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Review

Chlorophyll breakdown in higher plants and algae

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Abstract. Leaf senescence is accompanied by the metabolism of chlorophyll (Chl) to nonfluorescent catabolites (NCCs). The pathway of Chl degradation comprises several reactions and includes the occurrence of intermediary catabolites. After removal of phytol and the central Mg atom from Chl by chlorophyllase and Mg dechelatase, respectively, the porphyrin macrocycle of pheophorbide (Pheide) *a* is cleaved. This two-step reaction is catalyzed by Pheide *a* oxygenase and RCC reductase and yields a primary fluorescent catabolite (pFCC). After hydroxylation and additional species-specific modifications, FCCs are tautomerized nonenzymically to NCCs inside the vacuole. Different subcellular compartments participate in Chl catabolism and, thus, transport processes across membranes are required. This review focuses on the catabolites and the individual reactions of Chl breakdown in higher plants. In addition, the pathway is compared to Chl conversion to red catabolites in an alga, *Chlorella protothecoides*. Finally, the significance and regulation of Chl degradation are discussed.

Key words. Breakdown; catabolites; catabolic pathway; chlorophyll; detoxification; pigment; senescence; vacuole.

Introduction

The disappearance of green color in the leaves of deciduous trees during autumn is one of the most impressive natural phenomena. Research on chlorophyll (Chl) breakdown can be dated back to 1912 when Arthur Stoll discovered chlorophyllase [1]. Except for this early work, there was rather little progress in the understanding of degreening in plants. This is manifested in a review by George Hendry and co-workers 12 years ago in which Chl breakdown is described as a biological enigma [2]. In the last decade the situation has changed as the biochemistry underlying breakdown of Chl during developmental processes, such as leaf senescence and fruit ripening, gradually has been elucidated (for reviews see [2–11]).

A major difficulty in understanding the problem of Chl catabolism has been ignorance about the products of Chl breakdown. In analogy to bile pigments, the products of heme degradation, attention has been focused on colored catabolites. Radiolabelling of Chl and tracing the label during senescence eventually led to the discovery and isolation of intermediary and final catabolites [12]. Surprisingly, most of them were colorless, and therefore they had been overlooked for a long time. The structural elucidation in 1991 of a final nonfluorescent Chl catabolite (NCC) from barley marked a major breakthrough [13]. It provided an opportunity to compare the chemical structures of Chl with its final degradation products and thus enabled predictions of the reactions that are likely to occur during breakdown. Also, the availability of stay-green mutants [14, 15] led to substantial progress on the mechanism of Chl breakdown and allowed the verification of results obtained upon the study of enzymic reactions in vitro. Most of the biochemical steps of the catabolic pathway of Chl are now known, and the details will be outlined in this review.

The pathway of chlorophyll breakdown

The catabolism of Chl has been studied in senescing leaves and ripening fruits of a large number of plant species. However, most of these studies merely deal with the early steps of the pathway, i.e. with the transformation of Chl to chlorophyllide (Chlide), pheophorbide (Pheide) and other green pigments. Thus, information on subsequent breakdown steps and catabolites is rather scarce and is mainly available from investigations on senescent barley primary leaves and cotyledons of canola [6]. The pathway of chlorophyll breakdown as depicted in figure 1 summarizes current knowledge. The catabolic machinery comprises at least six reactions, whereby the first four are responsible for the conversion of Chl to a primary colorless catabolite (pFCC). Chl is first dephytylated to Chlide by the action of chlorophyllase, and subsequently the central Mg atom is removed by Mg dechelatase. The product of this reaction, Pheide, is the last colored (green) intermediary catabolite of the pathway. Its porphyrin macrocycle is oxygenolytically opened in a two-step reaction with a red catabolite (RCC) as an intermediate; the product, pFCC, is a fluorescent linear tetrapyrrole. This key step in Chl breakdown is catalyzed by the joint action of Pheide *a* oxygenase (PaO) and RCC reductase, and it formally occurs by the addition to Pheide *a* of two atoms of oxygen and four atoms of hydrogen. The structures of the final products of Chl breakdown, NCCs, from different plant species suggest that two further common reactions take place, namely hydroxylation at the $C(8^2)$ position of pFCC and (nonenzymic) tautomerization in ring D and the γ methine bridge of the fluorescent to the nonfluorescent catabolites.

In the following sections both the catabolites and the biochemical reactions of the pathway are described in detail.

Chlorophyll catabolites

Green pigments

Chlorins. Several putative porphyrinic degradation products of Chl have been identified. These include 13^2-hydroxy Chl *a* [16–18], pheophytin [19–21], pyropheophytin [19], pyropheophorbide [18, 22, 23], Chlide and Pheide (fig. 2) [18, 21]. Generally, during Chl breakdown these pigments occur only in trace amounts, suggesting that they represent either intermediates to other (colorless?) compounds [14] or artifacts of tissue extraction. Thus, e.g. release of Mg^{2+} from Chls or formation of pyro forms readily takes place under acidic conditions [21, 24]. 13^2-Hydroxy Chl, which has been considered as an intermediate of a so-called 'oxidative Chl bleaching pathway' [25]

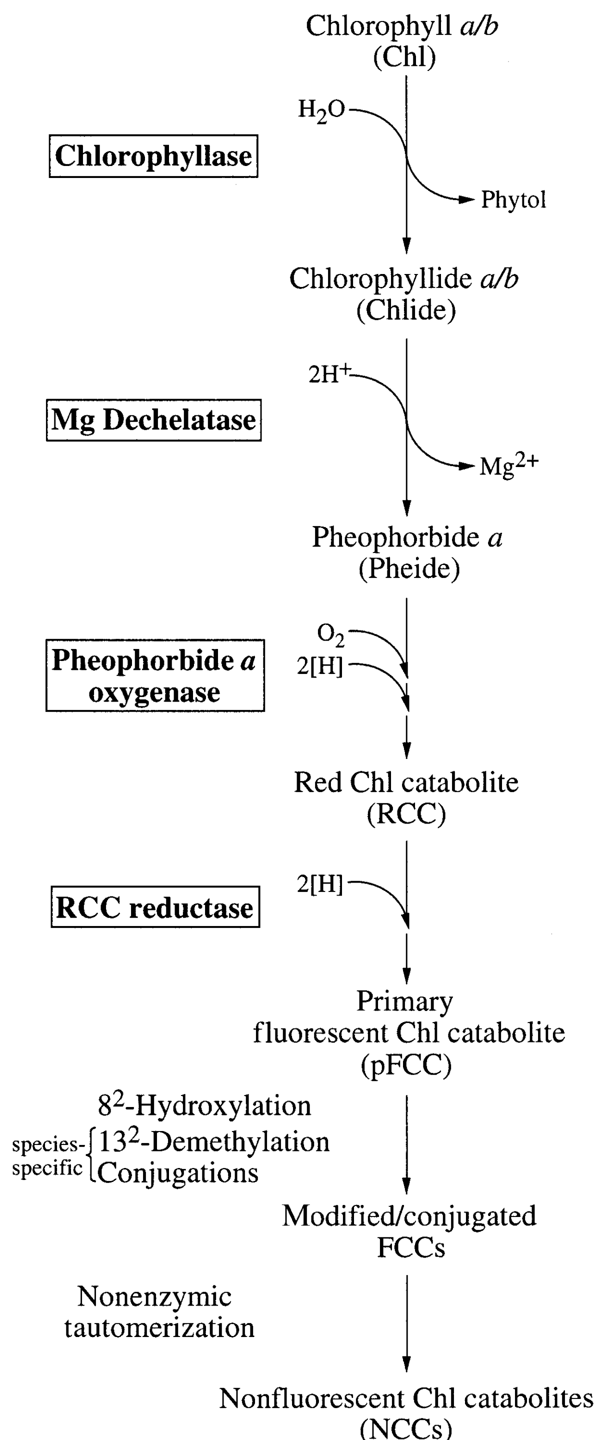
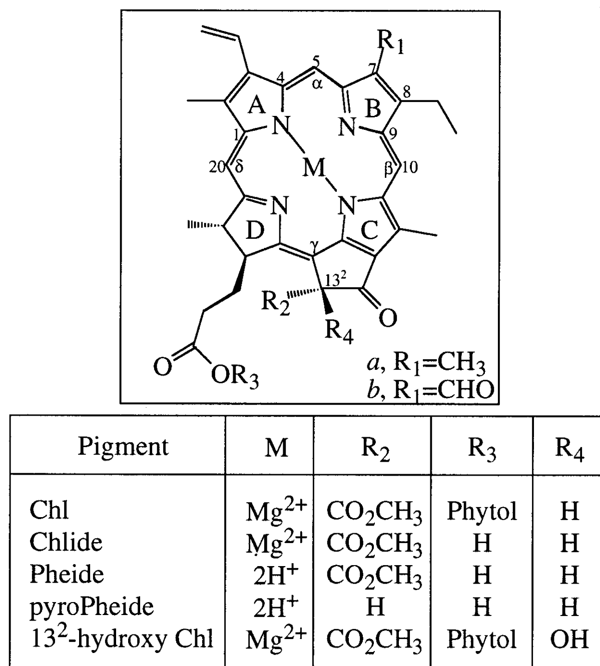


Figure 1. The pathway of Chl breakdown in higher plants. Enzymes identified and characterized to date are boxed.



