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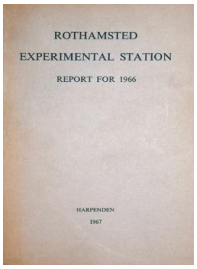

The Breakdown of Chlorophyll

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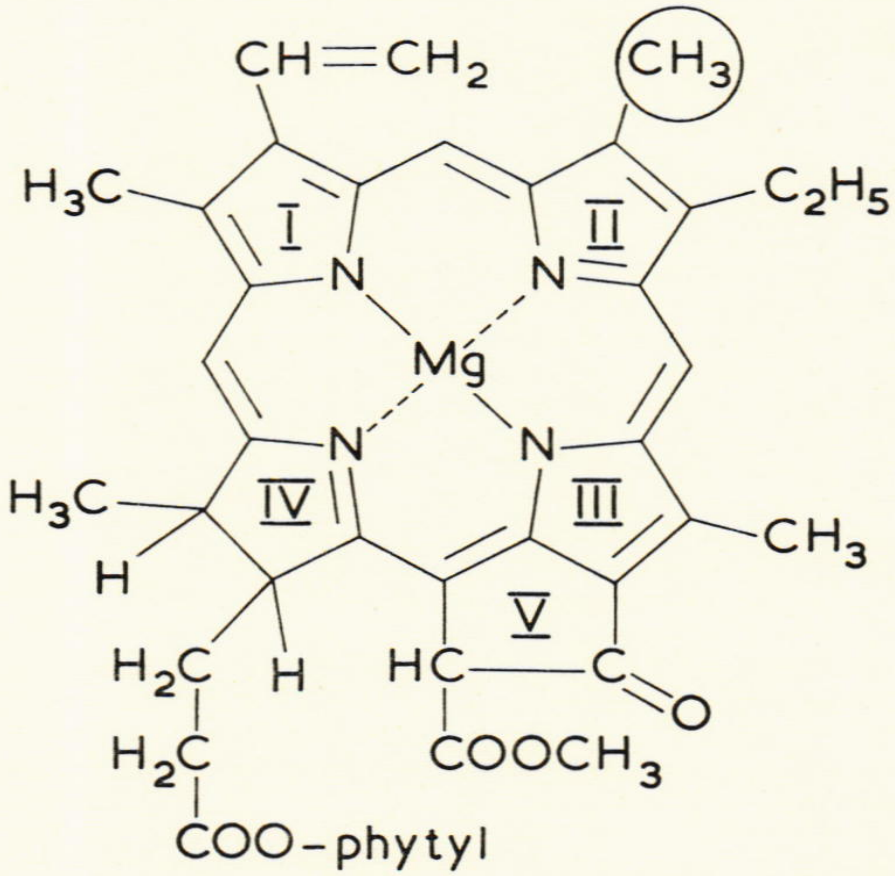
THE BREAKDOWN OF CHLOROPHYLL

MARGARET HOLDEN

The ability of flowering plants, conifers, ferns, mosses and algae to photosynthesise depends on two green pigments, chlorophyll *a*, whose structure is shown in the figure, and chlorophyll *b*, which differs from *a* in having the CH_3 group that is ringed in the figure replaced by CHO. Quantitatively, chlorophyll *a* is the more important, because there is usually three



times as much of it as of chlorophyll *b*. The chlorophylls break down in living tissues as part of normal physiological processes when leaves age or fruits ripen. Both also break down and thus diminish photosynthesis, because of many different adverse conditions, ranging from attacks by various pathogens to exposure to intense light, from deficiencies of nutrients to keeping in darkness and from lack of carbon dioxide to poisoning by various chemicals. Yellowing of leaves, the external sign of chlorophyll breakdown, is the commonest and often the first indication of ill health in plants.

