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CHLOROPHYLL DEGRADATION

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ABSTRACT

Although the loss of green color in senescent leaves and ripening fruits is a spectacular natural phenomenon, research on chlorophyll breakdown has been largely neglected until recently. This review summarizes knowledge about the fate of chlorophyll in degreening tissues that has been gained during the past few years. Structures of end- and intermediary products of degradation as well as the biochemistry of the porphyrin-cleaving reaction have been elucidated. The intracellular localization of the catabolic pathway is particularly important in the regulation of chlorophyll breakdown. None of the genes encoding the related catabolic enzymes has so far been isolated, which makes chlorophyll degradation an area of opportunity for future research.

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INTRODUCTION

A few months before the start of the twentieth century, Albert F. Woods addressed the American Association for the Advancement of Science in Columbus, Ohio, on the subject, "The destruction of chlorophyll by oxidizing enzymes" (185). His survey includes some spectacularly erroneous assertions (e.g. "It has long been known that chlorophyll could be readily converted by oxidation, into a yellow coloring matter, xanthophyll..."); but some of the enzymology he describes, and the questions he raises, are still keeping researchers busy, 100 years later. By 1912, the second major player in the tale of chlorophyll (Chl) breakdown, chlorophyllase, had been discovered by Arthur Stoll (155). And for most of this century, in spite of the appearance of some repetitive research reports and a few reviews, there was little more to be said about the biochemical basis of Chl breakdown. The publication in 1987 of Hendry et al's review entitled "The degradation of chlorophyll—a biological enigma" (47) marks a revival of interest in the subject. By drawing on information from satellite imaging, tourism, petrochemistry, and archaeology, as well as mainstream biology and chemistry, the authors revealed a rich field of research possibilities. Aspects of the subject have been reviewed subsequently (15, 37, 38, 85, 89, 90, 133, 163), telling a story of growing understanding, as well as of continuing frustrations. The present account of Chl breakdown not only brings this story up-to-date but also tries to anticipate how current and imminent developments, such as genomics, will affect the study of Chl catabolism, just as they will revolutionize most other areas of experimental plant biology.

PLASTID DEVELOPMENT

Chloroplast to Gerontoplast Transition

Chlorophyll degradation is a symptom of transition of chloroplasts to gerontoplasts. A distinctive term for the senescence-specific form of plastids (148)

is justified because the metabolism of gerontoplasts, in contrast to that of all other forms of a plastid, is solely catabolic. The yellowing of senescent leaves is due to unmasking and partial retention of carotenoids rather than to the new biosynthesis of yellow pigments such as occurs when chloroplasts differentiate into chromoplasts, for example. Developing gerontoplasts persist and remain intact throughout leaf senescence (84, 117). They lose volume and density as a consequence of extensive losses of stromal components and thylakoids, and the number and size of lipophilic plastoglobules increase. Thus, fully developed gerontoplasts consist of a still intact envelope surrounding a number of large plastoglobules. It is well established that gerontoplast development is under nuclear control and that the expression of plastid-encoded genes plays only a minor part, if at all (see 84). In rice coleoptiles and leaves, ctDNA is extensively degraded even before Chl breakdown marks the onset of differentiation of gerontoplasts (149, 150). On the other hand, leaf senescence in many species is reversible (31, 95, 104, 176), which suggests that fully developed gerontoplasts retain enough genetic information to support regreening and chloroplast reassembly.

Chloroplast to Chromoplast Transition

Chromoplasts are the typical form of Chl-less plastids in fruits and petals (16). Under conditions of light stress, chromoplast-like organelles may also develop in green algae (6). Transitions of chloroplasts into chromoplasts or gerontoplasts are comparable as far as Chl breakdown and the disappearance of thylakoids are concerned. A distinctive feature of chromoplast development is the incorporation of a new set of proteins, encoded in both nuclear and plastidic genomes, which functions in the synthesis of secondary carotenoids and their incorporation in fibrillar and globular structures (16).

DEGRADATION WITHOUT NET LOSS

Chlorophyll Turnover

When cells accumulate a particular metabolite, or maintain it at a steady level, there is usually flux through the related pool. Chl is probably no exception, but there are very few reliable reports in the literature on its rates of turnover. This section gives a picture of a highly incomplete and poorly understood area of plant metabolism, but at least it may point out fruitful research possibilities.

The estimation of rates of Chl synthesis and degradation during the greening of etiolated cereal leaves by following pigment accumulation in the presence and absence of an inhibitor of aminolevulinate (ALA) synthesis resulted in calculated half-lives of as little as 6 to 8 h for leaves in the early stages of deetiolation, increasing to more than 50 h as greening became complete (48, 154). Considerable incorporation of radiolabeled acetate into the Chl of mature dicot

leaves was observed, but little into the pigment of monocots (122). Some Chl turnover during photosynthesis and photoinhibition is likely associated with the short half-life of the D1 polypeptide, part of the heterodimeric reaction center of photosystem II (28, 124). Appreciable rates of Chl turnover were also observed in algae (39, 126).

The Chl biosynthetic pathway is detectable until very late in the lifespan of photosynthetic cells (56). The capacity to bleach the pigment appears also to be built into such cells. Thus, the potential for the simultaneous anabolism and catabolism of Chl is present; but mostly we do not know if this potential is realized. It seems likely also that degradation of Chl during net accumulation or at the steady state is only one aspect of a general requirement to regulate the family of macrocyclic tetrapyrrole intermediates in the biosynthetic pathway en route to photosynthetically functional pigment complexes (180).

Degradation During Chlorophyll Biosynthesis

The pathway of Chl synthesis comprises the action of more than a dozen enzymes to convert glutamate into Chl (180). All intermediates from uroporphyrin III onwards are potentially phototoxic. Hence, "traffic" restrictions along the biosynthetic route that result in the accumulation of such intermediates, e.g. as provoked artificially by antisense transformation at the level of uroporphyrin III decarboxylase (102), cause susceptibility of plants to photodamage. It would make sense if diversionary routes were provided to shunt such intermediates away harmlessly in the event of traffic restrictions farther along the main pathway leading to Chl. In one such detour, phototoxic precursors are exported from the plastid and further processed in the cytosol or at the plasma membrane (62). The *tigrina* series of barley mutants provide genetic evidence of at least four loci regulating metabolic flux between the C5 precursor ALA and protochlorophyllide (Pchlide), the intermediate immediately preceding the light-requiring step at Pchlide oxidoreductase (180).