Pigment	M	R_2	R_3	R_4
Chl	Mg^{2+}	CO_2CH_3	Phytol	H
Chlide	Mg^{2+}	CO_2CH_3	H	H
Pheide	$2H^+$	CO_2CH_3	H	H
pyroPheide	$2H^+$	H	H	H
13^2-hydroxy Chl	Mg^{2+}	CO_2CH_3	Phytol	OH

Figure 2. Chemical structure of Chl and of Chl catabolites with an intact chlorine macrocycle. Relevant carbon atoms, rings and methine bridges are labelled.

formed by Chl oxidase, an enzyme induced by linolenic acid [26], most likely is not relevant to Chl breakdown during senescence [3, 18]. The comparison of yellowing in normal and stay-green phenotypes, respectively, of species such as *Festuca pratensis* [14] and *Phaseolus* [27] was very helpful for the identification of the natural intermediates. It has been shown that Chlides *a* and *b*, as well as Pheide *a*, accumulate in the nondegreening genotypes which have a lesion with respect to the porphyrin ring opening. These dephytylated species can, therefore, be considered as early (green) breakdown products of Chl.

Phytol. After hydrolysis of Chl only small proportions of phytol are lost during senescence [28], possibly by photooxidative processes [29]. Reutilization for the synthesis of α-tocopherol which accumulates during senescence [30] has been suggested [31]. This is rather unlikely as it would require the activation of phytol to phytyl pyrophosphate, the cosubstrate of condensation with homogentisic acid [32, 33]. In barley, a large proportion of phytol remains localized in plastoglobules [34], which during the transition of chloroplasts into gerontoplasts [35] are present in increasing number and size [3]. Phytol either remains free or is esterified, e.g. with acetic acid [34] or with various fatty acids [36, 37].

Intermediary tetrapyrrolic catabolites

Fluorescent Chl catabolites. In vivo ^{14}C-radiolabelling of Chl was an important prerequisite for identifying nongreen catabolites of Chl breakdown. Specific labelling of the porphyrin macrocycle of Chl was achieved by feeding 4[^{14}C]-δ aminolevulinic acid (ALA) to excised expanding cotyledons of rape [38] or to etiolated barley primary leaves [12]. With ALA labelled in the C(4) position, radioactivity is specifically incorporated into the pyrrole units of the porphyrin moiety of Chl [12]. After maturation of the leaves, the incorporated label was followed during dark-induced senescence. With progressing senescence increasing amounts of ^{14}C-label were recovered from the water-soluble fraction of leaf extracts [12, 38]. Among the labelled nonfluorescent catabolites (see below) subsequently identified by high-performance liquid chromatography (HPLC) and by thin-layer chromatography was a fluorescent compound which was regarded to represent an early catabolite of Chl [39]. Blue fluorescing, colorless pigments (FCCs) were also found to occur in trace amounts in senescent rape [38, 40] and in *Phaseolus vulgaris* [15]. In the case of *Festuca pratensis* wild type, FCCs were demonstrated to be readily oxidized to 'pink pigments' [41], whereas in the nonyellowing mutant Bf993 these catabolites were absent [42]. Collectively, these findings suggested that fluorescent catabolites represent intermediary rather than final products of Chl catabolism [43].

An FCC (pFCC-1 [44]) from rape has been synthesized enzymically from Pheide *a* in vitro [40, 45], and after purification to homogeneity by HPLC its chemical constitution has been determined by mass spectroscopy and ^1H-nuclear magnetic resonance spectroscopy [46]. It has the structure of a 4,5-seco-4,5-dioxophytoporphyrinate, indicating that it is derived from Pheide *a* by the oxygenolytic opening of the macrocycle between pyrroles A and B (fig. 3B). Of the four stereogenic centers of pFCC-1, three have the same relative configuration as Pheide *a*, suggesting the absolute configuration to be the same as in Pheide *a*. The absolute configuration at C(1) has not yet been established, but structural analysis of the primary FCC from sweet pepper which is slightly less polar than pFCC-1 [47] revealed that they are stereoisomeric at C(1) (W. Mühlecker and B. Kräutler, unpublished). All FCCs, including pFCCs, have identical ultraviolet/visible spectra with a major absorption maximum at ca. 320 nm and a minor peak at around 360 nm (fig. 3B) [15, 38, 46]. The blue fluorescence has an emission maximum at 450 nm (excitation 320 nm) and is due to the Schiff's base configuration of the unsaturated γ methine bridge linking rings C and D.

Red Chl catabolites. In rape and in barley, pFCC-1 represents the first identifiable product of Pheide *a* breakdown [40, 46, 48]. Ring cleavage takes place in two consecutive steps with a red catabolite intermediate, RCC, which only occurs in minute amounts [48]. RCC has been chemically synthesized from Pheide *a* methyl ester [49] and was demonstrated by photospectroscopic and chromatographic means to be identical with the intermediary product of porphyrin cleavage. In contrast to pFCC-1, the δ methine bridge of RCC is oxidized so that a conjugated system extends over three of the four rings (fig. 3A). In addition to an absorption maximum at 316 nm, the spectrum of RCC is distinguished by a peak at ca. 485 nm.

Red derivatives of Chl corresponding to RCC are also known to be produced in *Chlorella protothecoides* [24, 50–52] and *C. kessleri* [4] during enforced degreening under N-deficiency and heterotrophic metabolism. In these green algae RCCs represent the final catabolites of Chl breakdown which are excreted into the medium [50–52]. Because acidic conditions were originally used for extraction, the red pigments were obtained as pyro derivatives [52, 53], but later it was confirmed that in the RCCs produced during *Chlorella* degreening the methoxycarbonyl function at $C(13^2)$ is demethylated [24]. In contrast to higher plants in which the final catabolites (NCCs) are exclusively derived from Chl *a*, an RCC with *b* configuration has been identified from *C. protothecoides* [54].

Final catabolites

NCCs were first identified in barley as compounds that were present only in senescent but not in presenescent primary leaves. In the native form these catabolites were colorless, but they were readily oxidized in air to rust-colored pigments of which the most abundant was referred to as RP14 according to its retention time on reversed-phase HPLC [55]. Radiolabelling of Chl with $4[^{14}C]$-ALA during greening of etiolated barley and subsequent senescence in permanent darkness confirmed RP14 as a derivative of Chl [12]. In the meantime several NCCs have been isolated from various plant species [15, 47, 56], and thus a convenient nomenclature was necessary [38]. For instance RP14 is now named *Hv*-NCC-1, whereby the prefix and the suffix indicate the plant species (*Hordeum vulgare*) and the relative retention on reversed-phase HPLC, respectively. Since

