Phytochemistry, Vol. 22, No. 11, pp. 2443–2446, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00

THE APPEARANCE OF CHLOROPHYLL DERIVATIVES IN SENESCING TISSUE

MARTIN J. MAUNDERS†, STANLEY B. BROWN and HAROLD W. WOOLHOUSE*†

Department of Biochemistry, University of Leeds, Leeds, LS2 9JT, U.K.; *Department of Plant Sciences, University of Leeds, Leeds, LS2 9JT, U.K.

(*Revised received* 10 *January* 1983)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; *Hordeum vulgare*; Gramineae; senescence; chlorophyll degradation; chlorophyll metabolism.

Abstract—Chlorophylls *a*-1 and *b*′, which are breakdown products of chlorophylls *a* and *b* respectively, were found in senescing leaves of *Phaseolus vulgaris and Hordeum vulgare* following excision from the plant. Chlorophyll *a*-1 was not detected in healthy plants, in senescing attached leaves or in chlorophyll–proteins isolated from senescent tissue. Chlorophyll *a*-1 formation in excised leaves increased with time for up to 10 days as chlorophyll *a* levels fell.

INTRODUCTION

Senescence in plants is normally characterized by a decrease in pigments, total protein and RNA, accompanying loss of structure in the chloroplasts and cytoplasm. The structural changes may result in the pigments becoming accessible to cellular acids and degradative enzymes. Loss of chlorophyll reaches completion within a few weeks but major breakdown products or intermediates have not been observed and the mechanism of degradation is unknown. This is not the case for the other major class of natural macrocyclic tetrapyrroles, the haems, the degradation of which proceeds via an initial hydroxylation reaction to linear tetrapyrroles [1].

The loss of chlorophyll from senescent tissue may be directly linked to decay of its ultrastructural environment, comparable to the loss of haemoglobin following removal of the red blood cell from the circulation. Light and oxygen are normally available during chlorophyll loss, though it may also occur in darkness following excision of leaves, a common method of inducing senescence. Loss of chlorophyll from a tissue is often taken as a reference parameter for following the progress of the senescence process, although in some instances senescence can proceed without pigment loss, for example in plants with mutant membrane structure [2]. Hence, chlorophyll loss may not be inseparably linked to the overall senescence process.

Products of chlorophyll breakdown are poorly characterized. Chlorophyll allomers accumulate in tissues following non-physiological treatments, such as boiling or impregnation with organic solvents [3], and oxidative enzymes can cause the formation of hydroxychlorophylls [4]. Such studies, however, are complicated by the formation of new compounds during the isolation procedures and few degradation products have been shown conclusively to occur in leaves prior to extraction. In the present work, we describe the isolation of pigments from senescent leaves which appear to be direct breakdown products of the chlorophylls.

RESULTS AND DISCUSSION

TLC of the pigments from bean and barley sampled after excision showed the presence of a novel band with mobility slightly greater than that of chlorophyll *b*. The pigment was easily observable and could be resolved from chlorophyll *b* using increasing percentages of propan-1-ol in petrol as developing solvent, though at 2% v/v and above, the bands lost definition. The visible spectrum of this novel pigment appeared to be almost identical to that of chlorophyll *a* (Table 1) and showed a similar red fluorescence under UV light. It was designated pigment X.

Addition of a few drops of acid to an ether solution of pigment X produced a phaeophytin with a spectrum almost identical to that of phaeophytin *a* (Table 1). Chlorophyllins (Fig. 1) were formed by the addition of five drops of 30% potassium hydroxide in methanol to 1.5 ml of a diethyl ether solution of pigment X or chlorophyll *a*. The 'chlorophyllin X' so formed exhibited a spectrum similar to, but not identical to, that of chlorophyllin *a* (Table 1; Fig. 2). A drop of concentrated hydrochloric acid converted 'chlorophyllin X' to a magnesium-free chlorin, with a significantly different spectrum to that of chlorin e_{6a} (Fig. 2). The Molisch phase test (one drop of 30% potassium hydroxide in methanol to an ether solution) proved negative for pigment X, i.e. no brown interface was visible as is observed with chlorophylls *a* and *b*. The negative phase test indicates the absence of a free hydrogen at C-10. These results are consistent with the properties of chlorophyll *a*-1 [5] (see Fig. 1) formed by hydroxylation of chlorophyll *a* at C-10. Literature spectral properties of chlorophyll *a*-1 are shown in Table 1. This compound has been identified on boiling of leaves or by treatment with organic solvent [3], and following allomerization of chlorophyll *a* [6]. Its visible spectrum is almost identical to that of chlorophyll *a* [6] and the spectrum of its phaeophytin would be expected to be similar to that of phaeophytin *a*. However, the chloro-