It is unlikely that the catabolic system degrading Chl during senescence is responsible for turning over Chl or its derivatives in pre-senescent tissues. End-product catabolites that accumulate in senescent cells have never been found in cells in which the pigment is undergoing net synthesis or steady-state turnover (P Matile's group, unpublished data). Moreover, non-yellowing senescence mutants display neither abnormal accumulation of Chl nor extended pigment synthetic capacity (4, 110). Turnover of the D1 protein during photosynthesis is also perfectly normal in the *Festuca* senescence mutant Bf993 (49).

There are a few reports of enzyme activities that may function in fine-tuning levels of macrocyclic intermediates in the Chl biosynthetic pathway. The first Mg-containing intermediates are Mg protoporphyrin IX and its monomethyl

ester (MgProtoMe); etiolated *Phaseolus* leaves were shown to contain heat-labile and oxygen-dependent activity able to bleach MgProtoMe (55). A similar enzyme from barley was purified (33); developmental changes in its activity suggested that it may be responsible for pigment bleaching during greening as well as during senescence-associated degreening. These interesting observations appear not to have been pursued further. A somewhat similar activity was demonstrated in preparations from cucumber cotyledons (184). The enzyme was shown to utilize MgProtoMe, to be heat-labile and oxygen-dependent, to correlate in a reciprocal fashion with MgProtoMe cyclase, and to decrease in activity as plastids differentiated. The authors presented evidence that this kind of bleaching process in plastid development is distinct from the peroxidative and fatty acid–dependent pigment oxidizing activities reported in connection with ripening, senescence, and pathological deterioration (see below).

Heme and Phytochrome

A branch of the C5 pathway that may be significant for the regulation of Chl biosynthesis leads from protoporphyrin IX via ferrochelatase to heme (181). The precursor of phytochrome chromophore synthesis is biliverdin IX, the product of opening the heme macrocycle (8). Mutants of pea, tomato, and *Arabidopsis* (159) that lack the ring-opening ferredoxin-dependent heme oxygenase (8) are yellow-green in color, but this is more likely to be a consequence of defective photocontrol of Chl biosynthesis than because Chl precursors have been rerouted. Before a specific Chl-degrading oxygenase was discovered, heme oxygenase was an object of fascination for those seeking to understand plant pigment breakdown. We now know that the reaction mechanisms of the two enzymes are quite distinct.

DEGRADATION ASSOCIATED WITH NET LOSS

Phenology of Net Loss

The loss of Chl is the preferred and most easily measured parameter for describing the yellowing of senescing leaves or color changes in ripening fruits. Degreening is clearly part of developmental processes taking place in fully viable cells. This can easily be appreciated, for example, by observing petal development, which in many species is associated with the rapid loss of Chl shortly before anthesis. The capacity of metabolic degreening is ubiquitous in the plant kingdom and is manifest not only in spectacular natural phenomena such as autumnal color changes in the foliage of deciduous trees but also in seeming evergreens, ferns, and algae.

Products of Chlorophyll Degradation

IDENTIFICATION Unambiguous identification of degradation products required the specific radiolabeling of Chl in the porphyrin moiety. Feeding of [4-^{14}C]ALA during greening of etiolated plantlets of barley (121) or to expanding and greening canola cotyledons (35) resulted in the specific radiolabeling of Chl for the study of catabolism. As Chl disappeared during subsequent senescence, the radiolabel was progressively recovered in the water-soluble fraction of leaf extracts, indicating that Chl degradation is associated with hydrolysis into the phytol and porphyrin moieties. In barley plantlets developing under natural conditions, the label incorporated into the pyrrole units of Chl was retained in the senescent leaves (119); there was no indication of export of radiolabel from the senescent primary leaf to other parts of the plant.

PHYTOL As Chl is degraded, substantial proportions of phytol remain esterified (83, 118, 120) with fatty acids (118) and with acetic acid (12). In senescent barley leaves, free and esterified forms of phytol are located in the plastoglobules of developing gerontoplasts (12). Losses of total phytol during leaf senescence have been attributed to photooxidative conversion into various isoprenoid compounds (132).

GREEN PRODUCTS OF DEGRADATION Several derivatives of Chl that have an intact macrocycle and are likely to represent intermediary products of degradation have been detected in one form or another: pheophytin, chlorophyllides (Chlide), and pheophorbide (Pheide) *a* (e.g. 3, 64, 127, 164, 186). During breakdown of Chl these compounds are detectable only in trace amounts (if at all), indicating that removal of phytol and the central Mg-atom is quickly followed by processes that cause the loss of green color. Dephytylation is commonly considered to be the first catabolic reaction, but it may be preceded by Mg-removal, as suggested by the occurrence of pheophytin in degreening leaves (3). Whether 13^2-hydroxy Chl *a*, which has occasionally been observed (e.g. 94, 186), belongs to the group of natural intermediary breakdown products is unclear.

TETRAPYRROLIC CATABOLITES Until recently, the fate of the porphyrin part of Chl in senescent leaves remained cryptic. For as long as the search for products of degradation that were expected to accumulate concomitant with the loss of Chl remained unsuccessful, it was speculated that a continuous, rapid breakdown to simple molecules such as CO_2 and NH_3 might explain the apparently traceless disappearance (47). In retrospect, the difficulties with the discovery of Chl catabolites are easily explained. Like bile pigments, all catabolites identified so far in senescent leaves have a linear tetrapyrrolic structure, but

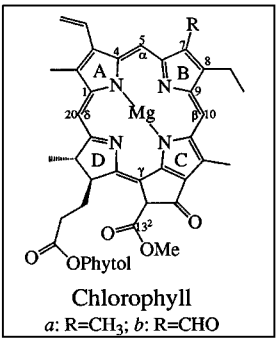
unlike the products of heme degradation, they are colorless and, hence, escaped detection.