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Although chlorophyll breakdown in living cells is such a common occurrence, little is known of its biochemistry, partly because it is difficult to study it in conditions where chlorophyll is perhaps also being synthesised. To gain information about the kinds of change chlorophyll may undergo, and the mechanisms that may be involved in its degradation, the reactions that take place in pieces of leaf and in plant extracts have been studied in detail. Three known products formed by small changes in the chlorophyll molecule are pheophytins, chlorophyllides and pheophorbides. Pheophytins are formed by removing the magnesium; this occurs when dilute acid is added to plant extracts and during prolonged heating of leaves. Pheophytins are brown or grey and are responsible for the unattractive colour of overcooked green vegetables. Chlorophyllides are formed when the phytyl side chain of the chlorophylls is removed by the enzyme chlorophyllase. This produces no colour change, because chlorophyllides have the same absorption spectra as chlorophylls. When acidified, chlorophyllides form pheophorbides, which are similar in colour to the pheophytins.

Separation of the chlorophylls and their coloured breakdown products. Methods for separating chlorophylls *a* and *b* and some of their coloured degradation products have been much improved by the use of chromatographic techniques, and were recently reviewed by Holden (1965a). Chromatography on columns of adsorbents is the obvious choice for preparing large amounts of pigments, and the introduction of powdered polyethylene for this purpose makes it possible to separate pigments occurring mixed in crude extracts. For separating small amounts, chromatography on paper (Holden, 1962) has now been largely superseded by thin-layer chromatography (TLC). Bacon (1965) described a method for separating the chlorophylls themselves, pheophytins, pheophorbides and chlorophyllides on thin layers of cellulose. Using this method, Bacon & Holden (1967) also separated a range of "changed" chlorophylls and pheophytins that are probably oxidation products.

Enzymes concerned with the breakdown of chlorophyll

Chlorophyllase. The first stage in the breakdown of chlorophyll *in vivo* might be the removal of the phytyl side-chain, a change catalysed by the enzyme chlorophyllase. A study of the properties of this enzyme seemed therefore to be the obvious starting-point for research on the enzymic degradation of chlorophyll. Chlorophyllase was discovered more than 50 years ago by Willstätter & Stoll, but little work had been done on it, and none at all using modern methods of enzyme chemistry. Sugar-beet leaves proved to be an excellent source of the enzyme, and soluble preparations were made and considerably purified (Holden, 1961, 1963) from acetone-powders of these leaves. The most noteworthy property of the enzyme is its stability, particularly in the presence of large concentrations of organic solvents. When leaves of most species are suspended in aqueous acetone (50–60%) for a few hours much of the chlorophyll is converted into chlorophyllide. Chlorophyllides are also formed by chlorophyllase action when leaves are heated at temperatures between about 45° and 80° C, but

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higher temperatures inactivate the enzyme and prevent chlorophyllides being formed.

Chlorophyllase is widely distributed (flowering plants, gymnosperms, ferns, mosses, algae and purple bacteria), and its presence is readily demonstrated by its hydrolytic activity on chlorophyll *in vitro*, but there is little evidence that it is directly involved in the breakdown of chlorophyll *in vivo*. The last stage in the biosynthesis of chlorophyll is the attachment of the phytyl group to the porphyrin ring system, and the enzyme responsible for this has also been called chlorophyllase, but whether this is the same enzyme that removes the phytyl group *in vitro* is not known.

A lipoxidase in seeds. Holden (1965b) described an enzyme system from legume seeds that converts chlorophyll into colourless compounds. In this system long-chain fatty acids are peroxidised by lipoxidase and other enzymes, and the hydroperoxides are then broken down by a lipoperoxidase leading to free-radical formation and the oxidation of the chlorophylls. The nature of the chlorophyll breakdown products is now being studied. As there is no reason for supposing that this is the only, or even the usual, way in which chlorophyll is broken down *in vivo*, other enzymes that will catalyse the degradation of chlorophylls are being sought.