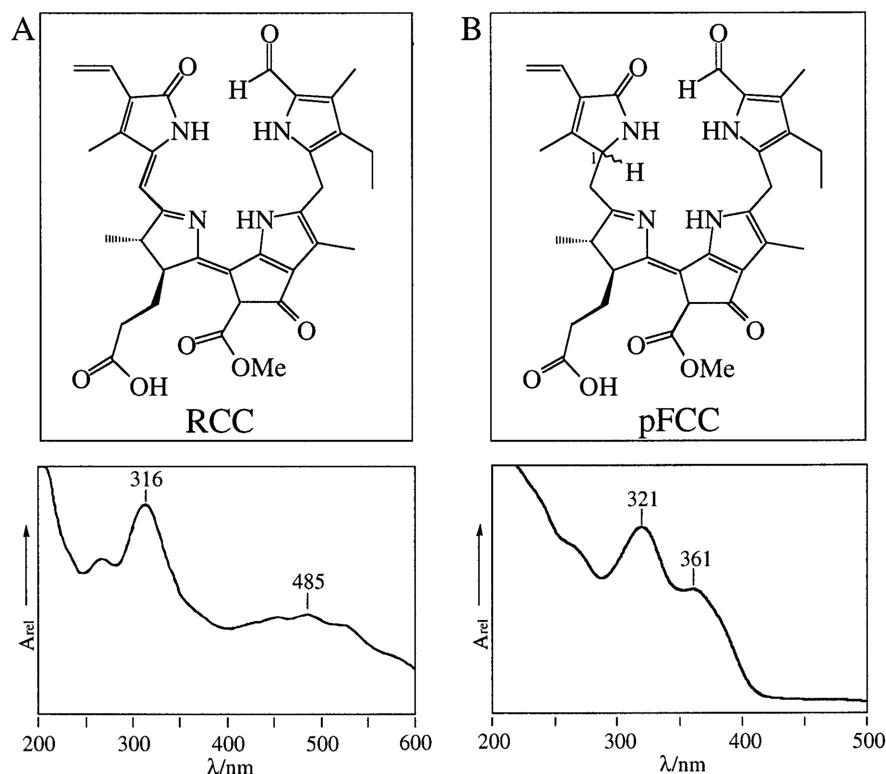
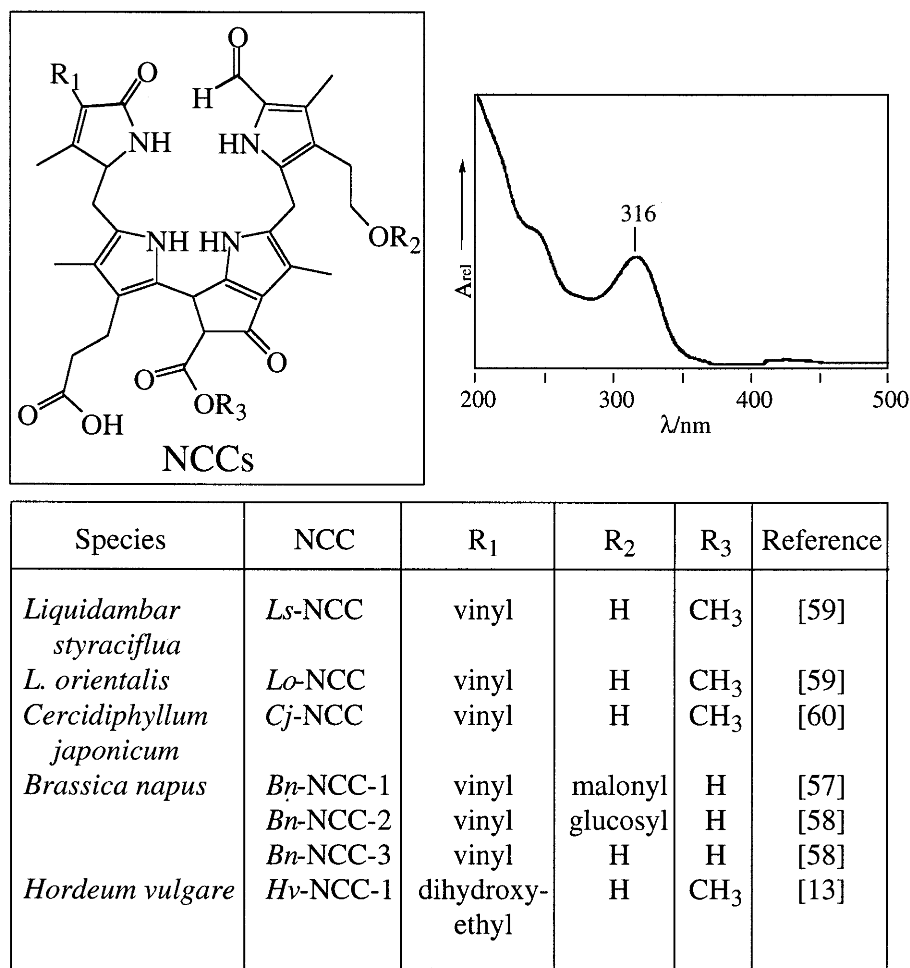


Figure 3. Chemical constitution of RCC (*A*) [49] and of pFCC (*B*) [46], and their ultraviolet/visible spectra. For labelling of relevant positions see figure 2. Note that the two pFCCs, pFCC-1 and -2, are stereoisomeric at C(1) [44].



Species	NCC	R_1	R_2	R_3	Reference
Liquidambar styraciflua	*Ls*-NCC	vinyl	H	CH_3	[59]
L. orientalis	*Lo*-NCC	vinyl	H	CH_3	[59]
Cercidiphyllum japonicum	*Cj*-NCC	vinyl	H	CH_3	[60]
Brassica napus	*Bn*-NCC-1	vinyl	malonyl	H	[57]
	Bn-NCC-2	vinyl	glucosyl	H	[58]
	Bn-NCC-3	vinyl	H	H	[58]
Hordeum vulgare	*Hv*-NCC-1	dihydroxy-ethyl	H	CH_3	[13]

Figure 4. Chemical constitution of NCCs from higher plants and the absorption spectrum of *Bn*-NCC-1 [58]. For labelling of relevant positions see fig. 2.

the first structure elucidation of *Hv*-NCC-1 in 1991 [13] the chemical constitution of a range of other NCCs from different sources has been established [57–60]. Their common structure is shown in figure 4. In all cases NCCs are derived from Chl *a*. In accordance with the structures of pFCC-1 and RCC (fig. 3), the porphyrin macrocycle is cleaved at the C(4)/C(5) mesoposition. All three remaining mesopositions are fully reduced, resulting in the complete disruption of the double-bonding system of Chls. Therefore, NCCs are nonfluorescent and colorless with a maximum of absorption at 315 nm (fig. 4) which is due to the α-formyl pyrrole moiety of ring B [13]. A further common feature of all NCCs structurally analyzed so far is the C(8^2) hydroxyl group in the ethyl side chain of pyrrole B. The NCCs from the autumnal foliage of *Cercidiphyllum japonicum* and of two *Liquidambar* species have this basic structure of final catabolites. In these species, the total amount of Chl present in the mature leaves is converted to a single type of NCC [59, 60]. In contrast, additional modifications occur in other species, such as e.g. hydroxylations at the vinyl side chain of ring A as in *Hv*-NCC-1 [13], or demethylation of the 13^2 methoxycarbonyl group in rape and the conjugation of the C(8^2) hydroxyl group with either malonic acid or with a β-glucose unit as in *Bn*-NCC-1 and 2, respectively [57, 58]. Whereas the three rape NCCs correspond to the total Chl of mature cotyledons broken down [38], radiolabelling in barley has demonstrated the presence of more than 10 additional NCCs whose chemical structures have not been determined [12].

Miscellaneous breakdown products of Chl and of heme

In all linear tetrapyrrolic derivatives of Chl that have been shown to occur in senescent leaves or in degreening *Chlorella*, C(5) of the α methine bridge is conserved as a formyl group attached to pyrrole B (figs 3 and 4).

In contrast, the corresponding catabolites of heme are 1,19-dioxobilanes, in which during porphyrin ring opening, the C(5) atom is lost as CO (fig. 5A) [61]. Biliverdin is not only the primary product of heme degradation but also a precursor of the phycobilins, phycoerythrin and phycocyan of blue and red algae [62].

Recently, water-soluble red pigments with properties of bile pigments have been isolated from a Chl *b*-less mutant of *Chlamydomonas reinhardtii* [63] and from *Bryopsis maxima* [64], but their relation to Chl degradation has not yet been clarified. Unusual tetrapyrrolic pigments are the light emitter molecules (fluorescent compound F and dinoflagellate luciferin, respectively) of krill (*Euphausia pacifica*) and *Pyrocystis lunula*, which are responsible for bioluminescence in these dinoflagellates [65, 66]. These compounds are derivatives of Chl, but in contrast to other Chl catabolites (and bile pigments), the porphyrin macrocycle is opened in the δ methine bridge rather than the α mesoposition (fig. 5B). The mechanism of ring opening responsible for synthesis of these photophores is unknown.