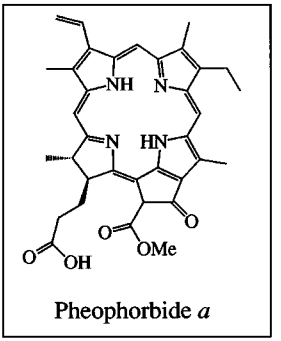
In every instance where the chemical structure of such a terminal Chl catabolite has been determined, it has been shown to be formally derived from pheophorbide *a* through the oxygenolytic cleavage of the macrocycle at the C4/C5 mesoposition (Figure 1). The methine bridge C5 carbon is preserved as a formyl group (a clear difference from heme catabolism, where this carbon atom is lost as CO), and a second O atom appears as a lactam group at C4. As well as the ring-opening mechanism, all known final catabolites share the complete eradication of the conjugated system between the pyrroles. Hydroxylation of the ethyl side chain in pyrrole B is another common structural feature.

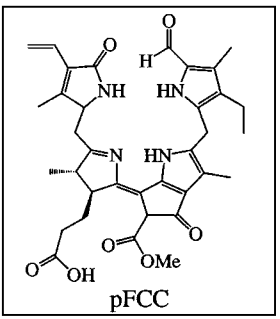
These final products have been termed NCCs (nonfluorescent Chl catabolites) and thereby distinguished from blue-fluorescing intermediary catabolites (FCCs, fluorescent Chl catabolites) and RCCs, the red-colored type of catabolite discovered in *Chlorella protothecoides* (26). Because the basic structure of NCCs is modified in some plant species, the following convenient nomenclature has been proposed (35). A prefix indicates the plant species (e.g. *Bn* = *Brassica napus*) and the individual compounds are numbered according to decreasing polarity as judged by retention times during reverse phase HPLC; e.g. *Bn*-NCC-3 represents the most apolar catabolite accumulated in senescent leaves of oilseed rape.

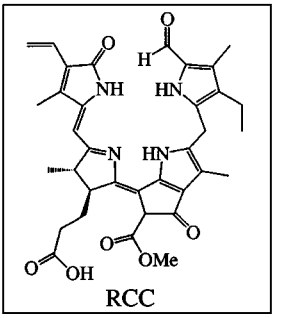
The structures of NCCs elucidated so far are illustrated in Figure 1. In the fully senescent leaves of *Cercidiphyllum japonicum* (18), *Liquidambar styraciflua*, and *L. orientalis* (61), the full complement of Chl-porphyrin is converted mole-for-mole into the basic structure of NCCs. In *Bn*-NCC-3 from oilseed rape this structure is modified as the 13^2-carboxymethyl group is demethylated (105), but in the major NCC of this species, *Bn*-NCC-1, the 13^2-hydroxyl group is esterified with malonic acid (106) and in *Bn*-NCC-2 it is glucosylated (105). The only structure of an NCC known so far from senescent barley leaves, *Hv*-NCC-1, has an intact 13^2-carboxymethyl group but is hydroxylated in the vinyl group of pyrrole A (69, 70). It may be anticipated that some or even many further structural variants in the family of NCCs remain to be discovered. Radiolabeling in barley has revealed the occurrence of about a dozen different NCCs (119, 121). It would not be surprising if, in addition to esterification with malonic acid, other conjugations reminiscent of secondary metabolites, e.g. with acetic or cinnamic acid, were to be discovered in various plant species.

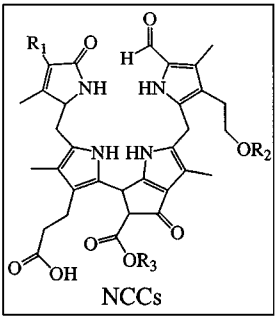
Hydroxylation and conjugation confer increased water solubility on NCCs, just as they do on secondary metabolites such as phenolics, which are dissolved in the vacuolar sap. Not unexpectedly, therefore, Chl catabolites are accumulated in the vacuoles of senescent mesophyll cells (13, 24, 50, 88).











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

Figure 1 Structures of intermediary and final Chl catabolites arranged according to the "pheophorbide *a* oxygenase" (PaO) pathway of chlorophyll degradation. Explanations, abbreviations, and references are presented in the text.

During periods of rapid Chl breakdown, trace amounts of colorless blue-fluorescing compounds are detectable in senescent leaves (23, 24). These FCCs were originally discovered in acidic extracts from leaves of *Festuca pratensis*: In an acidic milieu FCCs are readily oxidized to pink-colored compounds (85). Such "pink pigments" were identified as catabolites of Chl because they occurred only in senescent leaves of the wild-type cultivar Rossa but not in those of a mutant genotype, Bf 993, which is unable to degrade Chl (23, 87). Trace amounts of FCCs were found to occur only temporarily and to be positively correlated with rates of Chl breakdown. Such kinetics would be expected of intermediary products of a catabolic pathway, eventually leading to the progressively accumulating end products, NCCs.

FCCs have a fluorescence spectrum (excitation at 320 nm, emission at 450 nm) typical for Schiff-base structures $-N{=}C-C{=}C-N-$. The linear tetrapyrrolic structure of the canola FCC depicted in Figure 1 (107) suggests that the fluorescence is due to the unsaturated γ methine bridge linking pyrroles C and D. Such structures also occur in lipofuscin-like fluorescent compounds observed in senescent tissues (e.g. 96, 97) and suggested to be products of lipid peroxidation. The absence of lipofuscin-like compounds in senescent leaves of the stay-green meadow fescue (23) suggests, however, that they originate from Chl rather than from malondialdehyde. Incidentally, NCCs and FCCs react with acidic ninhydrin and may mimic proline (23): The accumulation of proline observed in senescent leaves (e.g. 125) in fact was probably due to the degradation of Chl.

Chlorella protothecoides excretes red pigments into the medium when cells are induced to bleach under appropriate conditions (115). The structure of such a pigment (Figure 1) not only reveals its origin from Chl but also the same regio-specific cleavage of the macrocycle (26) as in the catabolites from angiosperms. In contrast to Chl catabolism in senescent leaves, which seems to yield derivatives of Chl *a* exclusively, a red bilin (RCC) derived from Chl *b* has also been isolated from Chl-degrading cultures of *C. protothecoides* (60).

A special type of linear tetrapyrrolic catabolite, apparently derived from Chl *b* and ring opening at the C20/C1 mesoposition, has been identified as the light emitter in the bioluminescent dinoflagellate, *Euphausia pacifica* (108).

