Constituent Processes of Leaf Senescence in *Hordeum vulgare* cv. Dyan

THESIS

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IN SEARCH OF BEAUTY

"Beauty is the splendour of truth" wrote the English economist Schumacher. All of us are searching for truth, consciously and unconsciously, in our lives, in our associates, and in the world around us. What is plant physiological research but a search for truth in the living plant? The poet and artist are free to express their quest for beauty through a variety of media. Should not the research scientist also feel the joy and splendour of truth in a biochemical reaction, in a physiological process, or in the intricate design of a membrane that can be unraveled, comprehended, or probed? Surely one of the most satisfying personal rewards that I have experienced as a research scientist has been to identify a physiological problem, to formulate a specific set of questions to be answered, to anticipate the possible physiological reactions involved, to design a series of experiments to answer the questions posed, and to see the data fall into place as the experiments are carried out, this to me, is the "splendour of truth - beauty".

Ralph C Stocking

University of California (Davis)

This thesis is dedicated to

my mother, Julia Afitlhile

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ABBREVIATIONS

cv.	cultivar
d	day
GC-ECD	gas chromatography-electron capture detector
gFW	gram fresh weight
HPLC	high-performance liquid chromatography
i.d.	internal diameter
M	molarity
mCi	millicurie
min	minute
mRNA	messenger-RNA
N	normality
Pfr	phytochrome far-red absorbing form
RP 14	rust-coloured pigment with a retention time of 14 minutes
TBq	terabecquerel
tRNA	transfer-RNA
UVP	ultra violet programme
v/v	volume per volume
w/v	weight per volume

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ABSTRACT

Changes in chlorophyll content, carotenoid content and composition, abscisic acid and phaseic acid levels, hydrolytic enzyme activity and polypeptide pattern were monitored during senescence of the primary attached leaves of *Hordeum vulgare* L. cv. Dyan. Senescence occurred due to the normal course of leaf development or was induced by incubation of leaves in darkness. Loss of chlorophyll and total leaf protein was retarded in light whereas it continued rapidly in leaves from dark-incubated seedlings. Chlorophyll *a/b* ratio increased with the progression of senescence, suggesting that chlorophyll *b* was preferentially degraded during this process. Loss of total protein coincided with enhanced activity of acid and neutral proteases. In contrast, loss of chlorophyll was not accompanied by an increase in peroxidase activity, suggesting that this enzyme was not responsible for initiating chlorophyll breakdown.

Carotenoid and abscisic acid levels were monitored in the same tissue extracts. The results obtained show that the increase in endogenous levels of abscisic acid, induced by senescence, correlated with enhanced epoxidation of the xanthophyll cycle, ie., increased conversion of zeaxanthin to antheraxanthin and all-*trans*-violaxanthin. In addition, an increase in abscisic acid levels occurred concomitant with a decrease in all-*trans*-violaxanthin and 9'-*cis*-neoxanthin, suggesting an apparent 1:1 relationship on a molar basis. It is therefore proposed that enhanced abscisic acid production, due to foliar senescence, arises from fluctuations in carotenoid turnover.

Polypeptide patterns in isolated chloroplasts, purified thylakoid and stromal fractions were very similar for leaves incubated in either light or darkness. A decrease in intensity of bands was observed in isolated chloroplasts and stromal fractions. Intensity of bands in thylakoids remained unchanged with the progression of senescence. Protein standards of peroxidase and lipoxygenase co-migrated with proteins of the isolated chloroplast. Although tentative, some proteins of the chloroplast may be representative of precursors of hydrolytic enzymes which are known to increase during senescence.

CHAPTER ONE

1. GENERAL INTRODUCTION

Senescence is an integral part of plant development, and like any developmental process, it is subjected to direct genetic control (Thomas and Stoddart, 1980; Kar and Feierabend, 1984; Weidhase *et al.*, 1987b). Operation of the senescence programme is, however, delayed until the appropriate time in plant development. The development of leaf cells is an expression of the interaction between the genome of the nucleus and that of the chloroplast (Thomas and Stoddart, 1980). Chloroplast DNA codes for chloroplast components including ribosomal RNA (Kung, 1977), the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and certain structural proteins of chloroplast membranes (Joy and Ellis, 1975). The structural proteins of the chloroplast comprise photosystems I and II (PSI and PSII),cytochrome b_6-f, ferredoxin-NADP reductase and proton ATPase (Callahan *et al.*, 1989). These proteins together with plastoquinone and plastocyanin operate in series to harvest light energy, catalyze the primary charge separation and produce ATP and NADPH (Staehelin, 1986).

Biochemical and immunological studies have indicated that the abovementioned complexes are distinctly segregated on thylakoids. Granal lamellae are enriched in PSII proteins, while PSI and the proton ATPase are mainly located in stromal lamellae. PSI and PSII are involved in photosynthetic electron transport (Andréasson and Vänngard, 1988; Gamble and Mullet, 1989). The PSII complex which consists of a reaction centre, a water-splitting apparatus and a light-harvesting antenna complex, functions as a water:plastoquinone oxidoreductase. The PSII reaction centre comprise a 32 kDa protein, D2 and cytochrome b_{559} (Nanba and Satoh, 1987). The 32 kDa protein is apparently synthesized as a 33.5 to 34.5 kDa protein and is posttranslationally processed to the mature 32 kDa form exclusively on the stromal lamellae (Matto *et al.*, 1989). Following processing, the mature protein translocates to the grana (Mattoo and Edelman, 1987). The PSI reaction centre is a light-driven plastocyanin:ferredoxin oxidoreductase (Golbeck, 1992; Ikeuchi, 1992). The photoactive components include a primary electron donor (P_{700}), a primary electron acceptor (A_o), an

intermediate quinone acceptor (A_1) and three iron-sulphur centres F_x, F_A and F_B (Andréasson and Vänngard, 1988; Golbeck, 1992). It follows therefore that photosynthetic membranes of the chloroplast possess a highly complex protein fraction. The question is whether this photosynthetic apparatus is sustained during foliar senescence.