A lipoxidase in leaves. While investigating the behaviour of chlorophylls in leaf tissue kept in aqueous organic solvents, Bacon & Holden (1967) observed that most of the green colour disappeared from a suspension of chopped barley leaves in 50% acetone kept at room temperature in the dark overnight. Boiled suspensions remained green, suggesting that the bleaching was enzymic, and it seemed possible that these leaves contained a different chlorophyll-decomposing system. However, further work shows that a lipoxidase–lipoperoxidase system is again implicated (Holden, 1967). About 50 species were examined for chlorophyll bleaching by suspending leaf discs or strips in 50% aqueous acetone overnight; a few of them showed no loss of chlorophyll, but most lost some, and about one-third behaved like barley leaves and lost up to 80% of their green pigments. All these species were found to contain an active lipoxidase, although previously this enzyme was thought to be confined almost entirely to legume and cereal seeds. However, this lipoxidase differs in some of its properties from the one in legume seeds.

Glycollate oxidase. Kolesnikov (1948, 1949), who studied the oxidation of chlorophyll in suspensions of chloroplasts from barley leaves, found that when glycollic acid was added to suspensions kept in the dark the amount of chlorophyll oxidised was greatly increased. He suggested that oxidation was caused by organic peroxides of glyoxylic acid that were formed in the extracts. When the accumulation of peroxides was prevented by ascorbic acid or phenols the chlorophyll was not oxidised. However, he also observed that when carbonyl compounds were not formed a large amount of peroxide could accumulate without chlorophyll being oxidised. This suggests that a mechanism similar to the one in legume seeds was operating, with chlorophyll being bleached during breakdown of peroxides rather than by the peroxides themselves.

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Tolbert & Burris (1950), who confirmed that chlorophyll in the sap from barley seedlings was bleached when glycollic acid was added, isolated two yellow pigments formed from chlorophylls *a* and *b* during glycollate oxidation. Bacon & Holden (1964) also confirmed that some chlorophyll is bleached during the enzymic oxidation of glycollate by barley and pea seedlings, but did not isolate any breakdown products.

Bleaching of chlorophyll by hydrogen peroxide. Noack (1943) found that fresh leaves turned white within 6 hours when treated with 3% H_2O_2 whereas boiled leaves remained green for more than two days. Pretreatment of the leaves with 4% diethyl ether prevented the chlorophyll bleaching, but in 3% H_2O_2 + 4% ether the loss of pigment was the same as with fresh leaves. Other compounds, such as phenylurethane, sodium bisulphite and 10% KNO_3 also inhibited bleaching by H_2O_2.

Noack did some *in vitro* experiments on chlorophyll bleaching using chlorophyllides made by chlorophyllase action. He found that chlorophyllides, unlike chlorophylls, were readily bleached by H_2O_2 in the presence of either $Fe(OH)_3$ or montmorillonite as a catalyst. He attempted to identify the colourless breakdown products, and with a short reaction time found methylethylmaleinimide, ammonia and oxalic acid. When the reaction was allowed to proceed for a long time he assumed total oxidation, with carbon dioxide, ammonia and water as the end products.

Noack envisaged that the breakdown of chlorophyll in ageing leaves took place in the following way: (1) accumulation of H_2O_2 because of a decrease in catalase activity; (2) breakdown of chloroplast protein; (3) splitting off of chloroplast iron as $Fe(OH)_3$; (4) activation of chlorophyllase and the conversion of chlorophyll to chlorophyllide; (5) oxidative breakdown of chlorophyllide by H_2O_2 with $Fe(OH)_3$ as catalyst. As all the reactions would take place simultaneously, intermediates would not accumulate.

This ingenious theory has not been confirmed, and although peroxides are involved in some *in vitro* reactions in which chlorophyll is broken down, there is no evidence as yet for such a mechanism *in vivo*.

Oxidation of chlorophyll by quinones. Keegel (1958) suggested that, during the fermentation of tea leaf, chlorophyll is oxidised to a brown pigment by quinones formed when catechins of the leaf are oxidised by polyphenoloxidase. When the magnesium in the chlorophyll is replaced by copper, which is said to be possible when tea bushes are sprayed with a copper fungicide against blister blight, the chlorophyll does not oxidise during fermentation and the leaf does not assume the colour of properly fermented tea.

