that catabolites are modified and conjugated in the same manner as many water-soluble secondary compounds that are also known to be deposited in cell saps. Disposal of catabolites in vacuoles is achieved by a specific ATP-dependent carrier in the tonoplast that has been demonstrated to function in the primary active mode (Hinder et al., 1996).

When isolated, intact gerontoplasts are incubated in the presence of ATP or Glc-6-P, FCCs are produced in organello (Schellenberg et al., 1990; Ginsburg et al., 1994). The most abundant FCC of barley gerontoplasts, Hv-FCC-2, is released into the medium if ATP is provided at the cytosolic face of the envelope (Matile et al., 1992). Thus, gerontoplasts appear to be equipped with a carrier that is responsible for the export of newly produced catabolites into the cytosol.

To further understand Chl breakdown and its regulation, experiments were aimed at the exact localization of catabolic enzymes within the plastids. We began with chlorophyllase; it has been known for a long time that its activity is latent. Although the enzyme is associated with chloro-

![Figure 5](https://www.plantphysiol.org/)
plast membranes apparently close to its substrate, hydro-
lysis of the endogenous Chl does not take place unless the
membranes are solubilized in the presence of an appropri-
ate detergent (Amir-Shapira et al., 1986) or acetone (e.g.
Garcia and Galindo, 1991). Recently, an unexpected expla-
nation for latency in the intact organelle has emerged;
chlorophyllase appears to be localized in the chloroplast
envelope (Matile et al., 1996). This spatial separation of
chlorophyllase from the thylakoidal pigment-protein com-
plexes is unchanged when Chl breakdown takes place in
developing gerontoplasts. Therefore, breakdown of Chl is
likely to require, in addition to the enzymes mentioned so
far, an additional tool that mediates between the site of Chl
in the thylakoids and the site of catabolic enzymes such as
chlorophyllase (Matile et al., 1996) and Pheide a oxygenase
(Matile and Schellenberg, 1996) in the envelope. There are
good reasons to assume that newly synthesized,
senescence-specific protein is required for the establish-
ment of contact between Chl and chlorophyllase. Thus, the
oxygenase-deficient stay-green lesion in F. pratensis causes
progressive accumulation of dephytylated species of Chl
during senescence, indicating that chlorophyllase and
dechelatase are served with substrate, and this phenome-
non is abolished when cytoplasmic protein synthesis is
inhibited in the presence of cycloheximide (Thomas et al.,
1989). We hypothesize that the protein(s) in question
(marked X in Fig. 5) function by luring Chl molecules out
of complexes in the thylakoids and carrying them to the
catabolic machinery in the envelope. It may well turn out
that such a putative Chl carrier provides an answer to the
enigma of thylakoid deconstruction in developing geron-
toplasts.

**SIGNIFICANCE OF ChL BREAKDOWN**

If it is justifiable to extrapolate from barley, rape, and a
few other species to higher plants in general, then it can be
stated that Chl is not catabolized beyond the level of linear
tetapyroles and that these catabolites are stored in the
mesophyll cells down to the end of the senescence period.
In other words, the four N atoms contained in each mole-
cule of Chl are unlikely to be recycled from senescent
leaves to other parts of the plant. They may contribute to
the diet of soil microorganisms when leaves are finally
shed. And yet, the question of why plants catabolize Chl at
all and in such a complicated and energy-consuming fash-
ion remains to be answered. A look at stay-green mutants
that are characterized by high retention of Chl in senescent
leaves or in ripening fruits (Thomas and Smart, 1993) is
enlightening. Chl stability in such plants is associated with
the persistence of Chl-binding proteins in the thylakoids
(Thomas and Hilditch, 1987; Guiamet et al., 1991; Cheung
et al., 1993; Bachmann et al., 1994). Apoproteins seem to be
protected from proteolysis as long as they are properly
complexed with pigments. Since the proteins of pigment
complexes in the thylakoids account for over 30% of the
total protein of chloroplasts, their recycling contributes
substantially to the overall N budget. When *F. pratensis*
plants are cultivated under nutritional conditions that pro-
mote internal N recycling, the stay-green line pays a sig-
nificant penalty compared with the wild type in terms of
rates of growth and development, largely because thyla-
koid protein N is immobile in senescent leaves (B. Hauck,
A.P. Gay, J. Macduff, C.M. Smart, and H. Thomas, unpub-
lished data). It would be expected, therefore, that stay-
green genotypes might be less competitive under natural
conditions of N limitation than are wild types with a more
efficient internal N economy. It is no wonder then that
stay-greens are found above all in cultivated plant species,
particularly in legumes such as soybean, French bean, and
pea (Thomas and Smart, 1993).