Catabolic Enzymes and Catabolic Pathway

CHLOROPHYLLASE In the course of the many decades since its discovery (155), chlorophyllase (Chlase, E.C. 3.1.1.14) has acquired a vast literature. Commonly, the hydrolysis of Chl into Chlide and phytol is regarded as the initial step of breakdown (Figure 2); and yet, despite detailed knowledge about catalytic properties of the enzyme as studied in vitro, the action of Chlase in vivo has remained mysterious.

Chlase is a hydrophobic protein of plastid membranes (e.g. 14, 32, 51, 157, 172) that is distinguished by its functional latency: In preparations of chloroplast membranes Chl is not dephytylated unless the membranes are solublized in the presence of detergent (e.g. 2, 93) or acetone at high concentrations (e.g. 32). Even during Chl breakdown in senescent leaves Chlase remains latent. Hence, all the properties of Chlase determined under highly unphysiological conditions in vitro, such as kinetic parameters, dependencies on pH and temperature, and so forth (e.g. 29, 66, 81, 100, 144, 173) are likely irrelevant for the understanding of dephytylation in vivo. The most intriguing problem of regulation of Chl breakdown at the level of Chlase concerns the mechanism by which the interaction between Chlase and its substrates is achieved. Latency of the enzyme may be explained simply by the spatial separation between Chl in the thylakoid pigment-protein complexes and Chlase, which appears to be located in the plastid envelope (93).

Chlases have been purified repeatedly but despite the availability of N-terminal amino acid sequences (172, 173) and a specific antibody (172), the corresponding gene(s) has so far been recalcitrant to molecular cloning. Such unexpected difficulty is not easily explained[1]. Still another puzzling feature of Chlase is its apparent glycoprotein nature as inferred from binding to concanavalin A (158, 173), indeed an unusual property of a component of chloroplast envelope membranes.

Under certain conditions Chlase can act as a transesterase and, therefore, it has occasionally been considered to have a function in the phytylation of Chlide (e.g. 30). After the elucidation of the last step of Chl biosynthesis (134), such a function of Chlase can now be disregarded.

Mg-DECHELATASE The enzymic release of Mg^{2+} from Chlide in exchange for $2H^+$ has been demonstrated in preparations from algae (116, 189) as well as from higher plants (145), but detailed knowledge about the properties of Mg-dechelatase is scarce. Under conditions preventing further catabolism of the reaction product, Pheide, the activity can be demonstrated by assessing Pheide accumulation in vivo as well as in isolated chloroplasts and chloroplast membranes (73). Apparent dechelatase activity can be assayed with chlorophyllin as substrate (63, 179). This activity is associated with chloroplast membranes and like Chlase seems to be constitutive. In detergent-solubilized membranes of barley chloroplasts, dechelatase activity (substrate: chlorophyllin) was localized in a distinct complex (135). Attempts to purify Mg-dechelatase have yielded unexpected results: The activity was heat-stable and associated with a

[1]Note added in proof: The gene for chlorophyllase has now been cloned from ethylene-treated orange fruit and expressed in *E. coli* (D Jacob-Wilk, Y Eyal, D Holland, J Riov & EE Goldschmidt, personal communication).

low-molecular-weight compound rather than with a protein (146, 177). Whether this "Mg-dechelating substance" (146) is identical with the activity responsible for the release of Mg that occurs in the catabolic pathway of Chl remains to be demonstrated.

PHEOPHORBIDE *a* OXYGENASE, RCC REDUCTASE The ring-opening step of the catabolic pathway is decisive for the loss of green color. The first identifiable colorless product, pFCC (Figure 1) is formally derived from Pheide *a* by the addition of two atoms of O and four atoms of H. The enzymic conversion of Pheide *a* into pFCC in vitro requires two protein components from gerontoplast membranes and stroma, respectively (36, 53, 137). Oxygenolysis of Pheide *a* is catalyzed by the membrane component, Pheide *a* oxygenase (PaO), and yields the red bilin RCC that in a channeled reaction is reduced by RCC reductase to yield pFCC (128) (Figures 1, 2). Chl breakdown in *C. prototheco*ides is terminated by the action of the oxygenase and RCC is released into the culture medium (26).

In *Chlorella* (20) as well as in *Brassica napus* (54), incorporation studies in the presence of $^{18,18}O_2$ showed that only the formyl oxygen originates from dioxygen, whereas the lactam oxygen at C4 is probably derived from water. The mechanism of the monooxygenase-catalyzed ring opening has not yet been elucidated in detail. A hypothesis has been proposed in which the initial step is regioselective C4/C5 epoxide formation, followed by hydrolysis and rearrangement of double bonds (20). Stereoselective final reduction of the C9/C10 double bond has been demonstrated (19).

Inhibition by appropriate chelators (35) as well as regeneration studies (53) suggest that PaO is an Fe-containing monooxygenase. Its redox cycle is driven by reduced ferredoxin (Figure 2); in the light, this reductant is generated by photosystem I (128), whereas in the dark, NADPH and a corresponding system involving the stromal oxidative pentose-phosphate-cycle and glucose-6-phosphate as ultimate e^- donor are required (36, 53, 128). In senescent leaves, all components of such a reducing system (177), including ferredoxin (128), are retained or even newly synthesized as long as Chl breakdown continues.

Among the properties of PaO, the absolute specificity for Pheide *a* as substrate (53) is noteworthy because it explains the exclusive occurrence of Chl *a*-derived final catabolites (Figure 1). Pheide *b* is a competitive inhibitor of PaO but is no more effective as a substrate than pyroPheide, protoporphyrin IX, and Chlide *a* (S Hörtensteiner, unpublished data). The oxygenase of *C. prototheco*ides seems to be less specific, as suggested by the production of an RCC derived from Pheide *b* (60).