The available information indicates that photosynthetic capability and net production of carbohydrates declines during foliar senescence (Kamachi *et al.*, 1991). Photosynthetic electron transport capability has also been reported to decline in senescing leaves (Holloway *et al.*, 1983). The decrease in non-cyclic electron transport is greater than the decline in activities of PSI or PSII, which suggests that intersystem electron transport becomes rate-limiting. The latter has been attributed to selective depletion of cytochromes f and b_6 complexes during senescence (Ben-David *et al.*, 1983). A study of the thylakoid proteins during leaf senescence in *Phaseolus vulgaris* (Roberts *et al.*, 1987) revealed that synthesis of a 32 kDa protein continued throughout the senescence process. By contrast, synthesis of the α and β subunits of ATPase, PSI reaction centre polypeptides, cytochromes f and b_6 and the structural apoprotein of the light-harvesting chlorophyll protein complex (LHCP) were reported to decline during senescence. An apparent lack of change in the turnover rate of a 32 kDa protein in senescing leaves was interpreted by Roberts *et al.* (1987), as suggesting that the degradation of this protein may not be mediated by electron transport.

Rubisco, the chloroplastic protein responsible for $C0_2$ fixation in photosynthetic organisms, is synthesized predominantly during leaf expansion (Friedrich and Huffaker, 1980). Levels of this enzyme have been observed to decline with the onset of senescence (Peterson and Huffaker, 1974, 1975; Friedrick and Huffaker, 1980; Wardley *et al.*, 1984). Therefore, the above information suggests that the photosynthetic apparatus is impaired in its functioning or it is degraded during the senescence process. However, the initial reactions which ultimately lead to the senescence syndrome are poorly understood. Studies with inhibitors of protein synthesis suggest that leaf senescence requires the synthesis of specific proteins in the cytoplasm (Thomas and Stoddart,

1980; Garcia *et al.*, 1983) and in the chloroplast (Cuello *et al.*, 1984; Martin *et al.*, 1986), and that synthesis or expression of these proteins could be mediated by changes in levels of plant growth regulators.

1.1 Plant Growth Regulators

Senescence of a whole plant is often under correlative control (Noodén, 1988a). Various parts of a plant influence each other in ways that coordinate developmental processes. As mediators of these controls, plant growth regulators play a central role in regulating senescence (Goldthwaite, 1987; Noodén, 1988a).

The commonly recognized classes of plant growth regulators are auxins (IAA), gibberellins (GAs), cytokinins (CKs), ethylene (CH_2CH_2) and abscisic acid (ABA). Cyclopentanone compounds such as (-)-jasmonic acid (JA) and its methylester (JA-Me) are considered putative plant growth regulators (Weidhase *et al.*, 1987a; Parthier, 1990). The exact role of each plant growth regulator and its possible interaction with others in the control of senescence is still unclear (Sabater *et al.*, 1981; Noodén, 1988a).

The available information on auxins in relation to senescence is very limited. Nonetheless, auxins usually retard senescence in many tissues (Noodén, 1988b). Auxins can alter senescence-related processes such as chlorophyll loss, RNA synthesis and degradation, protein synthesis and degradation, wilting, membrane breakdown and changes in the level of hydrolytic enzymes in ways that are consistent with a senescence-delaying effect (Noodén, 1988b). The level of endogenous auxins is known to decrease during senescence. The available information on the interaction between auxin and other plant growth regulators is limited. Evidence suggests that auxin acts together with cytokinin to retard senescence (Goldthwaite, 1987; Noodén, 1988b). Little information is available on the interaction of auxin and gibberellins in senescing systems. Nonetheless, gibberellins can retard chlorophyll loss and inhibit RNA and protein breakdown, processes associated with senescence (Noodén, 1988b). The endogenous level of gibberellins, like that of auxins, has been observed to decline prior to or during senescence. Exogenous gibberellins have been reported to be most effective at stages when the endogenous level is low (Noodén, 1988b), suggesting that a decline in gibberellin levels plays a role in senescence.

There is substantial evidence to suggest that roots are the major sites of cytokinin synthesis (Goldthwaite, 1987; Van Staden *et al.*, 1988; Moore, 1989). Cytokinins are apparently transported in the xylem to the leaves (Goldthwaite, 1987). Therefore, levels of cytokinin in leaves may be determined to a great extent by the rate of production in the roots. Thus, a decline of endogenous cytokinin in leaves may be due to metabolism, a decrease in biosynthesis or to diminished transport (Van Staden *et al.*, 1988). Cytokinins play an important role in controlling many of the processes that contribute to plant senescence (Noodén, 1980; Thimann, 1980). In particular, cytokinins have been implicated in the maintenance of chlorophyll, protein and RNA levels, all of which decline during senescence (Van Staden *et al.*, 1988). Cytokinins apparently retard senescence by stimulating the synthesis of secondary metabolites, namely, polyamines such as spermine and spermidine (Pell and Dann, 1991). These compounds play a role in scavenging free radicals, stabilizing membranes, tRNA and mRNA (Drolet *et al.*, 1986; Galston and K-Sawhney, 1987). Thus, cytokinin and polyamine play a role in regulating the process of senescence. Therefore, cytokinins may be regarded as antisenescence plant growth regulators. A better understanding of the mechanism by which cytokinins act, particularly in tissues where they retard senescence, should help to elucidate their role in senescence. One important aspect could be the characterization of cytokinin protein receptors and the localization of the receptors which mediate cytokinin action. This would reveal which tissues are the targets of cytokinin action.

