

# Solving the Riddle of Chlorophyll Breakdown<sup>†</sup>

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## Introduction

The disappearance of chlorophyll and emergence of the autumnal colors in the foliage of deciduous trees is one of the most manifest and fascinating natural phenomena. Despite the high visibility of these processes, biochemical information on the breakdown of chlorophyll in plants has been very scarce until recently.<sup>1</sup> Only in the past decade has chlorophyll catabolism yielded some of its mysteries.<sup>2,3</sup>

The chlorophylls have a special position among the natural porphyrins, due to their unique roles in the biological transformation of solar energy,<sup>4</sup> essential to the evolved living world. Indeed, the seasonal appearance and disappearance of the green pigments is probably the most visual sign of life on earth, observable even from outer space (see Figure 1). It is estimated that more than 10<sup>9</sup> tons of chlorophyll is biosynthesized and degraded every year on the earth.<sup>1</sup> Although considerable work has been done on the biosynthesis of the chlorophylls,<sup>5</sup> there has been a definitive lack of information on the fate of the green plant pigments.<sup>1</sup> This gap is all the more surprising because chlorophyll breakdown is so visible and of obvious ecological and economic importance. However, despite considerable research efforts, the chlorophylls seemed to disappear “without leaving a trace”<sup>6</sup> and therefore without yielding any remains that would allow a glimpse at the biological degradation of these pigments.

## Early Investigations of Chlorophyll Breakdown

An early contribution to our knowledge of chlorophyll breakdown was provided by A. Stoll,<sup>7</sup> who discovered

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chlorophyllase and the enzymatic hydrolysis of chlorophyll (Chl) to chlorophyllide (Chlide) and to phytol (see Figure 2). The lipophilic phytol anchor is crucial for the insertion of the green pigment–protein complexes into the thylakoid membranes of chloroplasts. As we know now<sup>3,8</sup> the loss of phytol sets the stage for further enzymatic degradation of both the chlorophyllide and the proteins.

Oddly enough, until recently chlorophyllase was the only known part of the catabolic system responsible for Chl degradation. As products of further breakdown were unknown, the subsequent steps remained enigmatic. The main reason Chl catabolites remained undetected turned out to be quite trivial: in contrast to what was generally expected their major representatives are colorless.

By analogy to heme breakdown, an oxygenolytic opening of the porphyrinoid macrocycle of the chlorophylls was commonly considered as the key step in Chl breakdown.<sup>1</sup> On the basis of experiences concerning the reactivity of chlorins, it was assumed that opening of the macroring would occur at the “western”  $\delta$ -meso position (next to the peripherally reduced ring D).<sup>1</sup> The photooxygenolysis of chlorins indeed was found to preferentially occur at the  $\delta$ -meso position and thus served as a model experiment.<sup>1</sup> The apparent relevance of this observation for Chl breakdown was strengthened further by the structural analyses by Kishi and co-workers<sup>9</sup> of luciferin from the dinoflagellate *Pyrocystis lunula* and of a luminescing compound from krill. Both compounds were found to be linear tetrapyrroles that were most likely derived from chlorophylls by opening at the  $\delta$ -position of the macroring (see Figure 3).

## Discovery of Chlorophyll Catabolites in Degreened Leaves

Nongreen Chl catabolites were first discovered in extracts of senescent leaves of a nonyellowing genotype of the grass *Festuca pratensis*.<sup>10</sup> When such extracts were analyzed by thin-layer chromatography and compared with those from the naturally degreening wild type, only in the case of the extracts from the senescent (degreened) wild-type leaves did pink- and rust-colored compounds appear on the silica gel plates as chemical degradation products of the colorless catabolites (therefore termed “rusty pigments”). Similar compounds were found in yellowing primary leaves of barley.<sup>11</sup> Surprisingly they were found in the vacuoles, rather than in the degreened chloroplasts, from where they must have originated.<sup>11b</sup> Incorporation of <sup>14</sup>C from [4-<sup>14</sup>C]- $\delta$ -aminolaevulinic acid provided evidence for chlorophyll as the precursor of these rusty pigments.<sup>11c</sup> A major rusty pigment, RP-14 (**4**), was unambiguously identified as a colorless catabolite of chlorophyll *a* (Chl *a*, **1a**) by a combined use of mass

<sup>†</sup> Dedicated to Professor Wolfhart Rüdiger on the occasion of his 65th birthday.