So far, the activity of PaO has been detected only in senescent leaves of several species (36, 53, 137, 167, 178) as well as in ripening fruits (1, 103). Indeed, PaO

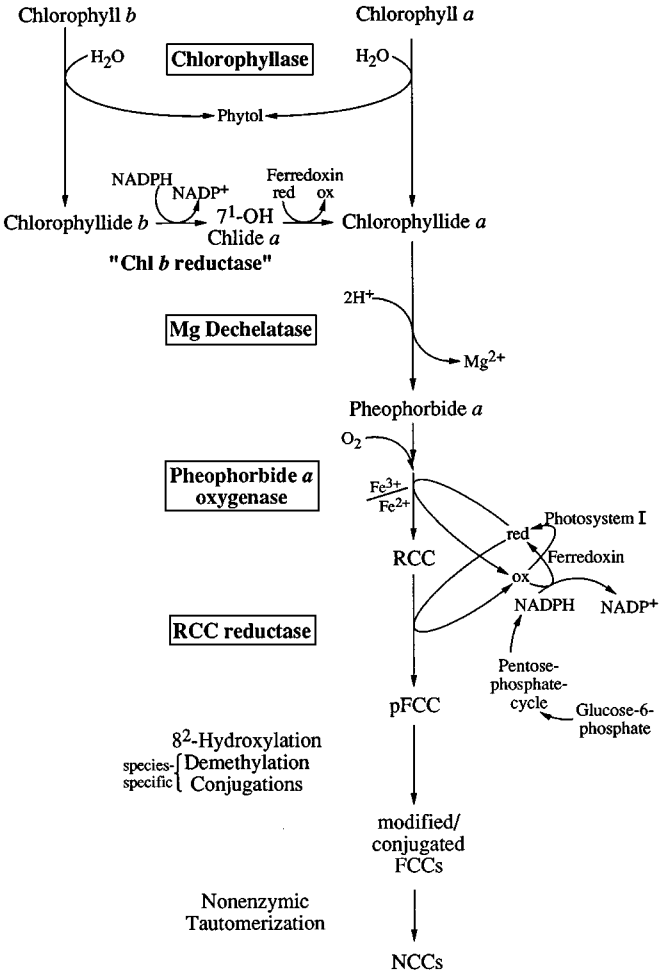


Figure 2 The "pheophorbide *a* (PaO) pathway" of chlorophyll degradation in senescent leaves. See text for discussion.

appears to be the only enzyme that plays a pace-making role in the breakdown of Chl, and the catabolic pathway (Figure 2) may, therefore, be designated as the "pheophorbide *a* pathway."

RCC is released from PaO only upon its reduction by RCC reductase in a reaction where reduced ferredoxin is again the immediate e^- donor (Figure 2). If, in the absence of PaO, RCC is employed as substrate of RCC reductase, pFCC is produced only under anaerobic conditions, suggesting that in situ a

channeled reaction takes place in an oxygen-free microenvironment at the site of PaO (128). Depending on the plant species, the cleavage of Pheide *a* yields one or the other of two C20-stereoisomers of primary FCCs. The species-specific type of pFCC is determined by RCC reductase, not by PaO (130, 177). RCC reductase is a soluble constitutive component of plastids that occurs not only in green tissues but also in etiolated leaves and even in roots (130).

METHYLESTERASE The structures of NCCs (Figure 1) demonstrate that the 13^2-carboxymethyl group may be demethylated. This modification is likely due to the action of a methylesterase sismilar to that discovered in *Chenopodium album* (147), which accepts Pheide as substrate. This constitutive "pheophorbidase" is absent from barley, the NCC of which (Figure 1) has an intact 13^2-carboxymethyl group (147).

HYDROXYLATION AND CONJUGATION All NCCs are hydroxylated in the ethyl side chain of pyrrole B. A cytochrome P_{450}-type enzyme may be responsible for the corresponding modification of FCCs. In senescent cotyledons of canola, the predominant final catabolite, *Bn*-NCC-1, is esterified with malonic acid (Figure 1). A constitutive cytosolic transferase catalyzes the esterification of *Bn*-NCC-3 with malonyl-SCoA as cosubstrate (52). A corresponding glucosyltransferase responsible for the formation of *Bn*-NCC-2 has not yet been identified.

TAUTOMERIZATION The conversion of FCCs into NCCs is due to a rearrangement of double bonds in pyrrole D and the adjacent γ methine bridge. This tautomerization takes place nonenzymically under slightly acidic conditions (S Hörtensteiner, unpublished data) and, therefore, is likely to occur upon the deposition of FCCs in the vacuole.

Chlorophyll b to Chl a Conversion

All final catabolites described so far not only are derived from Chl *a* but represent the breakdown products of Chl *b* as well (18, 35). In other words, degreening must be associated with the funneling of Chl *b* into the pool of *a*-forms. The corresponding reduction of the 7^1-formyl into the 7^1-methyl group was originally discovered in etioplasts (57) and is thought to be part of a Chl cycle by which the two forms of Chl are balanced in the photosynthetic apparatus (113). Reduction of Pheide *b* to 7^1-hydroxy Chlide *a*, Chlide *a*, and eventually to Chl *a*, has been demonstrated by infiltration of the Zn-analogue of Chlide *b* (138) or pyroChlide *b* (59) into etiolated leaves. "Chl *b* reductase" appears to consist of two components (Figure 2), an NADPH-dependent enzyme (58) producing the 7^1-hydroxy intermediate and a second reductase that is dependent on reduced ferredoxin (140).

In barley leaves the induction of senescence is accompanied by a marked increase in Chl *b* reductase activity (V Scheumann, unpublished data). Thus, Chl *b* reductase appears to play a role in the breakdown of Chl. The substrate specificity as determined in preparations of etioplasts (139) suggests that reduction may take place at the level of the dephytylated pigments, as indicated in Figure 2. Chlide *b* recycling must be very efficient in senescent leaves since inhibition of PaO activity leads to accumulation of only pheophorbide *a*, but never *b* (73, 178).

REGULATION OF BREAKDOWN

Subcellular Organization of the PaO Pathway

The PaO pathway of Chl breakdown extends over several intracellular compartments, starting in the thylakoids and inner-envelope membrane, and ending in the vacuole. As illustrated in Figure 3 it comprises not only stepwise enzymic breakdown but also transport processes within the senescent chloroplasts and across membranes. At least two enzymes of the pathway, Chlase (93) and PaO (91), have been localized to the plastid envelope. The location of the second enzyme of the pathway, Mg dechelatase, has not yet been established but it is not too speculative to assume that it is also located in the envelope. Since the ring-opening reaction occurs by the joint action of PaO and stromal RCC reductase, and since both enzymes depend on reduced ferredoxin (128) (Figure 2), the exact location can only be in the inner membrane of the envelope. Indeed, FCCs, including pFCCs, are present within gerontoplasts (36, 136, 137). Upon modification, presumably involving side chain hydroxylation(s), FCCs are eventually released into the cytosol. In isolated barley gerontoplasts, the export of *Hv*-FCC-2 was demonstrated to require the hydrolysis of ATP supplied in the medium, i.e. in the cytosol (92), suggesting that the inner membrane of the envelope is equipped with a corresponding carrier. Further metabolism of FCCs in the cytosol comprises species-specific modifications such as possibly demethylation of the 13^2-carboxymethyl group and conjugations such as the malonylation of *Bn*-NCC-3 in the case of canola (52).