Applied ethylene has been observed to promote the loss of protein, starch and chlorophyll, and this was associated with increased activities of ATPase, acid phosphatase, α-amylase, catalase and peroxidase (Mattoo and Aharoni; 1988). An increase in the endogenous levels of ethylene has been observed to coincide with the onset of senescence (Beyer and Morgan, 1971; Paulin *et al.*, 1986; Sylvestre *et al.*, 1989). The exact mechanisms by which ethylene brings about physiological responses and the subcellular site(s) where it acts remains to be elucidated. The results of Morgan *et al.* (1990) indicated that tissue sensitivity to ethylene plays a major role in inducing senescence. Senescence in plant tissues is accompanied by changes in membrane permeability (Droillard *et al.*, 1987) and such changes have been linked to increased ethylene production (Irigoyen *et al.*, 1992). The latter has been associated with activation of lipoxygenase, which increases lipid peroxidation and

peroxide content (Kacperska and Kubacka-Zelbaska, 1985). Therefore, an increase in the activity of catalase and peroxidase during senescence may be an enzymatic scavenging systems to prevent the damaging effects of free radicals (Irigoyen *et al.*, 1992).

The role of ethylene in senescence may also involve interactions with other plant growth regulators (Mattoo and Aharoni, 1988). As previously mentioned, endogenous level of auxin, gibberellin and cytokinin declines with the onset of senescence. It seems likely, therefore, that the onset of foliar senescence may be triggered by a decline in the level of these plant growth regulators. Such an event, in turn, probably releases control on the production of ethylene and ABA, and/or influences the ability of these two plant growth regulators to induce senescence.

Synthesis of ethylene and polyamines depends on the same precursor, S-adenosylmethionine (SAM). Polyamines apparently inhibit the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by reducing synthesis of ACC synthase and scavenging oxygen free radicals involved in the conversion of ACC to ethylene (Pell and Dann, 1991). In senescing tissues, polyamine synthesis is highly reduced, thus making more SAM available as a precursor for ACC (Galston and K-Sawhney, 1987). ACC is consequently converted to ethylene.

A group of naturally occurring compounds, cyclopentanones such as (-)-jasmonic acid (JA) and its methylester (JA-Me) have been suggested to represent putative plant growth regulators (Ueda and Kato, 1980, 1982; Meyer *et al.*, 1984; Weidhase *et al.*, 1987a; Farmer and Ryan, 1990; Parthier, 1990). Jasmonates are synthesized from polyunsaturated fatty acids such as linolenic acid (Vick and Zimmermann, 1986), probably as a result of membrane lipid peroxidation (Leshem, 1987). The physiological role and mode of action of jasmonates in foliar senescence are not well understood (Partheir, 1990). Furthermore, the primary site(s) of jasmonate action are unknown. However, jasmonates can apparently promote leaf senescence (Ueda and Kato, 1980, 1981). Jasmonates can also cause chlorophyll loss (Ueda and Kato 1980, 1981, 1982; Weidhase *et al.*, 1987b), stimulate ethylene biosynthesis (Saniewski *et al.*, 1987a, b), enhance peroxidase activities (Weidhase *et al.*,

1987b), reduce photosynthetic capacity (Popova *et al.*, 1988), and promote a rapid decline in levels of Rubisco protein (Popova and Vaklinova, 1988; Weidhase *et al.*, 1987a, b). Senescence symptoms caused by jasmonates are apparently restored by cytokinins (Ueda and Kato, 1982; Weidhase *et al.*, 1987b). These phenomena are identical with the symptoms of senescence. Therefore, the physiological function and mode of action of the endogenous jasmonates in plant senescence deserves attention especially as applied jasmonates and ABA induced the same proteins in leaf segments of *Hordeum vulgare* (Weidhase *et al.*, 1987a).

ABA accelerates senescence more in the dark than in the light (Thomas and Stoddart, 1975; Gepstein and Thimann, 1980; Garaizabal and Rodriguez, 1983). Gepstein and Thimann (1980) suggested that this may be due to light-activated breakdown of the ABA or decreased sensitivity of tissues in light. Thomas and Stoddart (1975) demonstrated that ABA promotes net protein breakdown in *Festuca pratensis* by enhancing the synthesis and/or activity of enzymes related to protein catabolism. Furthermore, ABA is known to inhibit the synthesis of nucleic acids and total protein, suggesting that ABA acts at the transcriptional and translational level. Studies have also indicated that ABA may alter membrane structure (Walton, 1980; Ho, 1983) and cause a decline in all cellular acyl lipids (Noodén, 1988b). According to Noodén (1988b), alteration in membrane structure may be an important aspect of ABA action on senescence, because membrane deterioration and leakiness are characteristics of the senescence process.