† Present address: John Innes Institute, Colney Lane, Norwich, NR4 7UH, U.K.

Table 1. Spectral data of chlorophylls isolated from senescent leaves and various derivatives

Pigment	λ_{max} (nm)	$\varepsilon_{Soret}/\varepsilon_{Red}$	Reference
Chlorophyll *a*	429,662*	1.3	[5]
Chlorophyll *a*-1	428,661*	1.3	[5]
Chlorophyll *a*	430,660*	1.31	This work
Pigment X	430,660*	1.30	This work
Phaeophytin *a*	408,508,528,608,667†	—	This work
'Phaeophytin X'	408,508,528,607,666†	—	This work
Chlorophyllin *a*	418,655‡	—	[9]
Chlorophyllin *a*	418,650§	—	This work
'Chlorophyllin X'	419,657§	—	This work
Chlorin e_{6a}	*ca* 400,660‡	—	[9]
Chlorin e_{6a}	407,656§	—	This work
'Chlorin X'	404,664§	—	This work
Chlorophyll *b*	452.5,642.5*	2.88	[8]
Chlorophyll *b'*	452.5,642*	2.86	[8]
Chlorophyll *b*	455,644*	2.92	This work
Pigment Y	455,644.5*	2.84	This work

*In diethyl ether.
†In acetone.
‡In methanol.
§In methanol–diethyl ether (1:6 v/v).

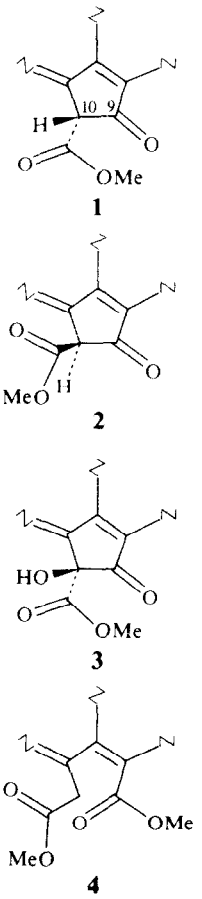

Fig. 1. Partial structures of chlorophyll derivatives. The region of the isocyclic ring only is shown. (**1**) Chlorophylls *a* and *b*; (**2**) chlorophylls *a'* and *b'*; (**3**) chlorophyll *a*-1; (**4**) chlorophyllin *a* (methyl ester form).