The final deposition of Chl catabolites in the vacuole is associated with the transport of these catabolites, presumably FCCs, across the tonoplast. Studies with isolated vacuoles from barley mesophyll cells have revealed the existence of a specific carrier that is directly energized by ATP hydrolysis (50). It may belong to the family of ATP-binding-cassette (ABC) transporters, as do multidrug resistance–associated proteins (MRP2, MRP3) from *Arabidopsis thaliana* that transport glutathione conjugates as well as an NCC (75, 171). Such a possible connection between detoxification of xenobiotics and the final disposal of Chl catabolites is anything but coincidental: It implies that Chl degradation might resemble Chl detoxification (see below in Significance of Degradation).

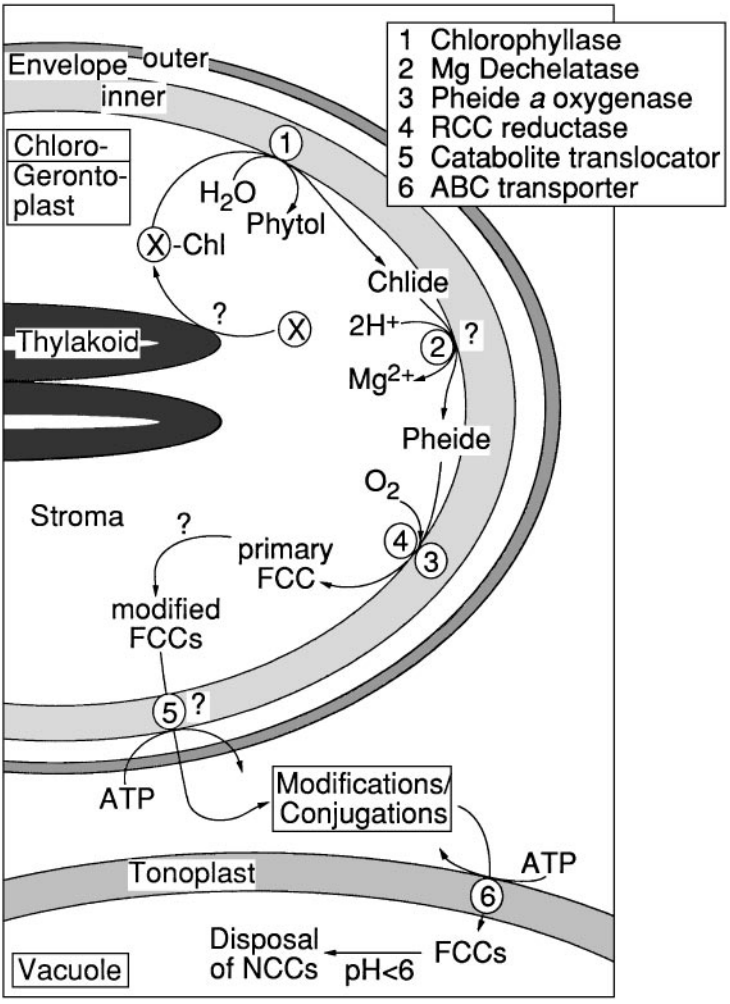


Figure 3 Intracellular organization of the pheophorbide *a* (PaO) pathway of chlorophyll degradation in senescent mesophyll cells. The hypothetical part of the catabolic system marked "X" is thought to be responsible for dismantling the thylakoid pigment-protein complexes and transport of the resulting Chl molecules to the inner-envelope membrane, site of the first catabolic enzyme, Chlase. Further explanations and details in the text and in Figure 2.

In isolated intact gerontoplasts, breakdown of Chl to FCCs depends on the supply of ATP in the medium (36, 136). However, none of the reactions of the PaO pathway required ATP. A similar discrepancy was observed in the case of Chl *b* to Chl *a* transformation, which in intact, but not in lysed etioplasts, depends on ATP (140).

Regulation of PaO Pathway

With the exception of PaO itself, all known components of the PaO pathway are constitutive enzymes. Activities may be modulated during development, as widely documented in the case of Chlase (e.g. 27, 64, 78, 80, 81, 98, 172), but PaO is detectable exclusively in senescent leaves (36, 53, 178), where its activity is positively correlated with rates of Chl loss (129).

It is not immediately clear why the overall regulation of breakdown should be at the third enzymic step of the pathway, rather than at the initial reaction. That there is some degree of feedback control is clear from experiments in which the action of PaO in senescent leaves is blocked by appropriate treatments. Under these conditions, Chlides and Pheide *a* accumulate (73, 120). This phenomenon can also be observed in a PaO-deficient mutant genotype of *Festuca pratensis* (178); but the accumulation of dephytylated Chls does not take place when senescent leaves of the mutant are treated with cycloheximide (164), which suggests that cytoplasmic protein synthesis is required for providing Chlase with its substrates. The respective protein(s) may be responsible for dismantling the thylakoid pigment-protein complexes and/or the transport of Chl to the site of Chlase.

Chlorophyll-Bleaching Enzymes of Unknown Functions

Chl is readily bleached in vitro by the action of oxidative enzymes such as peroxidases in the presence of H_2O_2 and a phenolic compound (e.g. 65, 71, 143, 188) or lipoxygenase in the presence of linolenic acid (e.g. 68). Chl is also bleached when chloroplast membranes (11, 76, 82) or isolated photosystem preparations (77) are incubated in the presence of unsaturated fatty acids. The significance of these reactions for Chl breakdown in senescent leaves is dubious. In any case, it is not justified to refer to "peroxidase" or "Chl oxidase" pathways (63, 187) as long as products of bleaching have not been identified as natural catabolites.