Studies have shown that endogenous levels of ABA rise before or during senescence in both excised and attached leaves and decrease during late senescence (Noodén, 1988b). ABA is apparently able to influence the endogenous level of other plant growth regulators and *vice versa* (Even-Chen *et al.*, 1978). During the course of senescence in a variety of tissues, ethylene production has been observed to rise before endogenous ABA levels (Sexton *et al.*, 1985), suggesting elevated ethylene leads to increases in ABA, which in turn may cause senescence. A study of senescence in leaves of *Arabidopsis thaliana* showed that chlorophyll breakdown is largely due to the action of endogenous ethylene (Zacarias and Reid, 1990). The acceleration of senescence by ABA was independent of the action of ethylene, even though ABA stimulated ethylene production. Zacarias

and Reid (1990) interpreted this as indicating that no single mechanism controls the rate of foliar senescence in *Arabidopsis*. Ethylene and ABA probably triggered senescence at different points in the sequence of events leading to the symptoms of senescence.

While ABA and ethylene act in the same direction, cytokinins, gibberellins and auxin act in a direction opposite to ABA. As previously mentioned, ABA apparently functions by altering the level of other plant growth regulators or *vice versa* (Even-Chen and Itai, 1975; Even-Chen *et al.*, 1978; Gepstein and Thimann, 1980). Even-Chen and Itai (1975) provided evidence for a relationship between cytokinins and ABA on the induction of senescence. A high cytokinin level increases the levels of bound ABA at the expense of free ABA (Noodén, 1988b). It appears that cytokinin, and possibly auxin and gibberellin may retard senescence by counteracting increases in the levels of ABA. This may be achieved by increasing the catabolism and/or conjugation of ABA, or by inhibiting ABA synthesis.

Current information suggests that ABA is produced by either the direct pathway involving a C-15 precursor derived from farnesyl pyrophosphate (FPP), or the indirect pathway involving a precursor derived from a C-40 carotenoid (Creelman, 1989). There is now considerable evidence to support an indirect apo-carotenoid pathway for ABA biosynthesis (for details, see Section 3.1.3).

By comparison, the catabolism of ABA in higher plant tissues has been extensively investigated and the products unequivocally identified (Milborrow, 1970; Zeevaart and Milborrow, 1976; Tietz *et al.*, 1979; Cowan and Railton, 1987; 1990). It is now accepted that ABA is catabolized via the unstable intermediate 8′-hydroxy ABA to phaseic acid (PA) which is then reduced to dihydrophaseic acid (DPA) and its 4′-epimer, 4′-epi-DPA (Figure 1.1).

In addition, both ABA and its acidic catabolites can be conjugated to yield their corresponding glucosyl ether and ester derivatives (Walton, 1980; Weiler, 1980). These water soluble conjugates appear to be the end

products of ABA catabolism which are then stored in the vacuole of mesophyll cells (Bray and Zeevaart, 1985). Their physiological role is unknown but it is thought that they might represent storage forms of ABA. Vacuoles, the storage sites of conjugates, are known to disintegrate during senescence (Thimann, 1987). It is therefore possible that disintegration of vacuoles may result in the flow of conjugates into the cytosol, where conjugates may be hydrolyzed to release free ABA. Thus, it is possible that the increase in endogenous levels of ABA during senescence results from hydrolysis of ABA conjugates.

1.2 Plant Pigments

In higher plants, carotenoids like chlorophylls are synthesized via the isoprenoid pathway (Figure 1.2) and are located exclusively in plastid structures (Malhotra *et al.*, 1982; Britton, 1988). In fact, chlorophylls are complex partners of carotenoids (Frosch and Mohr, 1980; Britton, 1988). Together, these pigments are non-covalently bound to intrinsic proteins of the thylakoid membranes (Oelmüller and Mohr, 1985) to yield functional holocomplexes which comprise the antenna or photochemical reaction centres (Malhotra *et al.*, 1982). However, each chlorophyll-carotenoid-protein complex has its specific pigment composition (Braumann *et al.*, 1982).

The biosynthesis of chlorophyll was previously thought to occur exclusively in the light (Frosch and Mohr, 1980; Virgin, 1981). However, evidence now exists supporting the idea that chlorophyll synthesis does occur in seedlings which have initially been exposed to light and then transferred to darkness (Walmsley and Adamson, 1989, 1990). Although chlorophyll synthesis was found to continue in the dark, the rate of chlorophyll accumulation decreases with time, because the reduction of protochlorophyllide to chlorophyllide is a light dependent enzymatic reaction (Viro and Kloppstech, 1982; Rudiger and Schoch, 1988).