Formation of "changed" chlorophylls. Strain (1954) described the formation of green oxidation products of the chlorophylls when chopped leaves were allowed to stand with alcohol or acetone in the presence of air. Chlorophyll *a* gave a pigment that was adsorbed more strongly on a column of sugar and hardly separated from chlorophyll *b*, which also gave a more strongly adsorbed pigment. Strain thought that these pigments

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were formed by enzyme action. Both these altered chlorophylls had absorption maxima at similar wavelengths to those of the pigments from which they were derived. Bacon & Holden (1967) found that two major "changed" pigments and some minor ones were formed from each of the chlorophylls when boiled leaves were kept in an aqueous alkaline medium and also when unheated leaves were kept in aqueous organic solvents. Bacon (1966) showed that these "changed" pigments are identical with pigments formed as artefacts on silica-gel chromatograms.

Loss of chlorophyll in senescing and detached leaves. In the chloroplast the chlorophylls are firmly attached to both protein and lipid, and until this linkage is broken the pigments are apparently not vulnerable to enzymic attack. *In vitro* the link can be broken by treatment with organic solvents such as acetone and alcohols or by heating. *In vivo*, concurrent breakdown of chloroplast protein seems to be necessary for chlorophyll degradation to occur. Several workers have correlated the disappearance of chlorophyll and protein in leaf tissues. Breakdown of lipid may be related even more closely than protein breakdown to chlorophyll degradation. Evidence to support this would be obtained if it could be shown that a mechanism involving peroxidation of lipids is actually responsible for the degradation of chlorophyll in some conditions.

The rate at which chlorophyll breaks down in detached leaves kept in the dark at room temperature depends on the species and age of the leaf and on the humidity. It also depends on pre-treatments such as freezing or boiling to which the leaves are subjected. Hoyt (1964) found that 90% of the chlorophyll was lost from Italian rye-grass after 4 days in a moist atmosphere, whereas in dry air chlorophyll degradation stopped after 2 days when the moisture content of the leaves had fallen from 76 to 33% and about half the chlorophyll still remained. Holden (1967) studied a wide range of species and found that leaves of purple sprouting broccoli lost 87% of their chlorophyll in five days under moist conditions. Most other species lost chlorophyll more slowly and, at the other end of the scale, Willow-herb (*Chamaenerion angustifolium*) lost only 5% in 10 days and *Paeonia* leaves none at all. Boiling or freezing of the leaves before keeping them in a moist atmosphere prevented chlorophyll breakdown. It is not unexpected that boiling should inactivate the chlorophyll-breakdown system, but it is of interest that freezing should also do so, because many enzymes can be frozen without loss of activity. However, it fits the observation that leaves of deciduous shrubs that are killed by a hard frost often remain green for a long period.

Detached leaves of about 30 plant species were kept moist and dark, and extracts were examined by TLC for the presence of coloured degradation products. Traces of chlorophyllides and pheophorbides were occasionally found, but usually only chlorophylls *a* and *b* in diminishing amounts were detected with no other coloured porphyrins. There is obviously a rapid conversion to colourless compounds without the accumulation of coloured intermediates.

Goodwin (1958) found that the remaining chlorophylls in autumn leaves of some deciduous trees, although having similar chromatographic

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properties to chlorophylls *a* and *b*, had different absorption spectra. The *a* pigment had a maximum in light petroleum at 652 mμ instead of 662 mμ and the *b* pigment at 635 mμ instead of 644 mμ. In samples of leaves taken from June to October the ratio of chlorophyll *a* to chlorophyll *b* remained nearly constant. Samples taken in November that contained only the altered chlorophylls had a much smaller ratio because the *a* derivative disappeared faster than the *b* pigment. So far we have been unable to confirm the formation of altered chlorophylls of this type in autumn leaves.