Genetics of Degradation

The number of enzyme activities known or suspected to open the ring of Chl or its derivatives, and to process the products of ring-opening, may approach 20. Unless it has been already identified for some other, apparently unrelated, metabolic function, not one of the corresponding 20 genes or gene families has been described in molecular terms. Either these genes remain to be isolated or they are among the anonymous ESTs and open reading frames emerging

from the various plant genomics projects. Functional analysis by, for example, transposon mutagenesis, should be a rewarding approach to assigning gene identities to those enzymes that bleach Chl, since mutant phenotypes will be easily screened. It is also likely that such mutations are tolerated and would not threaten the viability of the whole plant. Many naturally occurring mutations and genetic variants deficient in pigment-degrading capacity (referred to by the general term "stay-green") have been described (111, 168).

Despite the lack of molecular detail, by studying the characteristics of the different kinds of stay-green, some inferences can be made about the genetic mechanisms regulating expression of Chl degradation. Three classes of stay-greens are recognized. In the first group, the Chl degradation machinery is intact but is activated late. Alternatively, degradation may start on time but proceed abnormally slowly. In a third type of variation, initiation and rate occur normally but one or more steps in the degradation pathway may be deficient. These variations on the stay-green theme may be referred to as types A, B, and C, respectively (168). Stay-greens in a number of species have been classified, but only in the case of a few type Cs has Chl degradation been analyzed in terms other than simple pigment measurements.

The most intensively studied stay-green variant was originally identified in the pasture grass *Festuca pratensis* (169). Green tissues of the mutant retain Chl more or less indefinitely. The mutation is inherited as a single recessive nuclear gene (162). It is an example of a type C stay-green, since most of the senescence syndrome, including a decrease in photosynthetic capacity and degradation of soluble leaf proteins, proceeds normally (46, 160, 162, 169). Similar phenotypes have been observed in *Phaseolus vulgaris* (131) and *Pisum sativum* (the latter corresponding to the green cotyledon character originally described by Mendel), and in all three cases, pheophorbide *a* oxygenase activity is not detectable (4, 167, 178). The genetic lesion is likely to be situated either in the structural gene for PaO itself, or in a locus that regulates its induction at the onset of senescence. It would be a major step forward for the study of Chl degradation if the corresponding locus could be isolated.

The occurrence of this mutation in *Festuca* has some distinct advantages. Recombinant chromosomes resulting from introgression of *F. pratensis* genes into species of the related genus *Lolium* are readily identified by genomic in situ hybridization. This has allowed the stay-green locus to be physically mapped in the *Festuca-Lolium* genome (165, 170). Repeated backcrossing has reduced the size of the recombinant segment until it now has become feasible to identify the mutant locus, or at least genes tightly linked to it, by searching for *Festuca*-specific polymorphisms in genomic or cDNA clones. Because the stay-green phenotype is so easily screened, it is a very convenient character for testing the combined introgression/expression mapping approach to genetic analysis.

Not only deficiency regarding PaO but also lesions in Chlase, Mg dechelatase, or RCC reductase should result in a type C phenotype. But no corresponding angiosperm mutant has yet been described. Either it is necessary to increase the range of plant material to be screened, or else the constitutive nature of these enzymes means that knock-out mutations have as yet unforeseen lethal consequences for higher plants. We may need to isolate the corresponding genes and subject them to analysis by reverse genetics before we obtain any significant insight.

Legumes seem to be especially tolerant of mutations affecting Chl degradation, perhaps because nitrogen fixation compensates for the lower availability of internal nitrogen from which stay-greens tend to suffer. At least nine separate genetic loci have been associated with the stay-green phenotype and other senescence traits in soybean (168). One of particular interest is a cytoplasmic gene, *cytG*. The corresponding phenotype is characterized by near-normal loss of Chl *a* but comparative stability of Chl *b* during senescence (40). Chl *b* is likely converted to Chl *a* prior to degradation by the ring-opening oxygenase route (Figure 2). The product of the wild-type allele of *cytG* could well be one of the enzymes or controlling factors in the *b* to *a* conversion sequence. It would be interesting to examine the ctDNA of *cytG* soybean for a sequence anomaly that may identify the specific gene.

Stay-greens of types A and B are clearly not chlorophyll degradation mutants per se, although the variant genes are critical for the coordination of pigment loss with the other components of the senescence syndrome. Because of the agronomic significance of extended photosynthetic duration, type A/B traits have been studied physiologically and by linkage analysis. Quantitative trait loci (QTLs) related to foliar senescence are currently the subject of inheritance studies and linkage mapping in a number of species, including sorghum and millet (74, 156, 175, 182). As genes for components of the Chl degradation pathway and associated senescence processes are gathered by other approaches, it will be instructive to establish whether they map anywhere near the QTLs for leaf color and lifespan. Further developments on the molecular genetics of Chl degradation may also be anticipated following the isolation of three *Arabidopsis* stay-greens (112). Once the corresponding genes are classified and mapped, the powerful tools of *Arabidopsis* genetic analysis can at last be applied to the problem of Chl degradation, and rapid progress will surely follow.

SIGNIFICANCE OF DEGRADATION

Senescence Benefits from Chlorophyll Degradation

The amount of mobilizable material (principally Mg and nitrogen) invested in the Chl molecule is small relative to that available from other salvaged

cell constituents. For example, the 240-kDa photosystem II core complex is associated with about 36 Chl *a* molecules (44). If all the nitrogen in this complex were mobilized, less than 6% would be contributed by Chl. Moreover, isolated Chl is not particularly stable. And yet, at the end of its lifespan, the green cell takes special and metabolically expensive measures to degrade Chl because active catabolism of Chl is beneficial to the plant.

Both in vivo and in vitro, proper assembly of thylakoid pigment-protein complexes has an absolute requirement for Chls in precisely stoichiometric amounts (163). Thus, Chl has a constructional and stabilizing role in addition to its light-harvesting and photosynthetic functions. Building of Chl into complexes with other components, particularly carotenoids, is also important for controlling its photodynamism. But removing Chl from such complexes to recover protein nitrogen and lipid carbon is delicate and must be strictly coupled with the macrocycle opening by PaO and RCC reductase rendering Chl photodynamically harmless and preserving the viability of the senescing cells. The obligate relationship between pigment removal and nutrient recycling is convincingly demonstrated in certain type C stay-green plants, where a lesion in ring opening preserves Chl and significantly reduces the lability of associated proteins, lipids, and carotenoids (5, 41, 45, 46). In the stay-green mutant of *Festuca pratensis* the persistence of photostable Chl (166) must be accompanied by maintenance of processes that dissipate absorbed quanta other than through photosynthetic CO_2 fixation. Enhanced levels of photorespiration and carotenoids (10, 41) suggest that this mutant is attuned to the excitation state of Chl and adjusts its antioxidant status according to the need to channel excess energy away harmlessly.