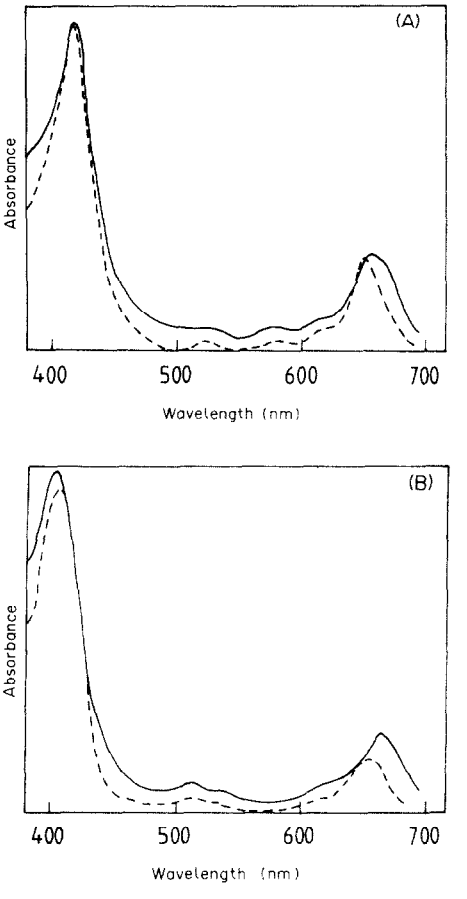

Fig. 2. Absorption spectra of chlorophyll derivatives. (**A**) Chlorophyllin *a* (---) and chlorophyllin X (~) in methanol–diethyl ether; (**B**) chlorin e_{6a} (---) and chlorin X (~) in methanol–diethyl ether.

phyllin and magnesium-free chlorin might be expected to differ from those derived from chlorophyll *a* in spectral properties since C-10 hydroxylation affects the products of cleavage of the isocyclic ring. Chlorophyll *a*-1 would be expected to be more polar and, hence, less mobile on TLC than chlorophyll *a*, in accordance with the observed properties of pigment X.

Agitation with an equal volume of aqueous 0.02 M potassium hydroxide was not adequate to extract pigment X from ether. Phytol was prepared from chlorophylls *a* and *b* by the method of Bacon and Holden [7]. A solution of each pigment in 7 ml 2.5% potassium hydroxide in methanol was incubated at 70° for 30 min. After cooling in ice 1.5 ml petrol was added, and the phases separated by addition of 10 ml water. The aqueous phase was re-extracted with a further 0.7 ml petrol and the combined extracts were spotted onto a silica gel GF TLC plate. After development with benzene–ethyl acetate (19:1, v/v) as solvent system the extracted lipids were visualized under short wavelength UV light, or by spraying with 0.25% aqueous potassium permanganate. A single compound, with an R_f value of 0.36 was isolated from both chlorophylls *a* and *b*, and this was assumed to be phytol. Similar treatment of pigment X released a single compound which behaved identically on TLC. These properties show that, apart from the probable C-10 hydroxylation, pigment X exhibits no other major structural differences from chlorophyll *a*. On the basis of these points we have, therefore, identified pigment X as chlorophyll *a*-1 (10-hydroxychlorophyll *a*).

Chlorophyll *a*-1 was not detected in young (10-day-old) leaves, nor in those leaves senescing while attached to the plant. This was true up to at least 30 days after the onset of pigment loss and in several different plant species (*P. vulgaris*, *H. vulgare*, *Cucumis sativa*, *Sinapis alba* and *Acer pseudoplatanus*). The pigment was also absent from freshly prepared chlorophyll–proteins from healthy or senescent leaves, though after 3 days storage at 4°, acetone extracts of both preparations contained traces of this compound.

Repeated extraction procedures and TLC development showed that chlorophyll *a*-1 was not an artifact produced by chlorophyll *a* breakdown during isolation but was present in the tissue prior to extraction. No chlorophyll *a*-1 could be detected before the leaves were excised. The progressive appearance of chlorophyll *a*-1 is shown in Fig. 3. It was first observed 6 days after excision though it may have been present earlier as total chlorophyll decline was already under way. Very low concentrations of chlorophyll *a*-1 would have been masked on TLC by chlorophyll *b*. The levels of chlorophyll *a*-1 rose for 4 days before declining (Fig. 3). Although the total level of 'chlorophyll *a* type' pigments decreased steadily, the percentage contributed by chlorophyll *a*-1 rose for at least 14 days (when it accounted for 9% of these compounds), after which the absolute level was too low to permit accurate calculation. The observation that the absolute level of chlorophyll *a*-1 rises, passes through a maximum and then decreases, shows that it is not a stable degradation product but is an intermediate which is itself degraded. However, the progressive rise in the proportion of chlorophyll *a*-1, shown in Fig. 3, indicates a slower rate of degradation than that of chlorophyll *a*.