The effect of growth substances, herbicides and pesticides. Bruinsma (1965) reviewed the effect of growth-regulating substances, herbicides and a range of pesticides on the chlorophyll content of plants. Many compounds, e.g. the herbicide 3-amino-1,2,4-triazole, affect the chlorophyll content by interfering with its biosynthesis in young tissue rather than by accelerating its degradation. However, substances such as thiouracil and chloramphenicol, which are inhibitors of protein synthesis, accelerate the breakdown of chlorophyll. By contrast, compounds such as kinetin (Richmond & Lang, 1957) and benzimidazole (Person, Samborski & Forsyth, 1957) delay senescence and the yellowing of detached leaves by maintaining protein synthesis in the tissues. The use of benzylaminopurine as a spray or dip for improving the keeping qualities of some horticultural crops has been suggested (Salunkhe, Dhalival & Boe, 1962).

Gibberellic acid delays the development of autumn colours in the leaves of many deciduous trees (Brian, Petty & Richmond, 1959), and the senescence of leaf discs of various species (Fletcher & Osborne, 1966).

Humphries (1967) found that (2-chloro-ethyl)trimethylammonium chloride, which diminishes the rate of shoot growth of dwarf-bean plants, enables the primary leaves to retain their nitrogen longer, and so delays both protein hydrolysis and chlorophyll breakdown.

The effect of some other substances on chlorophyll is more complicated and less well defined than with the examples given above. It depends on the species of plant, on its stage of development and also on various other factors. Even kinetin, which usually delays senescence, was found by Bruinsma (1966) to stimulate chlorophyll breakdown when potato-leaf discs were kept in the light instead of in the dark.

Some herbicides seem to have a specific effect on chlorophyll. Oxidation of chlorophyll has been postulated as the mechanism by which substituted urea herbicides act as plant poisons (van Overbeek, 1964). The herbicide apparently blocks the usual photosynthetic path by which electrons in chlorophyll are replaced, and the chlorophyll is therefore damaged by being irreversibly oxidised. Herbicides like diquat and paraquat are thought to act by their ability to pick up single electrons leading to the formation of free radicals (Cronshey, 1961). These in turn may give rise to peroxide radicals that damage the chlorophyll and other cell constituents (Calderbank, 1966).

Chlorophyll breakdown in soil. Hoyt (1964) reviewed the few papers on the degradation of chlorophyll in soils. He measured the amount of

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chlorophyll-type compounds in leaves, farmyard manure and other materials that are commonly deposited on or in soils. He then studied the rate at which chlorophyll decomposes in plant materials when added to soil, and found that the activity of soil micro-organisms speeds its decomposition. Chlorophyll *a* was more susceptible than *b* to the action both of tissue enzymes and of soil micro-organisms. In acid soils chlorophyll was quickly converted into pheophytin. In water-logged soil leaf enzymes were inactivated, and because conditions were also unsuitable for micro-organisms chlorophyll breakdown was very slow. When dried, ground rye-grass leaves were added to various Rothamsted soils in the laboratory only about 5% of chlorophyll-type compounds remained after 90 days.

In field experiments chlorophyll decomposed much faster in fresh tissues than in dried plant material in which the enzymes had been inactivated. At Rothamsted 90% of the pigments had disappeared from dried material after 150 days, but at Woburn only 50%. Chlorophylls *a* and *b* were still detectable 72 days after the materials were incorporated into the soil. At the final sampling (150 days) only pheophytin was detected.

All the soils Hoyt examined contained chlorophyll-type compounds (0·5–3 lb/acre). The top 2 in. of old grassland and woodland soils contained much more than topsoil of arable land, but lower down the profiles the amounts were similar in various types of soil.

Soils more acid than pH 4 had more chlorophyll-type compounds than alkaline soils, and the microbiological degradation of chlorophyll in acid soils was very slow.