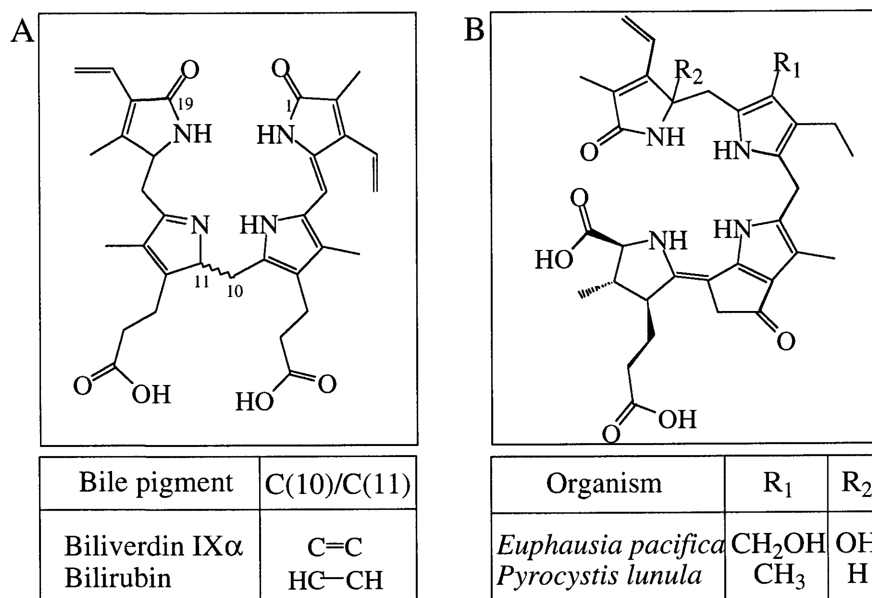
The biochemistry of chlorophyll breakdown

Dephytylation

During the last 80 years chlorophyllase has been thoroughly investigated, and its properties are documented by an innumerable number of publications and some review articles [67–69]. But despite these extensive efforts, the role of chlorophyllase in Chl breakdown has remained partially mysterious.

Chlorophyllase [EC 3.1.1.14] catalyzes the hydrolysis of Chl to Chlide and phytol. In addition, transesterification reactions with fatty acids, amino acids and Triton X-100 have been described [70, 71]. Taking advantage of its transesterase activity, chlorophyllase has been used as a tool to modify Chl with amino acids and peptides, e.g. in order to study Chl-protein interactions [72]. Chlorophyllase recognizes only porphyrinic substrates having a reduced ring D [73], but it acts on both Chl *a* and *b*, as well as pheophytins [74]. Chlorophyllase is generally believed to act as the first enzyme in Chl breakdown [75], but it has also been assumed to catalyze the phytylation step of Chl biosynthesis [71, 76]. Since the identification and cloning of chlorophyll synthase [77–80], biosynthetic function should no longer be attributed to chlorophyllase.

A puzzling feature of chlorophyllase is its latency: the enzyme is only active after extraction from chloroplast membranes with either detergents, such as Triton X-100 [75, 81], or with solvents such as acetone or methanol [67, 82]. The surprising localization of chlorophyllase in the inner envelope of chloroplasts [75, 83] may explain this structural latency with regard to spatial separation of chlorophyllase from its substrate Chl in the thylakoid membranes of mature green tissue. During senescence chlorophyllase remains latent [75], suggesting that a mechanism is necessary for establishing physical contact between Chl and chlorophyllase (see below). Nevertheless, chlorophyllase activity is modulated by factors affecting leaf and fruit senescence, such as ethylene and kinetin [81, 84, 85].



Bile pigment	C(10)/C(11)
Biliverdin IXα	C=C
Bilirubin	HC–CH

Organism	R_1	R_2
Euphausia pacifica	CH_2OH	OH
Pyrocystis lunula	CH_3	H

Figure 5. Chemical structures of heme degradation products (*A*) and of dinoflagellate luciferins (*B*). In *A*, carbon atoms are labelled according to the conventional bilin numbering system.

Chlorophyllase has been purified from several sources with contradictory results regarding its size. Thus, the enzymes of *Citrus* [81, 86] and *Chlorella regularis* [87] have an M_r of about 27 and 65 kDa, respectively, whereas the size is about 40 kDa in other species [88–91]. N-terminal sequences obtained from *Citrus* fruit [81] and from *Chenopodium album* [91] were completely different. Recently, a complementary DNA (cDNA) has been cloned from *Citrus* that confers chlorophyllase activity when overexpressed in *Escherichia coli* (E. Goldschmidt, personal communication).

Mg dechelation

The existence of a dechelating enzyme has been deduced indirectly from the occurrence of pheopigments during senescence (20, 21), in a stay-green genotype of *Festuca pratensis* [92] and during Chl breakdown in algae and photosynthetic bacteria [18, 19, 93]. Mg dechelatase activity has been characterized in a few systems to some extent [18, 92, 94–96]. It removes the central Mg^{2+} of Chlide in exchange with 2 H^+. For the assessment of Mg-dechelating activity, preparations of either Chlide [96] or of water-soluble chlorophyllin [97], obtained upon saponification of Chl [73], have been used.

Dechelating activity has been observed to be membrane-bound [97] and to be associated with a distinct complex from detergent-solubilized chloroplast membranes [98]. As with chlorophyllase, Mg dechelatase does not seem to be regulated during senescence. The activity is present in senescent as well as presenescent leaf tissue of barley [98] or rape [95]. Attempts to purify Mg dechelatase have produced surprising results: the activity turned out to be heat-stable [96, 99] and to be associated with a small molecule with a M_r of less than 5 kDa, which is referred to as Mg-dechelating substance (MDS) [96]. However, heat-labile dechelatase activities have been described as well [23, 94, 99], and hence it remains to be decided whether the soluble MDS is responsible for Mg^{2+} removal in vivo. It may be associated with a (labile) protein within chloroplast membranes.

Porphyrin ring cleavage

The initial steps of Chl catabolism, i.e. dephytylation and Mg dechelation yield catabolites which are still green. The first identifiable colorless product of porphyrin ring cleavage, pFCC (fig. 3B), is formally derived from Pheide *a* by the addition of two atoms of oxygen and four atoms of hydrogen [46]. pFCC-1 (formerly named *Bn*-FCC-2) is identical to the primary tetrapyrrolic catabolite of barley, *Hv*-FCC-4. In both systems the in vitro formation of the respective FCC has been shown to depend on the presence of two protein components of senescent chloroplasts, i.e. chloroplast membranes and stroma protein [40, 45, 100]. Likewise, in the ripening fruit of sweet pepper, production of a primary FCC from Pheide *a* required the presence of both chromoplast membranes and soluble protein [47]. The membrane-derived component, PaO, is specifically present in gerontoplast or chromoplast membranes but is absent in presenescent tissue [47, 100]. In contrast, the stroma factor, RCC reductase, is present at all stages of leaf development—including etiolated leaves—and is even found in roots [48]. Chelators such as 2,2′-bipyridyl or *o*-phenanthroline prevent Chl degradation and cause an artificial stay-green phenotype [92, 95]. Inhibitor studies showed that only the membrane fraction was affected, and inhibition of PaO could be reversed in vitro specifically by the addition of Fe^{2+} but not with other divalent cations [40]. These findings together with the failure of CO, an inhibitor of heme-containing oxygenases [101, 102], to inhibit pFCC formation in rape gerontoplasts [38] indicated that Pheide *a* oxygenase is a non-heme iron-containing enzyme. The requirement of two different proteins for pFCC formation from Pheide *a* is rationalized by the finding that the reaction occurs in two steps with RCC as the intermediate: after oxygenolytic cleavage by PaO, RCC is reduced at the C(1)/C(20) mesoposition to pFCC (fig. 6) [48]. As in vitro RCC does not accumulate to substantial amounts in the absence of RCC reductase, the overall reaction is likely to be metabolically channelled without intermediate release of RCC from PaO [48]. Both reactions depend on the presence in the assay mixture of reduced ferredoxin (Fd) [48], which cannot be substituted by any other electron donor [100]. Fd is kept in the reduced state either by reduced nicotinamide adenine dinucleotide phosphate (NADPH) via Fd NADPH oxidoreductase linked to the pentose-phosphate cycle or directly through light-dependent reduction at photosystem I (fig. 6) [48].