When isolated from tissue labelled with [^{14}C]ALA (5-amino laevulinic acid), a specific precursor of tetrapyrroles, chlorophyll *a*-1 exhibited a specific radioactivity

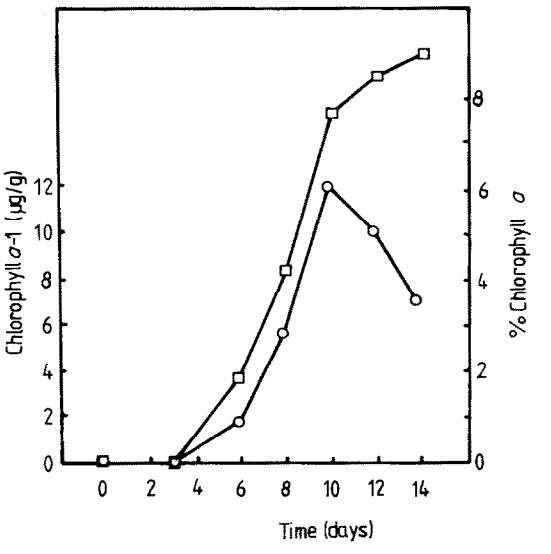

Fig. 3. The appearance of chlorophyll *a*-1 in senescing leaf tissue of *Phaseolus vulgaris*. Chlorophyll *a*-1 levels following excision (at day 0) are expressed in g/g leaf tissue (o) and as a percentage of the total 'chlorophyll *a*-type pigments' (□). Chlorophyll *a* declined from 156 μg/g tissue at day 0 to 80.2 μg/g tissue at day 14.

of 8.5 μCi/mmol, comparable to 8.7 μCi/mmol, for chlorophyll *a*, whilst that of chlorophyll *b* was 6.6 μCi/mmol. This indicates that chlorophyll *a*-1 is derived directly from chlorophyll *a*.

Another pigment was detected in bean leaves, in very small amounts, 10 days after excision. It was not detectable in barley, possibly because the yield of pigments was much smaller. This compound (pigment Y) was slightly more mobile on TLC than chlorophyll *b*, but of a similar olive-green colour. It possessed a spectrum very similar to that of chlorophyll *b* (Table 1) and showed a positive reaction to the Molisch phase test. It was provisionally identified as chlorophyll *b*′, the C-10 stereoisomer of chlorophyll *b* (see Fig. 1). No chlorophyll *b*-1 (analogous to chlorophyll *a*-1) was ever detected, illustrating the different susceptibilities of chlorophylls *a* and *b* to hydroxylation.

Our results show that chlorophyll *a*-1 appears to be a product of chlorophyll *a* breakdown in excised leaves, although it could not be detected as an intermediate of chlorophyll turnover in healthy leaves, or in attached leaves during natural senescence. That the pigment was not found in fresh chlorophyll–proteins from senescent leaves until they had been stored for some time suggests that degradation of chlorophyll follows or accompanies, but does not precede, dissociation from proteins, indicating a protective role for the latter. Chlorophyll *a*-1 has previously been detected in excised leaves following various treatments, such as boiling or organic solvent infusion [3, 7], which also suggests that the disruption of proteins or membranes promotes its formation. Oxidized chlorophylls previously detected in leaves [4] may include chlorophyll *a*-1.

The question arises as to whether the formation of chlorophyll *a*-1 in excised leaves represents the initial step of the pathway by which the bulk of the chlorophyll *a* is

degraded, or whether it represents a side reaction which accounts for only a small fraction of the chlorophyll *a*. Although it is not possible to fully answer this question from the present data, the indications are that the process accounts for a large proportion, if not all, of the chlorophyll *a*. This is evident from the fact that chlorophyll *a*-1 itself is degraded relatively rapidly (Fig. 3) yet, nevertheless, accounts for a significant proportion of the total chlorophyll *a* pigments after 8 days. It would not be surprising if hydroxylation was the first step in a chlorophyll breakdown pathway, since similar reactions initiate the catabolism of many cyclic molecules, including haem [1].