Chl is degraded not because its products are reusable but primarily because otherwise it would block access to more valuable materials. The nitrogen and carbon from which Chl is constructed remain in the cell for good. This is the price the plant pays for access to thylakoid proteins and lipids. The notion of Chl catabolism as being essential for salvage while not itself being a salvage process leads to a conclusion that may make sense for other aspects of the pathway. Not only is the Chl macrocycle converted into non-photodynamic linear forms, these are irreversibly transported to the vacuole and sequestered as oxidized and conjugated by-products (50, 75). In a sense, Chl is not so much catabolized as detoxified.

Chlorophyll Degradation May Be Beneficial During Cell Death

Discoloration, necrotic and chlorotic lesions, and other visible disfigurations are diagnostic of the pathological or terminal state of cells and tissues. During the hypersensitive (or a similar) injury response, rapidly propagated oxidation

is important to cauterize the traumatized zone (72, 123). When cells collapse there is neither the need nor the metabolic integrity to induce the oxygenase pathway of Chl catabolism, and rapid pigment destruction is likely to be due to the action of constitutive peroxidases, lipoxygenase, and fatty acid-dependent with Chl-bleaching activities (e.g. 76, 82, 141, 161).

EVOLUTION OF DEGRADATION

Chlorophyll-Degrading Organisms

When during evolution did autotrophic organisms gain the ability to degrade Chl? This question deserves attention in relation to the evolution of photosynthesis, coinciding with the appearance of the potentially toxic and photodynamic Chl molecule.

Chl-degrading processes have been observed in diverse species across the entire taxonomic range: in red algae (79), diatoms (152), green algae (22, 25), and prokaryotes (42, 99), and the leaves of many bryophytes, pteridophytes, and gymnosperms lose Chl during a process that closely resembles foliar senescence in angiosperms (7, 9, 21, 43, 86, 109, 114). In most of these instances of Chl bleaching, only Chlide, pheo-pigments (Pheide and Phein) and their pyroforms have been identified as degradation products so far (34, 79, 151, 189). Accordingly, data on enzyme activities are limited to Chlase (e.g. 144, 158) and Mg-dechelatase (116, 189). Thus, for many Chl-degrading systems the fate of Chl remains unclear, and we only can speculate as to whether non-angiosperms might be capable of generating Chl catabolites similar to the NCCs of higher plants.

Only in three cases have linear tetrapyrroles been identified as Chl breakdown products in lower plants (22, 26, 101). The structure of the main red bilin (RCC) excreted in *Chlorella prothecoides* (25) is identical to that of the intermediary product of Pheide *a* to pFCC transformation in higher plants (128). In addition, the mechanism of chlorin macrocycle opening in *Chlorella* and oilseed rape is identical with respect to oxygen incorporation (20, 54). The Chl breakdown mechanism of higher plants might have evolved from green algae, which are generally accepted as their phylogenetic ancestors (174). Thus, Chl catabolism appears to be an early innovation of photosynthetic organisms.

In contrast to *Chlorella*, where RCCs as the final catabolites are excreted into the surrounding medium, plants living in nonaqueous habitats have to degrade further the red, and thus still potentially photodynamic, RCC. RCC reductase, catalyzing the stereospecific reduction of the C20/C1 double bond of RCC to the primary FCC, is present in angiosperms (130) as well as in more ancient species such as *Selaginella* (S Rodoni & P Matile, unpublished data). It may be argued, therefore, that a crucial step in the evolution of the Chl catabolic pathway might have been the appearance of RCC reductase.

Plastid Transition and Evolution

Gerontoplasts and chromoplasts are plastids characteristic of the terminal, differentiated state of the above-ground organs of angiosperms. Mosses, ferns, and conifers are also likely to be genetically competent to carry out the conversion from chloroplasts to gerontoplasts. Ultrastructural modifications of thylakoid membranes during Chl breakdown have been described for unicellular algae (142), and even in *Synechococcus lividus* bleaching is accompanied by the formation of "vesicularized thylakoids" (99). Thus, the chloroplast-to-gerontoplast transition appears to be a common feature of the degreening process, which either predates the appearance of chromoplasts in evolution, or is at least derived from some common ancestral plastid form, perhaps of the kind observed in stressed cells of *Chlorella zofingiensis* (6). Color changes during leaf senescence can act as a visual signal or "fruit flag" (153, 183). We, therefore, speculate that chromoplast differentiation (16), a key process in entomophily, fruit dispersal, and angiosperm evolution, is a variation on the more ancient theme of plastid transition leading to gerontoplasts, and that the Chl catabolism pathway is a common, defining biochemical feature. The corolla was an innovation of mid-Cretaceous rosids (17). Before this, the stimulus that attracted insects to flowers was probably not visual. It may be, therefore, that the diversification of plastid differentiation pathways can be dated to somewhere around the late Cretaceous period—a time of rapid angiosperm diversification. It is tempting to link the two events, but fossils preserve neither color nor organelle structure, so we may never know whether this really happened.

CONCLUSIONS

A century after Albert F. Woods (185), we have made considerable progress in understanding some aspects of Chl degradation, though as recently as 20 years ago we could not have made this claim. We can now look forward to a period of accelerating progress, particularly if the goal of cloning the genes encoding PaO and the other catabolic enzymes is realized. The developmental and tissue specificities of gene expression, as well as complementation of PaO-deficient stay-greens, will tell us more about Chl catabolism by the PaO route and its regulation. Moreover, the PaO pathway is far from fully described; and as for alternative systems of Chl degradation, all we have at present are tantalizing glimpses of unknown physiological significance. One of the most important of the areas of present ignorance—important because it relates to N-recycling associated with leaf senescence—is the mechanism responsible for dismantling the pigment-protein complexes in the thylakoid and directing the resulting Chl molecules and apoproteins into catabolism. There is more than enough work here to keep researchers busy for another 100 years.

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