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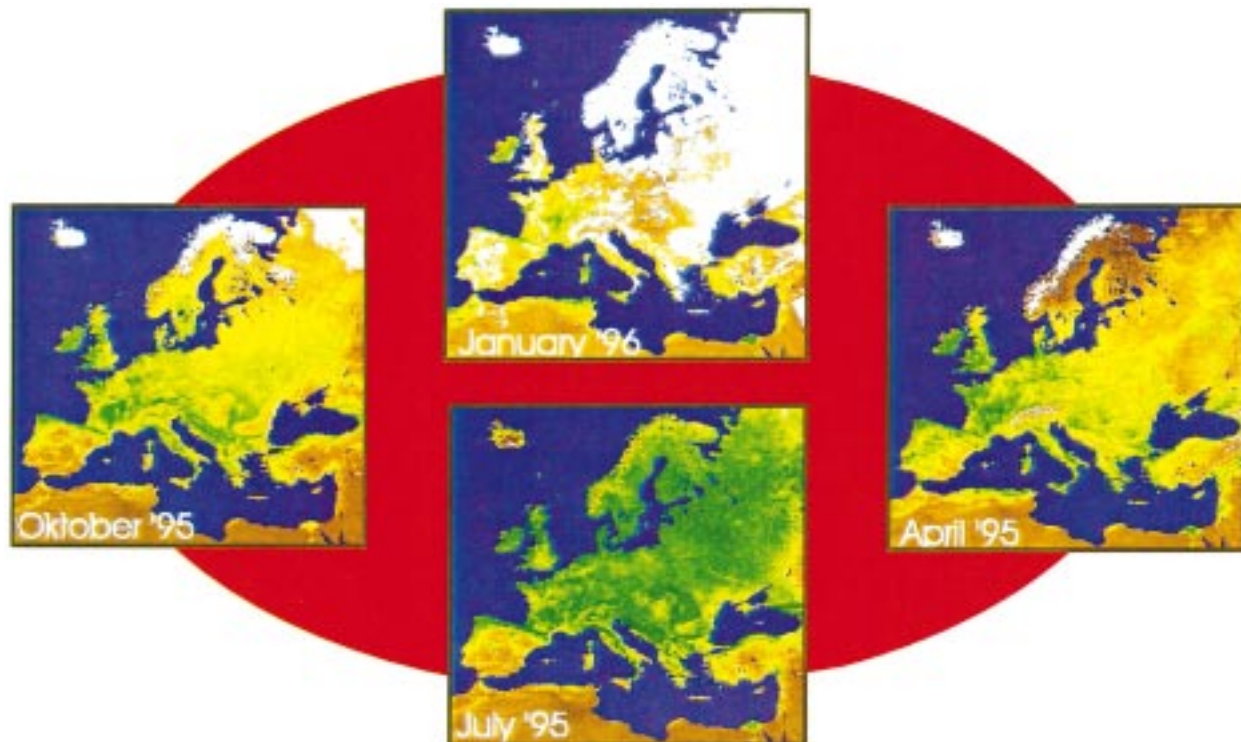


FIGURE 1. Seasonal appearance and disappearance of the chlorophylls as seen from a satellite monitoring the vegetation.

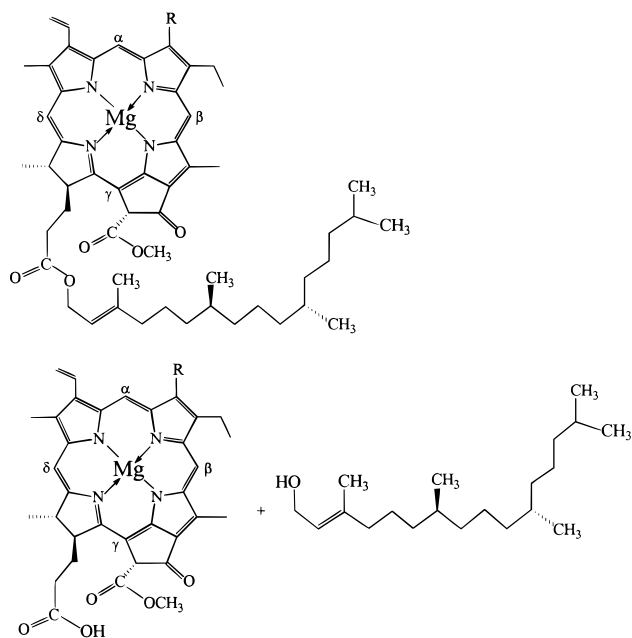


FIGURE 2. Structural formulas: (top) chlorophyll *a* (**1a**, R = CH<sub>3</sub>), chlorophyll *b* (**1b**, R = CH=O); (bottom) chlorophyllide *a* (**2a**, R = CH<sub>3</sub>), chlorophyllide *b* (**2b**, R = CH=O), and phytol.

spectrometry and nuclear magnetic resonance (NMR) spectroscopy, which allowed the constitution of rusty pigment **14** (**4**, see Figure 4) to be established.<sup>2,12</sup> This work, published in 1991, revealed the structure of a nongreen Chl catabolite from plants and gave the first clues as to the major structural changes occurring in the degradation of chlorophyll during plant senescence. It indicated, among other things, that an oxygenolytic open-

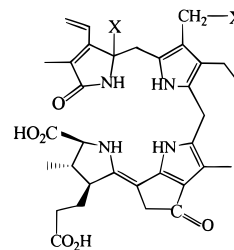


FIGURE 3. Structural formulas of luminescing tetrapyrroles from the dinoflagellate *P. lunula* (X = H) and from krill (*Euphausia pacifica*) (X = OH) according to Kishi and co-workers.<sup>9</sup>