Two atoms of oxygen are introduced into RCC, pFCC-1 and the corresponding red catabolites of *Chlorella protothecoides* [24, 46, 49], and production of pFCC-1 from Pheide *a* has been shown to require dioxygen [56]. Thus, it was tempting to investigate the specificity of PaO for incorporation of $^{18}O_2$ oxygen into Pheide. In pFCC-1 of rape [103] (fig. 6) as well as in *Chlorella* [104] ^{18}O was only found in the formyl group of pyrrole B, and hence the respective enzymes are monooxygenases. The origin of the lactam oxygen in pyrrole A remains unclear, but most probably it is derived from H_2O.

Properties of PaO have mainly been studied in the coupled reaction with RCC reductase from Pheide to pFCC-1 [40, 45, 47, 100]. The enzyme is specific for Pheide *a* with the *b* form being a competitive inhibitor [40, 47]. This substrate specificity appears to be respon-

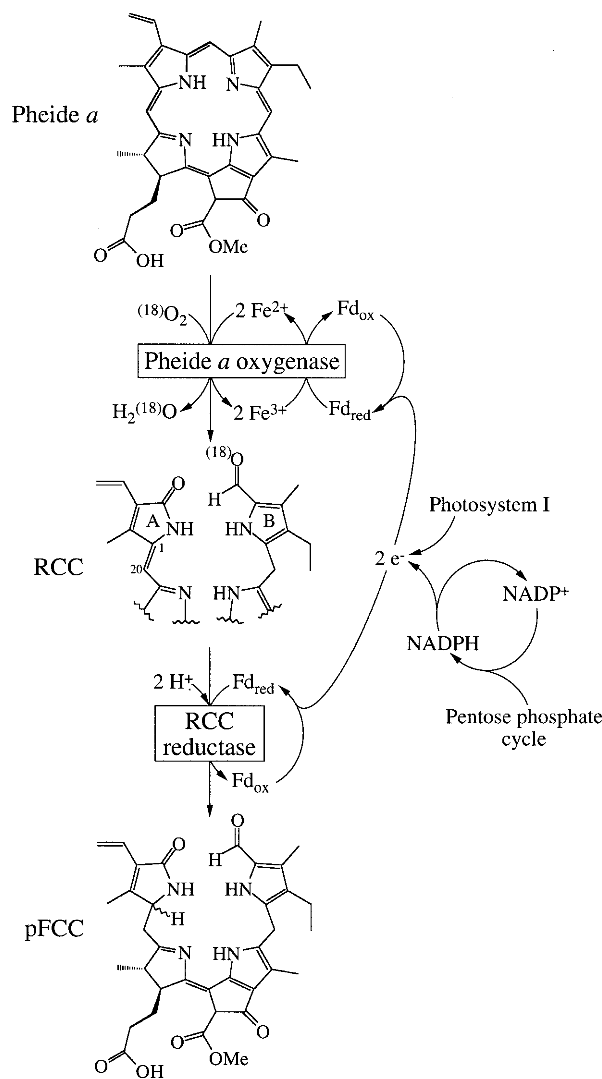


Figure 6. The key step in Chl breakdown, i.e. conversion of Pheide *a* to pFCC by Pheide *a* oxygenase (PaO) and RCC reductase. The intermediate, RCC, is not released, but metabolically channelled. Note that reduced Fd functions as an electron donor in both partial reactions.

sible for the fact that all NCCs structurally characterized so far from higher plants are derived from Chl *a* [13, 57–60]. On the other hand, it also raises the interesting question of the fate of Chl *b* during Chl breakdown (see below). PaO has been partially purified from senescent rape cotyledons starting with solubilized gerontoplast membranes [103], but unfortunately attempts to purify the protein to homogeneity and to clone the gene have failed so far. Purification of RCC reductase was more successful. It has been purified to near homogeneity from senescent barley leaves, and its properties were analyzed [44]. When RCC chemically synthesized from methyl Pheide *a* [49] was employed as substrate, three different FCCs were formed. Two of them were identified as pFCC-1 and pFCC-2 found in *Capsicum annuum* [47]. These FCCs have identical constitutions but differ in the absolute configuration at C(1) (fig. 3B). The structure of the third FCC remains unclear [48]. A survey of RCC reductases from different plant species revealed stereospecificity towards reduction of C(1). RCC reductase activity can be demonstrated in mono- as well as in dicotyledons [44], and is also found in pteridophytes and gymnosperms (S. Rodoni and M. Schellenberg, personal communication). Thereby, within a plant family RCC reductases from different genera and species have the same stereospecificity.

An interesting feature of RCC reductase is its sensitivity towards oxygen: in vitro pFCC formation from RCC occurs only under anoxic conditions [44, 48]. In contrast, the coupled in vitro assay (formation of pFCC from Pheide *a*) requires oxygen for incorporation into the substrate [103]. Thus, it is tempting to speculate that in the metabolic channelling of the two partial reactions, PaO creates an oxygen-depleted microenvironment which allows the action of RCC reductase.

Transition of pFCC into NCC

Hydroxylation at C(8^2). A hydroxyl function in C(8^2) is a common feature of all final Chl catabolites identified so far. To date there is no direct proof of an enzyme activity which catalyzes the respective hydroxylation reaction. Indirect evidence for the production of a hydroxylated FCC is given by radiolabelling of a polar FCC (*Bn*-FCC-1) present in senescent rape chloroplasts [56] which is produced in organello upon incubation with glucose-6-phosphate or adenosine triphosphate (ATP) [45]. This FCC, which is also present in isolated evacuolated rape mesophyll protoplasts (S. Hörtensteiner, unpublished), is produced in small quantities in an in vitro assay employing chloroplast membranes and stroma protein [45]. It can be utilized as a substrate of NCC malonyltransferase (see below), and thus most likely represents the C(8^2) hydroxylated form of pFCC-1. Hydroxylation reactions are generally accepted to be catalyzed by cytochrome P450-type monooxygenases [101]. However, formation of *Bn*-FCC-1 in organello was insensitive to CO, a common inhibitor of such monooxygenases [45]. On the other hand, the location in chloroplast membranes of allene oxide synthase, a P450-dependent monooxygenase, has recently been demonstrated [105, 106], and surprisingly it is insensitive to CO [105, 107]. Thus, it is likely that plastids may also contain a specific oxygenase which is responsible for the hydroxylation of pFCC-1 at C(8^2).

Malonylation. In rape, the major NCC, *Bn*-NCC-1, is the malonyl ester of *Bn*-NCC-3 [57, 58]. After senescence under natural conditions, it accounts for more than 90% of the total NCCs accumulated in cotyledons [38]. Malonyltransferase activity has been demonstrated in protein extracts of rape cotyledons with malonyl coenzyme A as the cosubstrate. It is constitutive and specifically accepts tetrapyrrolic Chl catabolites with a hydroxyl group at $C(8^2)$ as substrates [108]. Thus, NCCs from different sources were suitable substrates, and *Bn*-FCC-1, which is suspected to be hydroxylated in $C(8^2)$ (S. Hörtensteiner, unpublished). NCC malonyltransferase, partially purified from rape, has a temperature optimum of 34 °C [108] similar to other malonyltransferases [109].

Glucosyltransferase activity would be required for the conjugation of rape *Bn*-NCC-3 into *Bn*-NCC-2 [58]. This catabolite occurs in minor quantities mainly during dark-induced senescence [56]. A corresponding transferase activity with uridine diphosphate-glucose as cosubstrate has not been demonstrated up to now [108].