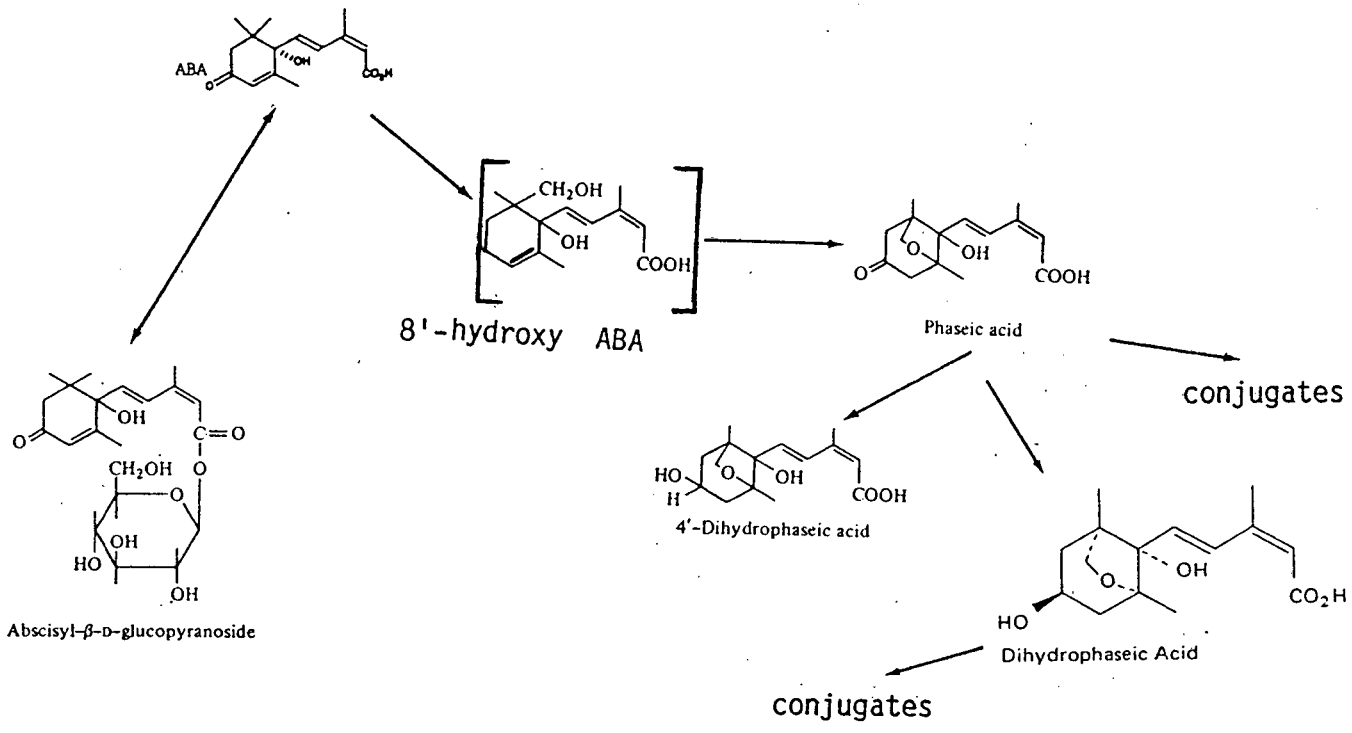

Figure 1.1 Characterized products of the abscisic acid catabolic pathway.