A further question concerns the lack of observation, in the present work, of chlorophyll *a*-1 in attached senescent leaves exposed to natural light. Two explanations for this are suggested. First, it is possible that natural senescence of chlorophyll in the attached leaf occurs by a mechanism not involving chlorophyll *a*-1. Secondly, degradation, whether light-induced or not, may proceed via hydroxylation to chlorophyll *a*-1, but further catabolism in the attached leaf might be so fast that chlorophyll *a*-1 is not detectable. Such further degradation may involve enzymes or may be photo-degradation, since chlorophyll *a*-1 itself is probably light-labile.

EXPERIMENTAL

Plant growth and senescence. Beans (*Phaseolus vulgaris* L. cv 'The Prince') were germinated in compost in darkness for 5 days at 25° prior to transfer to a 16 hr day regime for a further 21 days. The primary leaves were then excised and held in darkness at 25°, floating on H_2O. For radiolabelling expts, the beans were germinated and etiolated for 12 days before transfer to tubes of hydroponic nutrient soln [10] for a further 12 days. Each tube carried two plants and contained 2 μCi [^{14}C]ALA in 20 ml soln. Barley (*Hordeum vulgare* L. cv 'Ark Royal') was dehusked and germinated in darkness for 5 days before transfer to the 16 hr day regime. After a further 9 days the flag leaves were excised, cut into 1 cm lengths and floated on H_2O in darkness.

Tissue sampling. At intervals, triplicate samples of tissue were taken, the pigments extracted [11] and purified by TLC on cellulose using a petrol–Me_2CO–propan-1-ol (90:10:0.45 v/v) solvent system [12]. Further resolution was achieved by a second TLC using 1.5% v/v propan-1-ol in petrol as the solvent. Loss of pigment during this second development was corrected for by running a known amount of chlorophyll *a* on each plate. Pigment concns were determined spectrophotometrically [13, 14]. Radiolabelling of the pigments was measured by liquid scintillation counting to an efficiency of 90–95%. Presence of phytol in the compounds was determined by the method of ref. [7]. Chlorophyll–protein complexes were prepared from the tissues by the method of ref. [15].

Acknowledgement—We thank the Science Research Council for the award of a studentship (to M.J.M).

REFERENCES

1. Brown, S. B. and Troxler, R. F. (1983) in *Bilirubin Metabolism* (Heirwegh, K. P. M. and Brown, S. B., eds.) Vol. II, pp. 1–39. C. R. C. Press, Boca Raton.
2. Thomas, A. and Stoddart, J. L. (1975) *Plant Physiol.* **56**, 438.
3. Holden, M. (1970) *Phytochemistry* **9**, 1771.
4. Pennington, F. C., Strain, H. H., Svec, W. A. and Katz, J. J. (1964) *J. Am. Chem. Soc.* **86**, 1418.
5. Bacon, M. F. (1966) *Biochem. J.* **101**, 34c.
6. Pennington, F. C., Strain, H. H., Svec, W. A. and Katz, J. J. (1967) *J. Am. Chem. Soc.* **89**, 3875.
7. Bacon, M. F. and Holden, M. (1967) *Phytochemistry* **6**, 193.
8. Hynninen, P. H. (1973) *Acta Chem. Scand.* **27**, 1487.
9. Oster, G., Broyde, S. B. and Bellin, J. S. (1964 *J. Am. Chem. Soc.* **86**, 1309.
10. Hoagland, D. R. and Arnon, D. I. (1938) *Calif. Agric. Exp. Stn. Circ.* **347**, 1.
11. Strain, H. H. and Svec, W. A. (1966) in *The Chlorophylls* (Vernon, L. P. and Seeley, G. R., eds.) p. 22. Academic Press, New York.
12. Bacon, M. F. (1965) *J. Chromatog.* **17**, 322.
13. Comar, C. L. and Zscheile, F. P. (1942) *Plant Physiol.* **17**, 198.
14. Smith, J. H. C. and Benitez, A. (1955) *in Modern Methods of Plant Analysis* (Paech, K. and Tracey, M. V., eds.) Vol. IV, p. 142. Springer, Berlin.
15. Markwell, J. P., Reinman, S. and Thornber, J. P. (1978) *Arch. Biochem. Biophys.* **190**, 136.