Demethylation of the $C(13^2)$ methoxycarbonyl function. $O(13^4)$ demethylated, tetrapyrrolic Chl catabolites have been described so far only from rape [58] and from *Chlorella* [24]. In *Chlorella*, these catabolites were originally isolated as the respective decarboxylated pyro forms [52, 53]. Pyro Pheides have frequently been described as early catabolites of Chl degradation [18, 22, 23], and the enzyme involved was referred to as decarbomethoxylase [22, 23]. Recent investigations in *Chenopodium album* suggest that only demethylation of Pheide *a* is enzyme-catalyzed, whereas the subsequent decarboxylation to the pyro form occurs nonenzymically and is accelerated in the presence of acetone at high concentration [110]. This observation is interesting with regard to the instability of the free β-oxocarboxyl group if exposed to acids or if chromatographed on silica gel plates [24, 111], and raises a question about the relevance of pyro pigments in biological Chl breakdown. The enzyme catalyzing the formation of $O(13^4)$-demethyl Pheide *a* represents a methylesterase which has been tentatively named pheophorbidase [110]. In a recent study, hydrolysis of the $C(13^2)$ carboxymethyl ester of pFCC-1 has been demonstrated to be catalyzed by a soluble, most probably cytosolic, enzyme from rape cotyledons [112]. The identity of the reaction product with $O(13^4)$-demethyl pFCC-1 was confirmed by mass spectroscopy (S. Hörtensteiner and B. Kräutler, unpublished). Analogous to pheophorbidase of *Chenopodium album*, which accepts only chlorins (oxidized at ring D) but not porphyrins as substrates [110], the rape enzyme was specific for FCCs; NCCs with a carboxymethyl ester group such as *Cj*-NCC were not hydrolyzed (S. Hörtensteiner, unpublished). Esterases commonly used for hydrolysis of $C(17^2)$ carboxymethyl esters failed to hydrolyze the ester at $C(13^2)$ [49, 60], suggesting that the latter reaction is catalyzed by a rather specific enzyme.

Tautomerization involving ring D and the γ mesoposition. NCCs have been shown to be exclusively localized in vacuoles of senescent mesophyll cells [55, 113, 114], and the transport system responsible for the sequestration at the tonoplast has been identified (see below). Structural analysis of pFCC-1 from rape [46] has led to the presumption that NCCs are derived from FCCs through nonenzymic tautomerization under acidic conditions [24, 115]. This hypothesis could be verified employing purified pFCC-1 [112] (S. Hörtensteiner, unpublished): upon incubation at pH 4, pFCC-1 was converted to an NCC as judged by a change in optical properties to a spectrum typical for NCCs (fig. 4). Therefore, tautomerization of FCCs to NCCs most likely occurs nonenzymically after disposal of FCCs into the vacuole.

Miscellaneous modifications. Radiolabelling of Chl in barley and tracing of the label during senescence have demonstrated the occurrence of about a dozen labelled NCCs with different polarities on reversed-phase HPLC [12]. The structure of the most abundant NCC, *Hv*-NCC-1, is distinguished by its dihydroxyethyl group attached to pyrrole A [13]. Among the NCCs structurally analyzed so far, this modification is unique, but it raises speculations about possible structures of other NCCs of barley. A feasible type of modification would be the conjugation of one of the hydroxyl groups with sugars, amino acids or carbonic acids. Regarding conjugations, it is worth mentioning that the major NCC from senescent tobacco leaves has a molecular mass of 892 (S. Hörtensteiner and B. Kräutler, unpublished), i.e. 248 mass units in addition to the mass of a basic NCC such as *Cj*-NCC [60].

Reduction of Chl *b* to Chl *a*

In the photosystems of higher plants, algae and some photosynthetic procaryotes, Chl *b* is a component of the antenna complexes and occurs at variable ratios to Chl *a* [116, 117]. During biosynthesis of Chl *b*, the C(7) methyl group of Chl *a* is oxidized to formyl via 7-hydroxy Chl *a* [118], whereby the formyl oxygen is derived from atmospheric O_2 [119, 120]. Recently, Chl *a* oxygenase, which catalyzes oxidation to 7-hydroxy Chl *a*, has been cloned from *Chlamydomonas* [118], but details about the electron transfer system are still unknown [118, 120]. There are good reasons to believe that a reversal of Chl *b* biosynthesis, i.e. conversion to Chl *a*, is part of the degradation pathway. First, all NCCs of higher plants identified up to now are derived from Chl *a* [13, 57–60]. Second, the amounts of NCCs synthesized during senescence represent the totality of Chl

degraded [56, 60]. Third, PaO reacts exclusively with Pheide *a*, whereby Pheide *b* is a competitive inhibitor [40, 47]. Fourth, the interruption of breakdown at the level of PaO, whether caused by a genetic lesion [14, 92] or through inhibition by appropriate chelators [92, 95], results in the accumulation during senescence of both forms of Chlide but only Pheide *a*. Collectively, these results suggest that on its way to final colorless NCCs, Chl *b* must be fed into the Chl *a* pool before the oxygenolytic cleavage through PaO.

Conversion of Chl *b* to Chl *a* via 7-hydroxy Chl *a* has recently been demonstrated in etioplasts of barley [121, 122] and cucumber [123, 124]. The reduction to Chl *a* is likely to be specific for the dephytylated form of Chl [121, 125], although the reduction of Chl *b* has also been considered [124]. The conversion in organello requires ATP [122, 125], but similar to formation of FCCs in senescent chloroplasts [126], in vitro the reactions were independent of the supply of ATP. Dissection of the two consecutive reductions in lysed etioplasts has revealed another analogy to pFCC formation in vitro [48]: like PaO and RCC reductase, the reduction of 7-hydroxy Chl(ide) *a* to Chl(ide) *a* requires reduced Fd as cofactor. Thus, the stroma component which has been shown to be required [124] can be replaced by reduced Fd [125]. In contrast, the first reduction of Chl(ide) *b* to 7-hydroxy Chl(ide) *a* depends on NADPH [125]. The two partial reactions are catalyzed by two different enzymes. In chloroplasts, Chl *b* reductase is located in thylakoid membranes, and its activity is enhanced severalfold during senescence of barley leaves (V. Scheumann, personal communication).

The interconversion of Chl *a* and *b* is referred to as the Chl cycle (fig. 7) [118] and has been suggested to play a role in the reorganization of the photosystems for adaptation to varying light conditions [127, 128]. It is almost certain now that it also has an important role in Chl *b* breakdown during senescence.

Chlorophyll breakdown in *Chlorella* compared with catabolism in higher plants

Chlorella has been widely used as a unicellular model system for the study of photosynthesis and its regulation [129]. As first observed in 1969, an interesting feature of *Chlorella protothecoides* is coloration of the medium with red pigments when cells are grown under heterotrophic conditions with glucose or acetate as car-

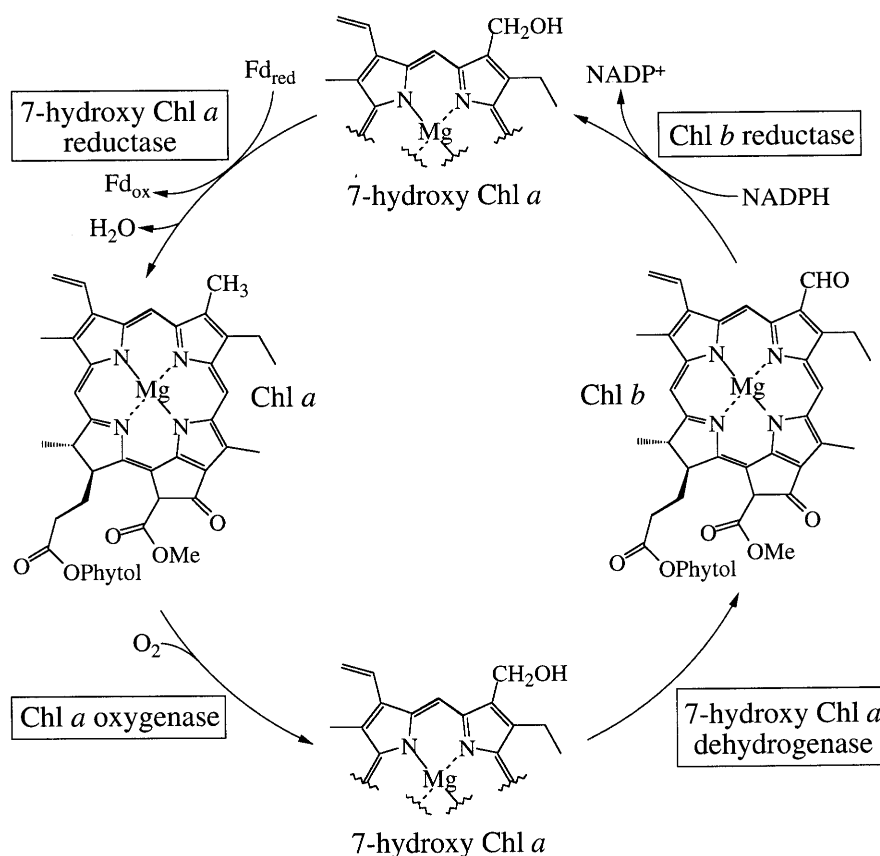


Figure 7. The chlorophyll cycle of higher plants, whereby Chl *a* and *b* are interconverted via 7-hydroxy Chl *a*.

bon sources and simultaneous starvation for nitrogen [50]. The release of red pigments is correlated with the loss of Chl only if the cells are kept in the dark; these pigments are neither produced in light-grown cells [51] nor in the dark if a source of nitrogen is provided (S. Hörtensteiner, unpublished). The chemical properties of the excreted pigments suggested that they represent bile pigments deriving from Chl [50]. Only 22 years later this assumption was proven by structural elucidation of the main pigment [52].