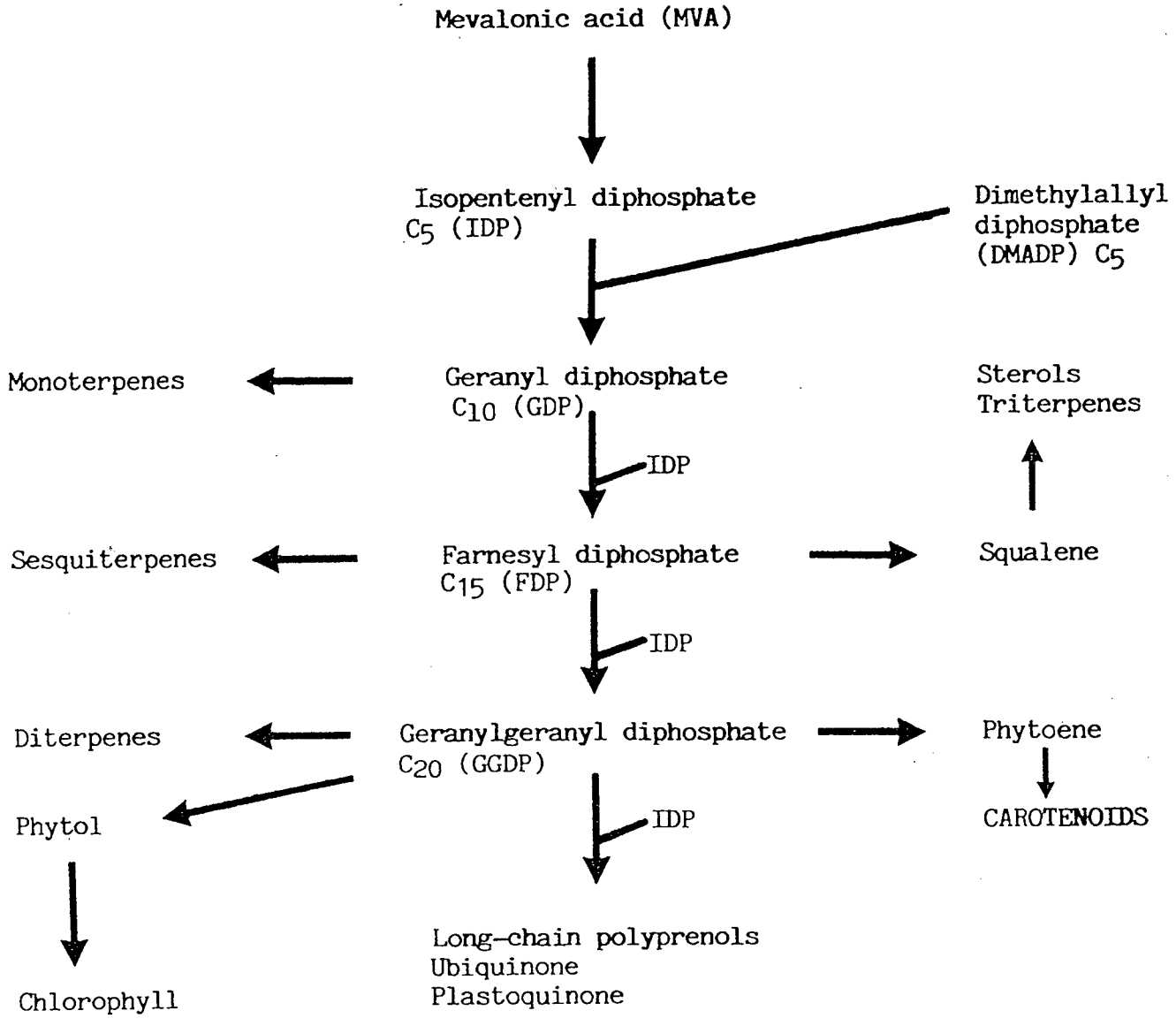

Figure 1.2 Schematic representation of the isoprenoid pathway.

Carotenoids are synthesized in both light and dark, even though the total amount is less in the dark (Grumbach, 1981). The accumulation of larger amounts of carotenoids occurs if chlorophyll is available (Frosch and Mohr, 1980; Malhotra *et al.*, 1982). This accumulation of carotenoids can be linked to the protection of chlorophyll against photooxidation (Cogdell, 1988; Rau, 1988; Ben-Amotz *et al.*, 1989). The accumulation of β-carotene is also associated with a decline in chlorophyll synthesis in both higher plants (Grumbach, 1984) and algae (Ben-Amotz *et al.*, 1989). Because senescence is characterized by the loss of chlorophyll (Maunders *et al.*, 1983; Bortlik *et al.*, 1990; Schellenberg *et al.*, 1990), accumulation of carotenoids during this phase is therefore probably not entirely dependent on the availability of chlorophyll.

Plant carotenoids can be classified into carotenes, namely, α- and β-carotene and xanthophylls, which are hydroxylated and epoxidated derivatives of α and β-carotene. α- and β-Carotene are synthesized via the isoprenoid pathway (Britton, 1988). As shown in Figure 1.3, synthesis of the carotenes from a C_{40} compound, phytoene, involves four desaturation and two cyclization steps. Xanthophylls (Figure 1.4) are oxidation products of α- and β-carotene (Lichtenthaler, 1987; Sandmann, 1991). Hydroxy groups are introduced in position C-3 and C-3′ of the ionone rings and epoxy groups are subsequently formed at the 5,6 and 5′,6′ position (Sandmann, 1991). According to this author, all the oxygen groups originate from molecular oxygen. Lutein, one of the major xanthophylls in plant chloroplasts, is derived from α-carotene by introduction of hydroxy functions. As shown in Figure 1.5, the xanthophylls violaxanthin, antheraxanthin, zeaxanthin and neoxanthin (not shown) are derived from β-carotene via β-cryptoxanthin by a combination of hydroxylation and epoxidation steps (Lichtenthaler, 1987; Sandmann, 1991). Carotenoids of the functional chloroplasts include β-carotene, lutein, violaxanthin and neoxanthin as major and regular components of the photochemically active thylakoids in higher plants and green algae (Lichtenthaler, 1987). Antheraxanthin and zeaxanthin, however, are regular but minor carotenoid components.

Zeaxanthin, antheraxanthin and violaxanthin are components of the xanthophyll cycle (Demmig-Adams, 1990; Thayer and Björkman, 1990). Under conditions of high light stress, violaxanthin is de-epoxidized via the