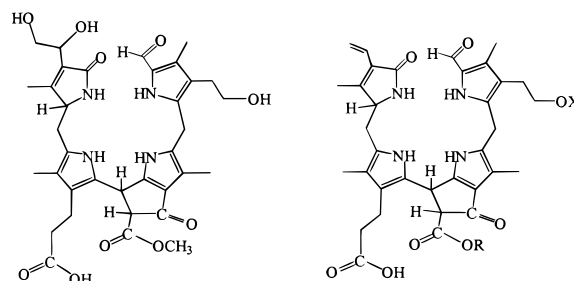


FIGURE 4. Constitutional formulas of nonfluorescent Chl catabolites (NCCs) from degreened plants: (left) *Hv*-NCC-1 (**4**) from barley (*H. vulgare*); (right) *Bn*-NCC-1 (**5**, X = C(O)CH<sub>2</sub>CO<sub>2</sub>H; R = H), *Bn*-NCC-2 (**6**, X =  $\beta$ -glucopyranosyl, R = H), and *Bn*-NCC-3 (**7**, X = H, R = H) from canola (*B. napus*); *Ls*-NCC or *Cj*-NCC (**8**, X = H, R = CH<sub>3</sub>) from *L. styraciflua* and from *C. japonicum*.

ing of the porphyrinoid macroring had occurred, not at the  $\delta$ -position, but rather at the "northern"  $\alpha$ -meso position.<sup>2</sup>

Whereas primary leaves of the monocot barley (*Hordeum vulgare*) were forced to degreen in permanent darkness, the cotyledons of the dicot canola were allowed to senesce under natural growth conditions. Three color-

less and nonfluorescent Chl catabolites (NCCs) could be detected in senescent cotyledons of canola (*Brassica napus*).<sup>13</sup> These three *Bn*-NCCs (**5**, **6**, **7**) were found to account for practically all of the chlorophyll broken down in the cotyledons of canola. They were isolated and were all shown to have the same basic structure as *Hv*-NCC-1 (**4**), and to differ from the catabolite of barley only by the peripheral (re)functionalization.<sup>14,15</sup> Most notably the *Bn*-NCCs proved to be linear tetrapyrroles, which were again derived from Chl *a* by an oxygenolytic ring opening at the  $\alpha$ -meso position (see Figure 4).<sup>14,15</sup>

Compounds with spectral characteristics similar to those of the NCCs were also discovered in senescent leaves of other species: the autumn leaves of trees, such as sweet gum (*Liquidambar styraciflua*)<sup>16</sup> and *Cercidiphyllum japonicum*,<sup>17</sup> were found to be rich in **8**. Thus, all NCCs isolated so far from a variety of degreened plants<sup>2,12,14–17</sup> represent linear tetrapyrroles of a uniform basic buildup (see Figure 4) and relate to Chl *a* (**1a**) rather than to Chl *b* (**1b**).<sup>18</sup> The structures of these nongreen Chl catabolites contradicted the assumed relevance<sup>1,19</sup> of enzymatic transformations at the substituted cyclopentanone unit of the Chls, except for that of an enzymatic hydrolysis of the methyl ester function.<sup>20</sup> This hydrolysis produces  $\beta$ -keto carboxylic acids which are prone to decarboxylation, and some of the pyropheophorbides and their products<sup>1</sup> may arise from nonenzymatic transformations.<sup>15</sup>

## Fluorescent Chlorophyll Catabolites from Senescent Leaves

In extracts of senescent cotyledons of canola the intermediary occurrence of tiny amounts of fluorescent compounds was observable, when the rate of Chl breakdown was high. They were named “fluorescing Chl catabolites” (FCCs) because <sup>14</sup>C-labeling identified them as porphyrin derivatives.<sup>21</sup> As none of these compounds accumulated in vivo, they were considered to represent early or even primary products of porphyrin cleavage. This assumption was supported by the finding that FCCs were located in and, under appropriate conditions, were released from intact chloroplasts isolated from senescent barley leaves.<sup>21b</sup> To prepare sufficient quantities of the “primary” FCC for structural analysis,<sup>21a,22</sup> an extract of the chloroplast membranes from senescent cotyledons of *B. napus* was used that contained the enzymatic oxygenating activity and converted pheophorbide *a* (**3a**, Pheide *a*) into the major (less polar) FCC, originally named *Bn*-FCC-2 (**9**).<sup>23</sup> The constitution of *Bn*-FCC-2 (**9**) was again elucidated by mass spectrometry and NMR spectroscopy.<sup>23</sup> It indicated that **9** was derived rather directly from Pheide *a* (**3a**), formally by the addition of O<sub>2</sub> and two mole equivalents of dihydrogen (see Figure 5). The structure supported the view that **9** could be considered the primary FCC and is therefore called pFCC.<sup>23</sup>

Another important piece of information concerning Chl breakdown was supplied by the discovery that Pheide *a* (**3a**), but not pheophorbide *b* (Pheide *b*, **3b**), accumulated