Degreening (bleaching) in *Chlorella* is accompanied by the loss of proteins [130] and is associated with ultrastructural changes of the chloroplasts such as the loss of thylakoids and production of granular bodies (plastoglobules) [131]. These changes resemble the changes occurring during the transition of chloroplasts into gerontoplasts in leaf senescence [132, 133]. Whereas in higher plants degradation of proteins in senescing plastids [5, 134] and the proteases involved [135, 136] have been investigated extensively, these aspects have not adequately been considered in the case of degreening *Chlorella* [130, 137].

In *C. protothecoides*, the final degradation products of Chl are excreted into the surrounding medium [50, 52], whereas in higher plants they are deposited in the vacuoles of mesophyll cells [113, 114]. Thus, in both systems they are removed from the physiologically active part of the cell, a fact which seems to be important in respect to the relevance of Chl breakdown (see below). Compared with higher plants, where the biochemical pathway, and particularly the key step of Chl breakdown, i.e. porphyrin ring cleavage, has recently been elucidated, the knowledge of the analogous reaction in *Chlorella* is rather limited. Thus, in *Chlorella* the actual substrate and the reductant required for cleavage of the porphyrin macrocycle are unknown. Nevertheless, a comparison of the respective reactions in the two systems is possible. In both cases, the oxygenase catalyzing porphyrin cleavage is a monooxygenase [103, 104]. In *Chlorella*, a mechanism with intermediary formation of a C(4)/C(5) epoxide and subsequent hydrolytic cleavage and prototropic rearrangements has been proposed [7, 104]. Thereby, the second rearrangement at C(10) has been demonstrated to be highly stereoselective [138].

Disregarding the possible common mechanism of macrocycle cleavage in *Chlorella* and higher plants, respectively, there are significant differences. One of them concerns substrate specificity as indicated by the occurrence in *Chlorella* of catabolites of both Chl *a* and *b* [54]. In addition, the first identifiable product of the cleavage reaction is a red rather than a colorless compound as in higher plants. This suggests that in *Chlorella* porphyrin cleavage does not require the joint action of a monooxygenase and a reductase as is the case in higher plants [48].

The data so far available for *Chlorella* in terms of Chl degradation and degreening render it an interesting model of a primitive, unicellular 'plant'. Easy manipulation of growth conditions [131], as well as of mutagenesis [139] and the possibility of genetic transformation of *Chlorella* [140], might thus provide attractive opportunities of research on chloroplast senescence.

Significance of Chl breakdown

Regulation

Plant hormones. The most important hormones that regulate senescence in higher plants are cytokinins and ethylene. Cytokinins delay senescence in many plant species [5], and it has been shown repeatedly that endogenous cytokinin levels decrease at the onset of leaf senescence [e.g. 141]. Tobacco transformed with a gene of *iso*-pentenyltransferase, the first enzyme of cytokinin biosynthesis, retained Chl longer than wild-type plants [142]. The effects of cytokinin on metabolic processes include delays in the degradation of Chl and protein [143, 144] concomitant with decreased activities of chlorophyllase [145] and PaO [144] in senescing barley leaves.

In contrast to cytokinin, ethylene hastens many metabolic processes of leaf senescence [5] and is an inducer of fruit ripening, an effect that in tomato or banana is commercially very important [146]. In this respect it is worth mentioning that in transgenic tomato fruit ripening can be delayed by antisense expression of either 1-aminocyclopropane-1-carboxylic acid (ACC) synthase [147] or of ACC oxidase [148], the two last enzymes in ethylene biosynthesis. Ethylene has been shown to accelerate Chl breakdown and to cause a great increase of chlorophyllase activity in *Citrus* fruits [81]. In many dicotyledoneous species endogenous ethylene production only occurs at stages of rapid loss of Chl [149], whereas in oat it precedes breakdown of Chl [150].

The roles in Chl breakdown of the three other classical phytohormones, abscissic acid (ABA), auxins and gibberellins cannot be generalized [5], although the latter two have been shown to retard senescence in several systems [151, 152], and ABA is suggested to have regulatory functions in drought- or salt-stressed plants which show symptoms of senescence [153]. In addition, ABA caused an increase in PaO activity during senescence of barley leaves in the light [144], but it remains to be clarified whether PaO expression is directly modulated by ABA, as is the case in ABA-responsive genes cloned from many sources [153].

Jasmonates represent a class of plant hormones that are considered to play a role in the regulation of stress-induced metabolism [154]. Some effects of jasmonates

also resemble senescence. Thus, methyljasmonate causes a rapid loss of Chl and degradation of protein in barley primary leaves in the light [155]. However, jasmonates may participate in stress signalling rather than in the modulation of senescence [156]. In response to jas monates, species-specific sets of proteins are synthesized [156]. Interestingly, pFCC formation from Pheide *a* in barley is inhibited by stroma protein(s) specifically occurring in jasmonate-treated leaves [144]. Thus, methyljasmonate may indirectly be involved in the regulation of Chl breakdown.

Gene expression. Leaf senescence and fruit ripening are processes that are genetically controlled and require cytoplasmic protein synthesis. This is demonstrated by the inhibition of Chl degradation by cycloheximide or other inhibitors of cytoplasmic protein biosynthesis [3]. Differential screening of cDNAs from tomato [157], *Arabidopsis* [158], rape [159], strawberry [160], barley [161], maize [162] and *Chlorella protothecoides* (S. Hörtensteiner, unpublished) have led to the isolation of a number of different senescence-related genes [8]. Some of these genes turned out to have no homology to known sequences. Hopefully, one or another of them will eventually be identified as encoding a Chl catabolic enzyme.

In two cases of stay-green genotypes the biochemical defect has been attributed to deficient PaO activity [27, 92]. In *Festuca pratensis* it has been shown that the stay-green character is associated with a single recessive gene, *Sid* [163]. This locus has been transferred by intergeneric introgression into wild-type backgrounds of *Lolium multiflorum* and *L. perenne*; the corresponding stay-green genotypes are referred to as *Festulolium* lines [164]. The locus of the 'green gene' has been physically mapped [165], but it is still unclear whether *sid* directly encodes PaO or, more likely, regulates its expression during senescence. Genetic defects of the many other stay-greens [166, 167] have remained elusive, but among the types of stay-greens A, B and C defined by Smart [5], type C genotypes, such as *ore*11 of *Arabidopsis* [168] are possible candidates for a defect in Chl breakdown, since most senescence-related processes occur normally except for Chl metabolism [5].

Programmed cell death and reversibility. Many developmental processes of plants that lead to cell death, such as formation of tracheary elements [169] and senescence of flowers [170], are genetically programmed and resemble apoptosis described in animals [171]. Leaf senescence, including Chl breakdown, normally leads to cell death and is, therefore, interpreted as an example of programmed cell death [9]. This view is supported by the observation of DNA fragmentation in senescing leaves, a hallmark of apoptosis [172]. However, questions about the regulation of programmed cell death during leaf senescence have remained unanswered. Thus, none of the senescence-associated genes identified up to now appears to be a regulatory gene that has a function in initiation or control of senescence [5, 8]. On the other hand, leaf senescence is reversible. Reversion is possible not only during the early stages of senescence, e.g. when a short dark treatment is reversed by light [173], but regreening has also been demonstrated to take place even at a late stage of leaf senescence, in some species even when most of the Chl had been degraded [173, 174]. Rejuvenation is accompanied by a redifferentiation of gerontoplasts into chloroplasts including Chl biosynthesis and thylakoid membrane formation [175]. Reversibility of senescence is restricted to processes occurring before the 'point of no return', the timing of which has been a matter of debate [5]. Thus, Chl breakdown represents a rather early event in programmed cell death and requires intactness of the cell [9, 176]. From this point of view an irreversible step towards cell death can be assigned to autolysis starting with the disruption of the tonoplast [176, 177], as demonstrated for example in connection with tracheid formation in cultured *Zinnia* cells [169].