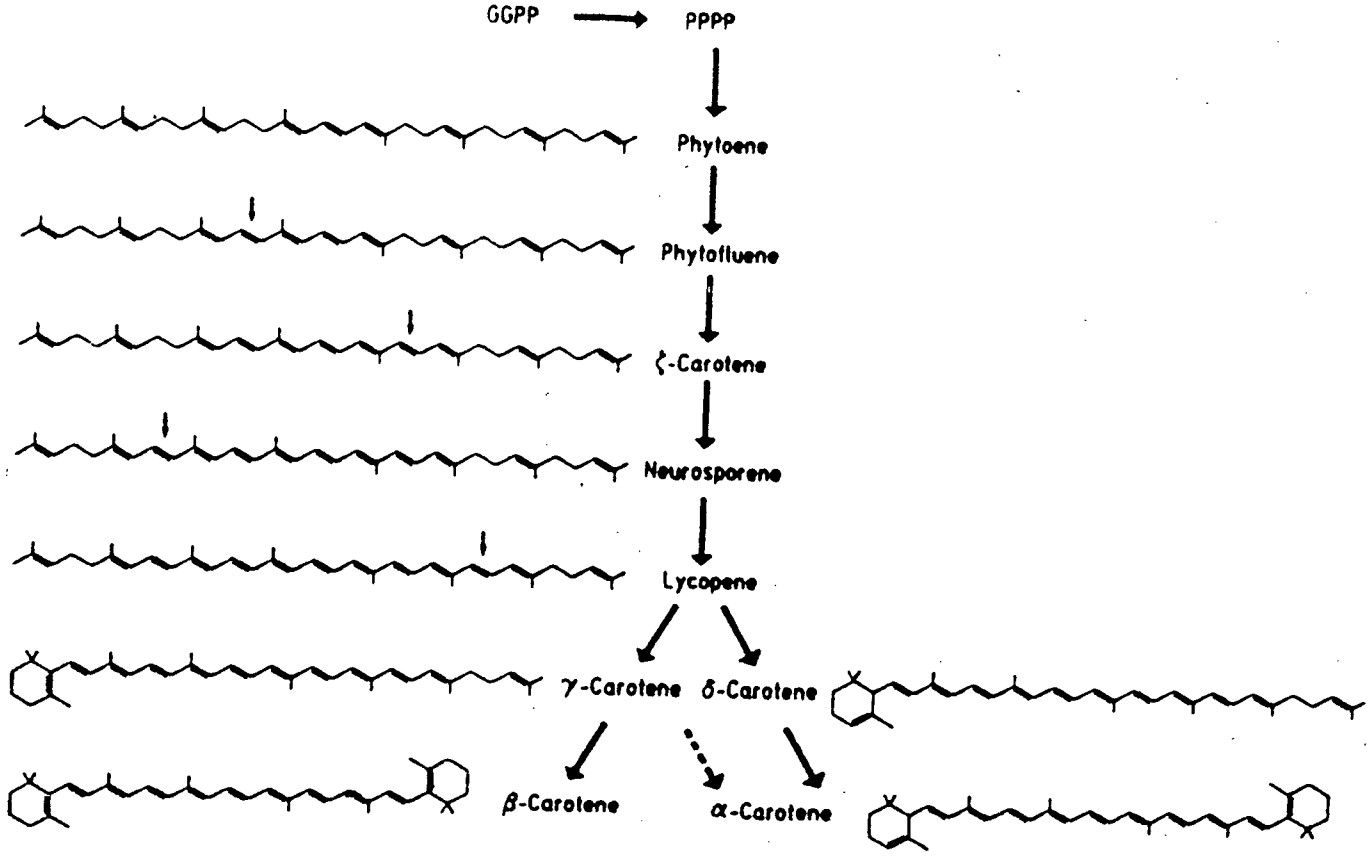

Figure 1.3 Biosynthetic pathway of the formation of α- and β-carotene from geranylgeranyl pyrophosphate. GGPP = Geranylgeranyl pyrophosphate, PPPP = prephytoene pyrophosphate . Small arrows mark the introduction of new double bonds.

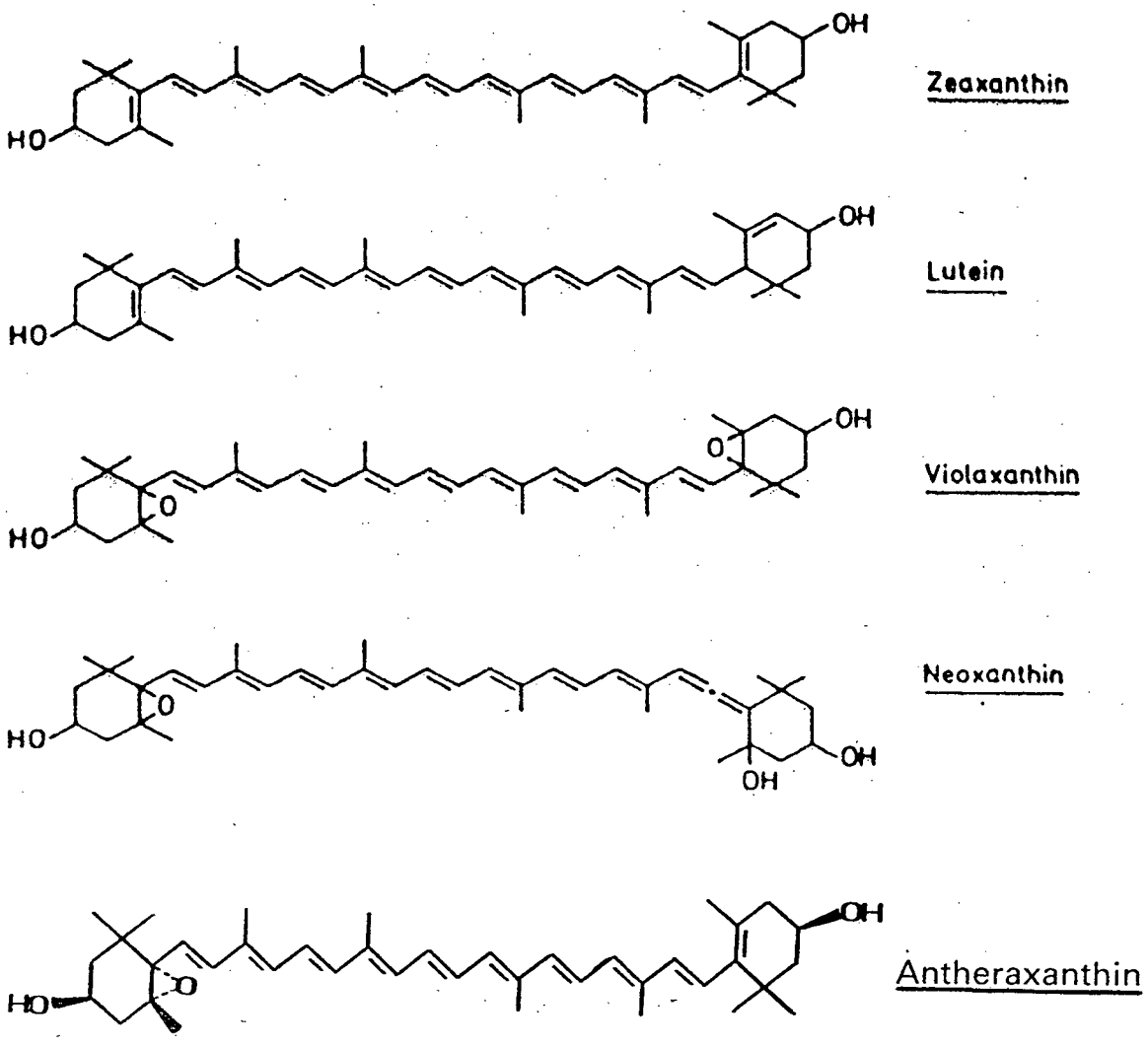

Figure 1.4 Structures of higher plant xanthophylls.