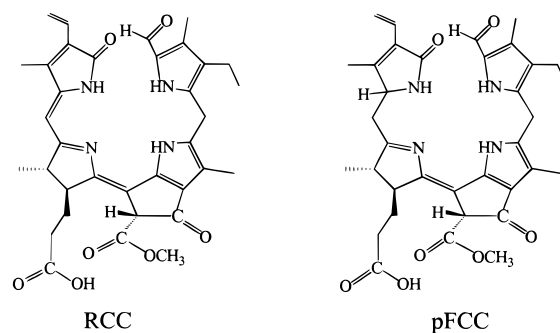


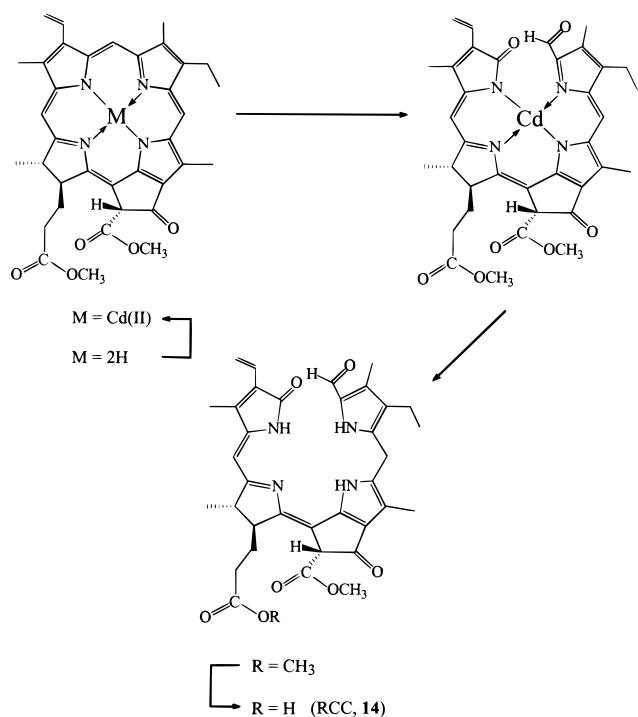
FIGURE 5. Structures of RCC (**14**) and of the fluorescing Chl catabolite pFCC (**9**).

in the absence of molecular oxygen,<sup>24</sup> suggesting the involvement of O<sub>2</sub> and **3a** as common substrates in an oxidative enzymatic step during Chl breakdown. In support of this, the putative oxygenase, an iron enzyme whose activity also depended upon the presence of reduced ferredoxin,<sup>25</sup> turned out to be remarkably specific for **3a**, while **3b** acts as competitive inhibitor.<sup>22</sup> These findings suggested that, in the course of degreening, Chl *b* might be reduced to Chl *a* (see below).<sup>3,26</sup>

In senescence-induced Chl breakdown, **3a** accordingly appeared to be the last intermediate with an intact chlorin macrocycle. Between **3a** and the NCCs there still existed a large structural and biosynthetic gap. The fluorescing catabolite pFCC (**9**) provided a crucial structural link.<sup>23</sup> It indicated that the oxygenolytic opening of the macrocycle of **3a** and the saturation of two of its three other meso positions preceded the other (re)functionalization reactions (minimally) involved in the path from the chlorophylls toward the NCCs.<sup>23</sup>

## A Red Tetrapyrrolic Compound as the Product of Oxygenolytic Ring Opening (and as an Elusive Intermediate in Chlorophyll Breakdown)

The structure of pFCC (**9**) and the other findings outlined<sup>23,24</sup> made it likely that the oxygenolytic cleavage of the ring would occur first and would be followed by a reduction step, leading to saturation of the western  $\delta$ -meso position.<sup>23</sup> Accordingly, the red tetrapyrrole **14** was suggested as a precursor of **9** and therefore as putative intermediate in Chl breakdown.<sup>23</sup> The tetrapyrrole **14** would be similar to red bilinones which had been found to be excreted as the final degradation product of the chlorophylls in the green alga *Chlorella protothecoides*.<sup>27</sup> The red tetrapyrrole **14** could be prepared by partial degradation of **3a** in a sequence of five chemical steps<sup>28</sup> (see Figures 5 and 6), based on a regioselective photo-oxygenolytic opening of the macrocycle of a cadmium pheophorbide.<sup>29</sup> The red compound **14** was then available for tracing experiments which showed that **14** was identical to RCC obtained from **3a** by enzymatic oxygenolysis. This was demonstrated by the incubation of washed membranes of senescent canola chloroplasts in the presence of Pheide *a* (**3a**). Under appropriate conditions, the elusive RCC was produced in trace amounts and



**FIGURE 6.** Preparation of RCC (**14**) by partial synthesis from Pheide *a* methyl ester.

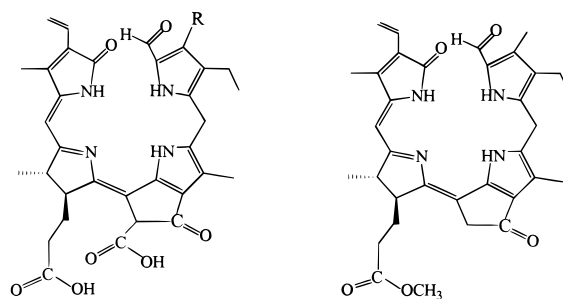
was identified by HPLC with synthetic **14**.<sup>30</sup> Incubation of chemically prepared **14** with a preparation of stroma proteins from chloroplasts resulted in the formation of three FCCs provided that reduced ferredoxin was furnished under anaerobic conditions.<sup>30</sup> One of these FCCs had chromatographic characteristics identical to those of **9**.