Chlorophyll derivatives in sediments and oil deposits. It is clear that in living tissues the porphyrin ring system of chlorophyll is broken easily, and colourless degradation products are formed. By contrast, in dead tissues, provided that the ring was intact when the plant died, it is remarkably stable, and coloured compounds are then found. Marine and fresh-water sediments up to 100,000 years old contain coloured degradation products of chlorophyll. Pheophytin, pheophorbide and chlorophyllide have all been found in sediments from Little Round Lake, Ontario (Vallentyne, 1960). Several factors seem to be responsible for preserving the porphyrin ring in wet sediments, of which lack of oxygen is one of the most important and others are lack of light, cold and the insolubility in water of the compounds in their natural state.

Petroporphyrins. The presence of porphyrins in oil deposits many million years old provides further evidence for the great resistance of the porphyrin ring to degradation. Thirty years ago Treibs described the separation of porphyrins from various bituminous materials, including oil, shale and coal. He identified etioporphyrin III (Etio) and deoxyphylloerythroetioporphyrin (DPEP) and put forward a degradation scheme by which hemin could be converted into Etio and chlorophylls *a* and *b* into DPEP. Six types of reaction were implicated: (1) removal of the metal; (2) saponification; (3) reduction; (4) aromatisation; (5) decarboxylation;

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(6) chelation with a metal such as vanadium. More recent work has in general confirmed Treibs' ideas, but mass spectrometry shows that there are many homologues present of both the deoxyphyllo and etio series and also small amounts of rhodoporphyrins. Baker (1966) suggested that yet another type of reaction must also be involved to account for the many products found. This might be transalkylation via an ionic or free-radical mechanism.

Chlorophyll breakdown in animals. In animals during the digestion process chlorophyll is mainly converted into pheophytin and pheophorbide, but phylloerythrin is also formed by bacterial action. In phylloerythrin the phorbin ring is still intact, but the phytyl side-chain, magnesium and the two hydrogen atoms on ring IV have been lost, the vinyl group on ring I has been reduced to ethyl and the methoxy carbonyl group on the cyclopentanone ring has been replaced by hydrogen. Phylloerythrin is responsible for one type of photosensitisation reaction in animals. Its effectiveness as a photosensitiser may depend on its conversion to an activated form, possibly with a free-radical structure (Slater & Riley, 1966). It is usually absorbed from the gut and excreted by the liver, and is not concentrated enough in the blood to cause photosensitisation unless the liver is damaged in some way. A disease of cattle in South Africa, called Geeldkoppe, is a phylloerythrin photosensitisation caused when a poisonous fodder plant *Tribulus* damages the liver.

Conclusion

Work on the enzymic breakdown of chlorophyll was started in the hope that it would explain the processes by which chlorophyll disappears from leaves as they age or suffer from various infections and deficiencies. The experimental conditions used for studies *in vitro* are, of necessity, somewhat artificial, and chlorophyllase and the lipid-peroxidising enzymes may function differently *in vivo*. It is, however, clear that the activity of these enzymes *in vitro* is not correlated with the activity of chlorophyll loss in detached leaves. Other enzyme systems, yet to be found, may be involved, or other factors, such as the effect of light on chlorophyll when the linkages between it and either protein or fat are broken.

One of the most obvious effects of many virus infections is mottling and yellowing of leaves. Synthesis and breakdown seem to be affected, depending partly on the age of the tissues. We do not know whether virus infections affect chlorophyll biosynthesis and degradation in a specific way or whether the processes are upset because of the general derangement of metabolism.

With mineral deficiencies chlorophyll may disappear from mature tissues or fail to be formed in young leaves. In young leaves the effect is clearly on biosynthetic processes, and this may also be true in mature leaves. In these the synthesis of chlorophyll may not be able to keep up with the breakdown because one or more of the enzyme reactions may require the mineral that is deficient.

Fungal infections also often cause yellowing, but whether the breakdown

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by leaf enzymes is stimulated or whether the pathogens themselves contain chlorophyll-splitting enzymes remains to be discovered.

There is no reason to assume that the same mechanisms operate when chlorophyll disappears from leaves subjected to different forms of stress. Some progress has been made in elucidating the changes that take place in killed tissues, but much more information is needed before the processes that control the disappearance of chlorophyll from the living plant can be explained.

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