Compartmentation and Chl detoxification

Breakdown of Chl in higher plants is initiated in the thylakoid membranes, and it ends with NCCs located in the central vacuole of senescent mesophyll cells. Hence, catabolism takes place in several subcellular compartments and requires transport processes across membranes. The topographical model shown in figure 8 summarizes the current knowledge of the catabolic pathway of Chl as organized within a degreening mesophyll cell.

The first steps of Chl breakdown take place within gerontoplasts as demonstrated by in organello production of FCCs in barley and rape [39, 45]. The corresponding enzymes chlorophyllase [75] and PaO [178] (and probably Mg dechelatase [97]) are located in the inner envelope membrane, whereas RCC reductase is a soluble protein of the stroma [44]. It has been speculated that a specific transport protein for Chl or plastoglobules plays a role in shuttling pigment molecules between thylakoids and the chloroplast envelope [6, 133]. This factor (X in fig. 8) appears to act upstream of chlorophyllase and is newly synthesized as Chl breakdown is induced. Treatment of senescing leaves of the stay-green mutant of *Festuca pratensis* with cycloheximide prevented accumulation of Chlide or Pheide, which under control conditions occurred as a consequence of a lesion at the level of PaO [14]. The nature of X is unknown. Recently, a water-soluble apoprotein of Chl has been cloned and characterized from cauliflower; the purified protein was able to remove Chl from pigment-protein complexes when incubated together with

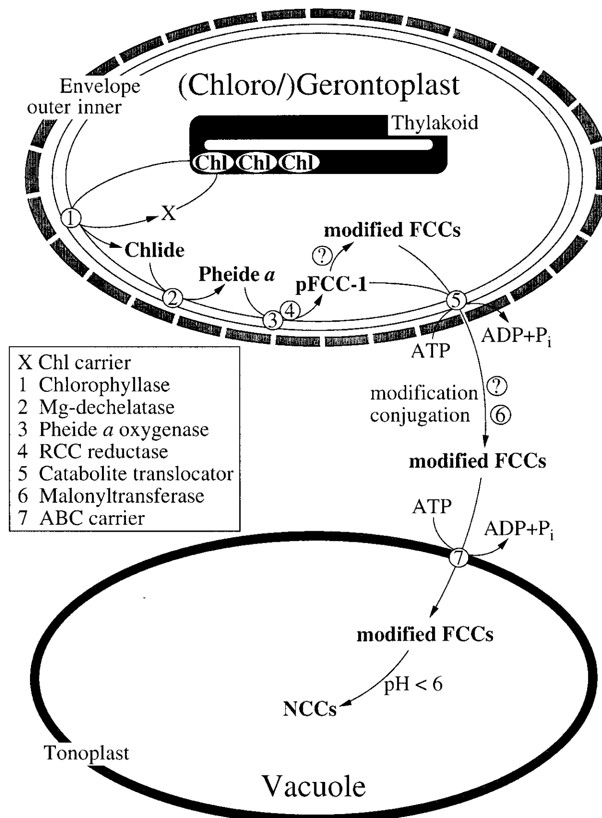


Figure 8. Topographical model of the pathway of Chl breakdown involving several cellular compartments. Characterized enzymic reactions and transporters are numbered. Question marks indicate proposed but as yet unknown reactions. An unidentified factor (X) is responsible for the removal of Chl from the thylakoid.

thylakoid membranes [179]. The protein is located in plastids, is induced under stress conditions, and thus has properties of a candidate of a putative shuttle for Chl from thylakoids to the site of chlorophyllase.

Incubation of isolated barley gerontoplasts with ATP not only induced the formation of primary and modified FCCs, but also release of the modified FCC *Hv*-FCC-2, from the organelles. Whereas FCC formation could be activated by uridine triphosphate or glucose 6-phosphate as well, export activity was specific for ATP [126]. This finding implies that export of FCCs is due to an ATP-dependent transport protein in the envelope of gerontoplasts. The structures of modified FCCs of rape (*Bn*-FCC-1 [45]) and barley (*Hv*-FCC-1 and -2 [39]) have not yet been determined, but their occurrence in gerontoplasts suggests that, in addition to the ring cleavage reaction catalyzed by PaO and RCC reductase, other reactions in the pathway may also be localized within gerontoplasts.

In rape, the presence of glucosylated and malonylated NCCs [58] requires the presence of enzymes catalyzing their respective modifications. Transmalonylation has been demonstrated in preparations of soluble proteins [108], most probably of cytosolic origin.

The final catabolites of Chl breakdown are localized in the vacuole of senescent cells [113, 114], and a specific carrier of the tonoplast is responsible for transport into barley vacuoles [114]. This carrier is a primary active ATPase and resembles the directly energized systems for the transport of glutathione S-conjugates [180] and bile acids [181]. The interesting finding of nonenzymic tautomerization of FCCs into NCCs in an acidic milieu [112] suggests that the entire metabolism of catabolites, including modifications and conjugations, takes place at the level of FCCs prior to transport into vacuoles. This view is corroborated by the finding that in barley the catabolite carrier of the tonoplast had a particularly high affinity for an FCC [114]. The disposal of NCCs in the vacuole is reminiscent of the stepwise detoxification in plant cells of xenobiotics and herbicides [182]. After hydroxylation by the action of P450-dependent monooxygenases located at the endoplasmic reticulum, these compounds are malonylated, glycosylated or conjugated with glutathione, respectively. Such modifications increase the water solubility of xenobiotics and also turns them into harmless conjugates. In a third step, xenobiotics are exported from the metabolically active part of the cell into the central vacuole. Transport of glutathione S-conjugates has been shown to be primary active [180]. The carriers involved in transport across the tonoplast have been identified as members of the family of ATP binding cassette (ABC) transporters. Thus, in *Arabidopsis* transport is mediated by multidrug resistance-associated proteins (MRPs) [183]. Overexpression of these MRPs in a yeast mutant-sensitive to Cd^{2+} not only restored Cd^{2+} resistance but also conferred transport activity for glutathione S-conjugates as well as for a Chl catabolite, *Bn*-NCC-1 [184, 185]. Since hydroxylations and modifications analogous to detoxification of xenobiotics also occur during Chl breakdown, it is reasonable to state that Chl is detoxified rather than metabolized during leaf senescence. Plants are attuned to efficiently remobilize nutrients, particularly nitrogen from senescing leaves and recycle them to sink or storage organs, such as expanding leaves or seeds. This is reflected by extensive degradation of proteins including the apoproteins of Chl [3]. In a mature chloroplast the proteins of pigment complexes account for as much as 30% of the total N content [133]. In stay-green mutants, impairment of Chl breakdown during senescence caused a retention of apoproteins [15, 186], suggesting that Chl stabilizes its apoprotein complex. Thus, a prerequisite of apoprotein degradation might be the removal of Chl. When Chl is released from the complexes, immediate abolishment of photodynamism is necessary to prevent damage of senescing

cells. This is achieved by the stepwise breaking up of the conjugated π-electron system to a nonexcitable level and by disposing the detoxified compounds in the vacuole. In contrast to higher plants, *Chlorella* does not need such a complicated pathway, because the first water-soluble product of macrocycle cleavage, RCC, can be directly excreted into the medium. From this point of view it can be argued that RCC metabolism by RCC reductase marks a major 'invention' during the evolution of Chl catabolism.

Conclusions

The fate of Chl during senescence has successfully been elucidated in the course of the last decade. Intermediate and final catabolites have been structurally analyzed, and the enzymes of the catabolic pathway are biochemically well characterized. Despite these efforts, some questions on different aspects of Chl breakdown remain unanswered. Thus, the factor(s) initiating and the mechanisms regulating Chl degradation are elusive. The isolation of the genes encoding the catabolic enzymes and the characterization of their respective promoters will be necessary for addressing these questions. Up to now, attempts to purify enzymes of the pathway have resulted in protein sequence information only in a few cases. Further biochemical work, but also molecular strategies, will be necessary to eventually clone the respective genes. Projects on functional genomics and introgression/mapping analysis of stay-green phenotypes will be helpful in that respect as well. It is not overly speculative to suggest that within the next decade there will be another major step forward in solving the 'biological enigma' of Chl breakdown.

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