During leaf senescence, the red Chl catabolite, RCC, is formed in trace amounts only, most probably in an enzyme bound state. Formally, RCC arises from Pheide *a* by the addition of 1 equiv each of dioxygen and dihydrogen. Reduction of RCC converts it into one of the FCCs.<sup>23,31</sup> Accordingly, the enzyme in the stroma fraction was identified as a reductase which converts RCC into pFCC and was therefore named RCC reductase.<sup>31</sup>

The (single) enzyme in chloroplast membranes from senescent cotyledons, which converts Pheide *a* (**3a**) by an effectively irreversible oxygenolytic cleavage of the porphyrinoid macroring into (a bound form of) RCC (**14**), is termed pheophorbide *a* oxygenase (PaO).<sup>30</sup> This enzymatic reaction depends on molecular oxygen and is apparently inhibited by the oxygenation product.<sup>30</sup>

### Red Tetrapyrrolic Compounds as (Apparent) Final Products of Chlorophyll Breakdown in the Green Alga *C. protothecoides*

Since the 1960s, the green alga *C. protothecoides* was known to excrete red pigments when grown in nitrogen-deficient and glucose-rich medium.<sup>32</sup> These pigments were subjected to extensive structural studies in the laboratory of Gossauer:<sup>27,33,34</sup> The red pigments from *C. protothecoides* were determined to be linear tetrapyrroles with the same basic skeleton as found in the colorless Chl



**FIGURE 7.** Left: Proposed structures **15a** ( $R = \text{CH}_3$ ) and **15b** ( $R = \text{CH}=\text{O}$ ) of Chl catabolites from *C. protothecoides*.<sup>34</sup> Right: Ester **16a**, an early isolation form.<sup>27</sup>

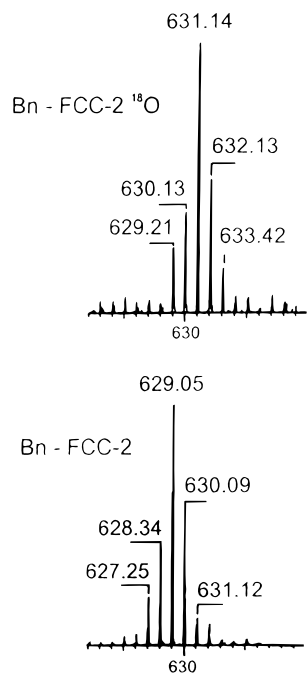
catabolite *Hv*-NCC-1 (**4**), i.e., also to be correlated to the chlorophylls by an oxygenolytic cleavage of the macroring at the northern meso position. In contrast to the plant systems, the red catabolites were found to be derived from chlorophyll *a* as well as from chlorophyll *b* (see Figure 7).<sup>33</sup> Subsequent results indicated that the diacids **15a** (and **15b**) were the authentic products of enzymatic catabolism in *C. protothecoides*,<sup>34</sup> rather than a monoacid, which was originally isolated and identified<sup>27</sup> as the methyl ester **16a**. A nonenzymatic decarboxylation of  $\beta$ -keto acids, such as **15a** and **15b**, may occur easily, and the methyl ester **16a** turned out to be an artifact of the isolation procedure.<sup>34a</sup>

Labeling studies with  $^{18}\text{O}_2$  and mass spectrometric analysis of the excreted pigment as the  $^{18}\text{O}$ -labeled methyl ester **16a** clearly indicated incorporation of only one  $^{18}\text{O}$  atom into **16a**.<sup>35</sup> From analysis of a fragment, the  $^{18}\text{O}$  label could be assigned to the formyl group which is derived from the *meso*-carbon of chlorophyll. This result suggested that the hypothetical ring-cleaving enzyme of the green alga was a monooxygenase,<sup>35</sup> whose direct substrate(s) and product(s) still are not known.

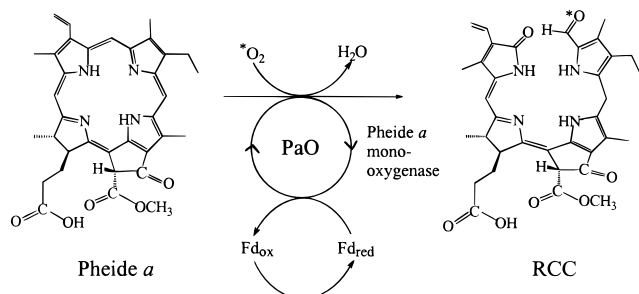
### A Monooxygenase as the Key Enzyme in Chlorophyll Breakdown in Senescent Plants

In contrast to the activities of chlorophyllase, as well as of several other enzymes contributing to Chl breakdown, pheophorbide *a* oxygenase (PaO) is detectable only in senescent leaves.<sup>24</sup> PaO catalyzes the crucial (and effectively irreversible) cleavage reaction of the porphyrinoid macrocycle and may be considered to represent the key enzyme of Chl breakdown. An *in vitro* assay, employing partially purified PaO as well as RCC reductase from canola, was used to characterize the reaction catalyzed by PaO.<sup>23,36</sup> In the presence of  $^{18}\text{O}_2$ , this mixture of partially purified enzymes converted Pheide *a* (**3a**) into  $^{18}\text{O}$ -labeled pFCC ( $[^{18}\text{O}]\mathbf{9}$ ) which contained one  $^{18}\text{O}$  atom per molecule of catabolite, as determined by mass spectrometry (see Figure 8).<sup>36</sup> From mass spectral analysis of fragment ions of  $[^{18}\text{O}]\mathbf{9}$ , the isotopic label could be assigned to the formyl group. These results indicate the incorporation of one oxygen atom from  $\text{O}_2$  at the  $\alpha$ -*meso* position of **3a**.

