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 thought to be 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 Chlide 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 Chlide 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 wild-type leaves (Matile et al., 1987, 1989). Subsequently, similar-colored compounds were found to be present in yellowing leaves of barley (Bortlik 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 possibly 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. *Ho* = *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, *Br*-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-oxo-bilane, as first found in *Ho*-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 *Ho*-NCC-1 to its putative Chl

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**Abbreviations:** Chl, chlorophyll; Chlide, chlorophyllide; FCC, fluorescent chlorophyll catabolite; NCC, nonfluorescent chlorophyll catabolite; Pheide, pheophorbide.
precursor revealed that an oxygenolytic ring-opening reaction between C-4 and C-5 of the northern mesoposition of the porphinoid macrocycle is likely to represent the crucial step of Chl breakdown in senescent leaves (Kräutler et al., 1991). In agreement with the colorless nature of all NCCs, the three mesopositions linking the four pyrroles are all saturated. The common UV/visible absorption maximum around 320 nm has been suggested to originate from the α-formyl pyrrole unit (Kräutler et al., 1991). The constitutions of the various NCCs differ from each other with respect to peripheral functionalities by way of the introduction of hydroxyl groups and other polar substituents. The three NCCs from rape constitute a particularly impressive series of catabolites, with the increasing polarities being due to the introduction of a hydroxyl, a β-glucopyranosyl, and a malonyl substituent in Bn-NCC-1, Bn-NCC-2, and Bn-NCC-3, respectively. In addition, in the Bn-NCCs, the characteristic methoxycarbonyl group of the Chls has been hydrolyzed to a free carboxylate function (Mühlecker et al., 1993; Mühlecker and Krautler, 1996), whereas it remained intact in the NCCs from other senescent plant sources (Kräutler et al., 1991, 1992; Iturraspe and Mayano, 1995; Curty and Engel, 1996). The ease of loss by decarboxylation of the β-keto-carboxylate function in the Bn-NCCs (Mühlecker et al., 1993) may provide a rationale 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 α (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 α 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 tetrapyrrole derived from Pheide a (Fig. 2). In Bn-FCC-2, the chromophoric system responsible for \( A_{365} \) 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 tetrapyrroles have functional traits of Chl α 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 tetrapyrroles 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-2, 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 Mühlcker et al. (1996). It has the molecular formula $C_{35}H_{30}N_2O_2$ (Pheide $a$, $C_{35}H_{29}N_2O_2$). 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.,

![Figure 3](https://example.com/figure3.png) Chlorophyll breakdown in senescent leaves: putative sequence of enzymic reactions.
Chlorophyll

Chlide

Photosystem I

O₂

Fd₄₉₃

Fd₄₅⁰

NADP⁺

NADPH

Intermediary compound

Chlide a

Pheide a (Oxygenase)

Pheide a

Stroma protein (Reductase)

NAD⁺

NADPH

Glucose-6-

phosphate

Primary FCC

Hv-FCC-4 = Bn-FCC-2

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 chloro-
plast 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 extrac-
tion, 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; Hörtenstein 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 senes-
cent 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
stoma factor required for the ring-opening reaction. Stu-
dents 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 ele-
ment of the catabolic system located upstream of chloro-
phyllase that is regulated in a senescence-specific manner.

COMPARTMENTATION OF BREAKDOWN IN THE CELL

An appreciation of the spatial organization within senes-
cent mesophyll cells and gerontoplasts is crucial for under-
standing 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 or-
ganello (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 cyto-
solic face of the envelope (Matile et al., 1992). Thus, geron-
toplasts appear to be equipped with a carrier that is re-
sponsible 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 cata-
bolic enzymes within the plastids. We began with chloro-
phyllase; it has been known for a long time that its activity
is latent. Although the enzyme is associated with chloro-

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plast membranes apparently close to its substrate, hydrolysis of the endogenous Chl does not take place unless the membranes are solubilized in the presence of an appropriate detergent (Amir-Shapira et al., 1986) or acetone (e.g., Garcia and Galindo, 1991). Recently, an unexpected explanation 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 complexes is unaffected 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 establishment 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 phenomenon 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 linking 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 gerontoplasts.

**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 tetrapyroles 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 molecule 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 fashion 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 promote internal N recycling, the stay-green line pays a significant penalty compared with the wild type in terms of growth and development, largely because thylakoid protein N is immobile in senescent leaves (B. Hauck, A.P. Gay, J. Macduff, C.M. Smart, and H. Thomas, unpublished 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 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 separation 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 photodynamic inactivation of Chl. In this sense, Chl breakdown may be regarded as a process of detoxification. It is significant that the metabolic sequence that transports Chl catabolites 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 corresponding reaction in *C. protothecoides* has been demonstrated 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 primary FCC in the presence of 18O2.

A most puzzling finding concerns the specificity of oxygenase for Pheide a. It may explain the apparently exclusive occurrence of NCCs derived from Chl b, 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 chloroplasts of cucumber cotyledons (Ito and Tanaka, 1996). This conversion, which utilizes Chlide b, may also occur in senescent leaves, since under conditions favoring the accumulation 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 (cytoplasmically 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
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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