Another important reason why the cellular machinery
for breakdown in senescent leaves needs to be so elaborate
concerns the photodynamic nature of Chl. Disassembly of
pigment-protein complexes is associated with the separa-
tion of Chl from the various mechanisms that, in the intact
thylakoid, prevent photooxidative damage by activated
oxygen. Therefore, it is crucial for the viability of senescent
mesophyll cells that dismantling of the photosynthetic
membranes remains tightly coupled with the photody-
namic inactivation of Chl. In this sense, Chl breakdown
may be regarded as a process of detoxification. It is signif-
ants that the metabolic sequence that transports Chl ca-
tabolites to the vacuole and sequesters them as conjugates
is also reminiscent of the fate of xenobiotics and toxic
secondary compounds in plant cells.

**AREAS OF CURRENT INTEREST**

Several question marks in Figure 5 point to unsolved
problems. The localization of dechelatase in the envelope is
not yet firmly established, nor is the catalytic function of
the stroma protein, which acts in conjunction with the
oxygenase. An important item concerns the mechanism of
oxidative opening of the porphyrin macrocycle. The corre-
sponding reaction in *C. protothecoides* has been demon-
strated to occur in two steps, epoxidation of the double
bond in the methine bridge followed by hydrolysis, so that
only one of the newly introduced O atoms originates from
dioxygen (Curty et al., 1995). Whether the mechanism in
higher plants is the same can only be settled by the in vitro
production and mass spectroscopical analysis of the pri-
mary FCC in the presence of $^{18}$O$_{2}$.

A most puzzling finding concerns the specificity of oxy-
genase for Pheide a. It may explain the apparently exclu-
sive occurrence of NCCs derived from Chl a, but it poses
the problem of how Chl b is catabolized. A possible answer
may be inferred from the recent discovery of Chl-b-to-Chl-a
conversion in etioplasts (Ito et al., 1993, 1994) and chloro-
plasts of cucumber cotyledons (Ito and Tanaka, 1996). This
conversion, which utilizes Chlide b, may also occur in
senescent leaves, since under conditions favoring the accu-
mculation of dephytylated Chls only Pheide a and never
Pheide b can be observed. A lesion in this pathway may
explain the phenotype of cytG, a stay-green (cytoplasmic
ally inherited) mutant of soybean in which Chl a is more
labile than Chl b during senescence (Guiamet et al., 1991).

Perhaps the highest priority must be given to purifying
the oxygenase for the purpose of cloning the gene. To our
knowledge, it is the only enzyme so far described to occur

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exclusively in senescent leaves. Genes expressed during senescence that have been identified to date have functions at other stages of plant development as well (Smart, 1994; Smart et al., 1995). We anticipate that the promoter of the oxygenase will turn out to have structural and functional features that can shed much-needed light on how senescence is regulated in space and time, as well as representing tempting biotechnological targets. Solving the mystery of Chl degradation has been a unique exercise in combining the disciplines of structural chemistry, cell biology, and genetics and has taken us to the verge of understanding and manipulating senescence itself, a process of the greatest importance for plant development and agronomy.

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