The ring cleavage step of Chl degradation in senescent plants is accordingly catalyzed by a monooxygenase (see

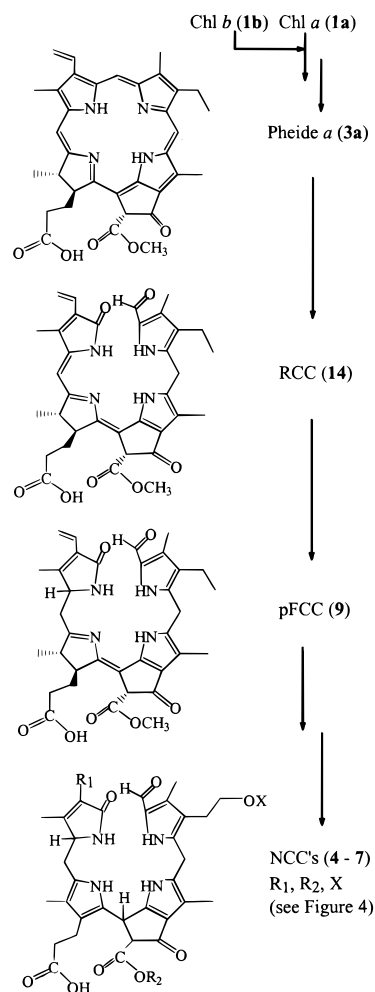


**FIGURE 8.** Section of the fast atom bombardment mass spectrum showing the molecular ion of  $^{18}\text{O}$ -labeled pFCC (*Bn*-FCC-2  $^{18}\text{O}$ , [ $^{18}\text{O}$ ]**9**, top) and unlabeled pFCC (*Bn*-FCC-2, **9**, bottom).



**FIGURE 9.** Pheide *a* cleavage to the red Chl catabolite RCC.

Figure 9).<sup>36</sup> Of the two oxygen atoms introduced in this reaction, one is derived from  $\text{O}_2$  and the other from another source, most likely (directly or indirectly) from water. Over all, the transformation of Pheide *a* (**3a**) into RCC is a remarkable structural change: Besides the incorporation of two oxygen atoms, the ring opening at the newly oxygenated sites with formation of two carbonyl functions and the saturation of the “eastern”  $\beta$ -meso position also occur during this step. At this stage, the mechanistic questions are not resolved concerning the hypothetical isomerization of the primary enzymatic oxygenation product to the ring-opened (enzyme-bound form of) RCC. The formation of the red bilinone **15a** in the green alga *C. protothecoides* has been suggested to result from hydration of an epoxide intermediate and subsequent rearrangement.<sup>34,37</sup> These and related mechanistic considerations require further critical experiments. The structural resemblance of the red intermediates and the apparent similarity of the oxygenation mechanisms in Chl breakdown in higher plants and in the green alga indicate a biochemical relationship. Hence, the Pheide oxygenases of higher plants and *C. protothecoides* may have comparable catalytic properties. Notable differences



**FIGURE 10.** Structural outline of the pathway of Chl breakdown in senescent plants.

concern the substrate specificity and the requirement of a second enzymic reaction (RCC reductase) in the case of breakdown in higher plants.

It is worth mentioning that the process catalyzed by PaO had a crucial impact on the development of the laws of genetics by Mendel in the last century.<sup>38</sup> The puzzling observation of the phenotype of a recessive allele in Mendel’s “green peas” is now known to be due to a deficiency of PaO.<sup>39</sup>

## Pathway of Chlorophyll Breakdown in Senescent Plants

In the last 10 years, significant steps toward the elucidation of Chl breakdown in higher plants have been achieved. A major contribution to this arises from the knowledge on the structure of the intermediates and of the (apparently) final products from the degradation of Chls in senescent plants (see Figures 10 and 11).<sup>2,3</sup> In this pathway, the crucial senescence-induced oxygenolytic cleavage of the porphyrinoid macrocycle occurs regioselectively at the northern methine bridge and with Pheide *a* (**3a**) as the substrate. This oxygenolytic ring-opening reaction represents the third step in the course of Chl breakdown when starting with Chl *a* (**1a**). Hydrolytic

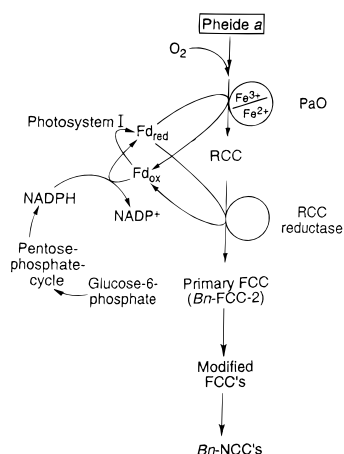


FIGURE 11. Flow diagram outlining Chl breakdown in senescent cotyledons of canola.

removal of the lipophilic phytol substituent and of the central magnesium ion of **1a** by the actions of chlorophyllase<sup>7,40</sup> and of a magnesium dechelate<sup>41</sup> are the two earlier steps in the degradation of Chl *a*.

Since the Chl catabolites from plants have all been found to be derived from Chl *a* (and none from Chl *b*) and as the crucial oxygenase accepts only Pheide *a* (**3a**) as substrate, Chl *b* is indicated as being degraded via Pheide *a* (**3a**). Recent work has provided evidence for the biochemical link from the *b*-type to the *a*-type chlorophylls and involving the reduction of the characteristic formyl group of **1b** to a methyl substituent.<sup>26,42</sup> In this way the minor Chl component in plants is also made available for the degradative path.

The oxygenolytic cleavage of the porphyrinoid macrocycle by the monooxygenase PaO is intriguingly specific for Pheide *a* (**3a**).<sup>22</sup> PaO is located in the chloroplast envelope,<sup>43</sup> and ferredoxin drives its redox cycle. Apparently, PaO is inhibited by its oxygenation product, RCC (**14**), and depends on the cooperation of RCC reductase, whose action is associated with the release of pFCC (**9**). RCC reductase is located in the stroma of plastids and also depends on reduced ferredoxin as electron donor. Thus, macrocycle cleavage in higher plants effectively occurs in two enzymic steps and may be an example of "metabolic channelling".<sup>30,31</sup>

The chemically rather labile, fluorescing FCC **9** has been shown not to accumulate during Chl breakdown. FCCs undergo a series of transformations which do not involve the site of cleavage, but rather a tautomerization to the chromophoric system of the colorless "nonfluorescing" Chl catabolites (the NCCs, such as **4–8**) and one or several peripheral refunctionalization reactions. All of these latter transformations introduce polar groups, such as the remarkable hydroxylation of the terminal position of the ethyl group on ring B, found in all the NCCs.<sup>2,14–17</sup> It appears to introduce an anchor point for further secondary refunctionalization with hydrophilic groups, such as in **5** and **6** from canola.<sup>14</sup> The esterification with malonic acid has been demonstrated with a protein preparation from canola cotyledons and malonyl-CoA as substrate.<sup>44</sup> Esterification and glucosylation (in **5** and **6**)

are reminiscent of many secondary plant metabolites which are, like NCCs, deposited in the vacuoles.<sup>45</sup>

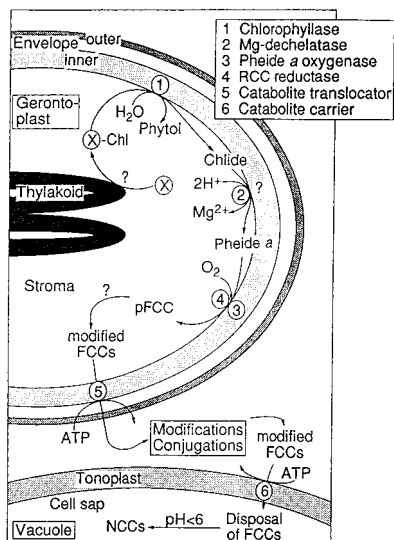
Another common feature of the *Bn*-NCCs is the presence of a free  $\beta$ -keto carboxylic acid group in the characteristic cyclopentanone moiety. In contrast, in *Hv*-NCC-1 (**4**)<sup>2</sup> and in **8**<sup>16,17</sup> the methyl ester function of the Chl precursor is still present. For the formation of the  $\beta$ -keto carboxylic acid grouping of the *Bn*-NCCs, the hydrolysis of the corresponding ester function is indicated to occur at the stage of the FCCs or later. This contrasts with Chl breakdown in *Chenopodium album*, where enzymatic conversion of Pheide *a* (**3a**) into 13<sup>2</sup>-carboxypyropheophorbide *a* has been detected.<sup>20</sup> Likewise, none of the red pigments from *C. protothecoides* still carry the  $\beta$ -keto ester functionality present in the Chls **1a** and **1b**.<sup>27,33</sup>

The complete deconjugation of the four pyrrolic units, characteristic of the tetrapyrrolic NCCs, finally comes about by a tautomerization reaction which converts the chromophoric system of the FCCs into that of the NCCs. This final stage in the transformation of the chromophoric system of the colored Chls into that of the colorless NCCs has some precedence from chemical model experiments<sup>46</sup> and may occur in the course of natural Chl breakdown under rather mild conditions and possibly even non-enzymatically.<sup>47</sup>

Clearly the result of the primary ring cleavage reaction and of the subsequent (coupled) step(s) can be envisaged as the rapid conversion of the chromophore of a photoactive and intensely colored chlorin, such as **3a**, into that of a colorless tetrapyrrole with deconjugated heterocyclic rings, such as **4**. Chl breakdown in senescent leaves may, therefore, be apostrophized as a detoxification process. A well-controlled and sequentially operating enzymic machinery dealing with a remarkably limited range of substrates appears characteristic of these early stages in the degradation of the chlorophylls. It is still unclear, however, in which order the later and terminal refunctionalization reactions occur: These reactions tend to increase the hydrophilicity of the tetrapyrrolic breakdown products but do not cause further cleavage into smaller fragments. Indeed, the nonfluorescing Chl catabolites (NCCs) appear to represent the final products of Chl breakdown in senescent plants.<sup>3</sup>

## Subcellular Organization of Chl Breakdown

Chl breakdown in yellowing leaf cells is associated with the development of the photosynthetically competent green chloroplasts into a specific senescent form of the organelle termed "gerontoplast".<sup>3</sup> The initial catabolic steps, particularly the opening of the porphyrin ring, take place within the developing gerontoplasts. At first glimpse it would seem logical that the corresponding catabolic enzymes are located together with their substrates in the internal membrane system of senescent chloroplasts. This view was supported, apparently, by the observation that both chlorophyllase and PaO reside in membranes.<sup>48</sup> It turned out, however, that they are components of the Chl-free plastid envelope, rather than of the green thyl-



**FIGURE 12.** Topographical model of Chl breakdown in senescent chloroplasts (gerontoplasts)<sup>3</sup> and disposal of catabolites in vacuoles.

akoids.<sup>40a,43</sup> Thus, breakdown of Chl appears to require a hitherto unknown mechanism by which Chl molecules are lured out of the pigment–protein complexes of thylakoids (marks “X” and “X-Chl” in the topographical model of Figure 12) and transported to the site of catabolic enzymes in the envelope.

Figure 12 also illustrates some other remarkable features of subcellular organization of Chl breakdown. A carrier residing in the envelope appears to be responsible for the export of FCCs from developing gerontoplasts into the cytosol.<sup>21b</sup> Conjugations of hydroxylated catabolites, such as the esterification with malonic acid in the case of *Bn*-NCC-1, take place in the cytosol,<sup>44</sup> and the final deposition in the sap of the central vacuoles is achieved by the action of a specific carrier in the vacuolar membrane.<sup>45a</sup> Since many known transporters with similar functions are energized indirectly by the pH gradient between the vacuolar sap (acidic) and the cytosol (neutral), it was quite unexpected that the transporter of Chl catabolites was found to be driven directly by ATP hydrolysis. Most probably the carrier recognizes FCCs as substrates which, upon release into the acidic cell sap, are tautomerized to NCCs.<sup>47</sup>

In the course of leaf senescence, the total content of phytol, one of the products of chlorophyllase action (see Figure 2), was found to be remarkably constant. Moreover, phytol remains largely esterified when chlorophyll is broken down.<sup>49</sup> In senescent barley leaves, phytol acetate (and other lipophilic remnants of the thylakoids) accumulate within the senescent chloroplasts,<sup>50</sup> in numerous small droplets (plastoglobuli), a feature of developing gerontoplasts.

## Regulation of Chlorophyll Breakdown

Very little is known about the factors and conditions that are responsible for the induction of Chl breakdown. In deciduous trees, photoperiodical control is very likely, but trees are most difficult experimental systems and regula-

tion has been studied primarily in herbaceous species. Hormonal control of leaf yellowing has been studied extensively, and inhibition or retardation of Chl breakdown as well as of other senescence processes by cytokinin has been established.<sup>51</sup>

Conversely, senescence processes including Chl breakdown have been demonstrated to be hastened by the phytohormones ethylene and abscisic acid.<sup>51</sup> And yet, the signals that launch the final phase of leaf development are still largely unknown. With regard to PaO and its important role in Chl breakdown, regulatory effects of the phytohormones cytokinin and abscisic acid have recently been demonstrated.<sup>52</sup>

Knowledge about the genes that are specifically expressed upon the induction of Chl breakdown is still scarce. None of the genes encoding known catabolic enzymes have so far been cloned.<sup>53</sup>

## Chlorophyll Breakdown and the Nutrient Economy of Plants

The development of gerontoplasts plays a most prominent role in the recycling of nutrient elements such as nitrogen from senescent leaves to other parts of the plant. A predominant proportion of the total leaf protein is located in the chloroplasts. During senescence, chloroplast proteins are broken down and amino acids are exported for reuse in developing leaves or for the filling of seeds with reserve proteins. Of all the nitrogen contained in mature chloroplasts, about one-third is represented by the proteins of the thylakoid pigment complexes. In mutants that are disturbed in Chl breakdown (stay-green genotypes) the apoproteins of chlorophyll are not degradable as long as they are complexed with the pigments.<sup>54</sup> Hence, such mutants have a disadvantage due to incomplete N-recycling during senescence.

As shown above, chlorophyll is not broken down beyond the stage of tetrapyrroles. Obviously, Chl breakdown is not aimed at the reuse of porphyrin N (which represents only a few percent of total leaf nitrogen) but rather at the dismantling of Chl–protein complexes as a prerequisite of protein degradation. In the complexed state, chlorophyll is stable and carotenoids act as quenchers of photoactivated chlorophyll and singlet oxygen. Dismantling of the complexes is, therefore, dangerous, and the sophisticated machinery of Chl catabolism must be interpreted as serving a vitally important detoxification process. Indeed, it is important that cells remain viable because the breakdown of protein and the recycling of nutrients depend on a perfectly organized metabolism down to the very end of the senescence period.

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