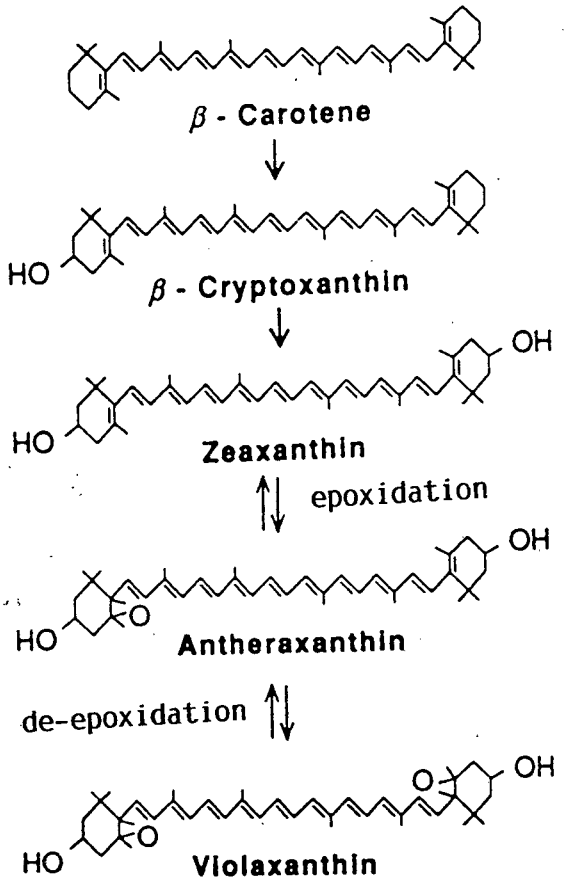

Figure 1.5 Possible biosynthetic pathway for the synthesis of zeaxanthin and related xanthophylls in higher plants.

intermediate antheraxanthin to zeaxanthin (Figure 1.5) (Bilger, *et al.*, 1989; Demmig-Adams, 1990; Morales *et al.*, 1990; Thayer and Björkman, 1990). This reaction is thought to occur in the lumen of thylakoids, catalyzed by violaxanthin de-epoxidase. The reverse reaction ie. from zeaxanthin to violaxanthin, is catalyzed by an epoxidase thought to be located in the stromal side. Zeaxanthin formation has been proposed to be the mechanism responsible for non-radiative energy dissipation (Demmig-Adams, 1990), thus protecting the photosynthetic reaction centres from photooxidative damage. Lutein and neoxanthin function as accessory pigments in photosynthetic light absorption (Lichtenthaler, 1987). Although β-carotene may serve as a light-absorbing pigment, its main function is the protection of chlorophyll from photooxidation in the reaction centre. Therefore, carotenoids have two major functions in photosynthesis. They act as photoprotective agents, preventing photooxidative damage and as accessory light-harvesting pigments (Cogdell, 1988; Rau, 1988).

1.3 Proteins and Enzymes

The protein population of plants is in a dynamic state of flux (Peoples and Dalling, 1988). The amount present in tissue is regulated by both synthesis and degradation (Huffaker and Peterson, 1974; Huffaker, 1990). Protein turnover, which is defined as the rate of synthesis or degradation, is able to adjust the level of key enzymes and other proteins within the cell in response to developmental changes such as senescence (Peoples and Dalling, 1988).

During the first stages of leaf senescence, protein synthesis remains active in both the cytoplasm (Thomas and Stoddart, 1980) and chloroplast (Garcia *et al.*, 1983; Martin *et al.*, 1986). Little is known about the genes expressed during senescence, although the effects of inhibitors of protein synthesis suggest that some chloroplast genes must be expressed in order for senescence to occur (Yu and Kao, 1981; Cuello *et al.*, 1984). Chloroplast gene expression is apparently controlled at the level of transcription (Lerbs *et al.*, 1984) and translation (Miller *et al.*, 1983; Monroy *et al.*, 1987). Nonetheless, the earliest changes associated with leaf senescence are known to occur in the chloroplast. Photosystem I (PSI), which is located mainly in the stromal thylakoids, has been reported to decline faster than PSII (Gepstein, 1988). The latter is localized mainly in the grana. According

to this author, the rapid decline in PSI could be explained by the fact that stromal thylakoids disintegrate faster than the granal thylakoids. In the thylakoids, formation of light-harvesting chlorophyll *a/b* proteins (LHCP) has also been reported to decline during senescence, whereas synthesis of the 32 kDa protein associated with PSII continued throughout the process (Roberts *et al.*, 1987).

Rubisco, a stromal enzyme, is a predominant protein lost during the initial stages of senescence (Kasemir *et al.*, 1988). The chloroplast contains a broad spectrum of hydrolytic enzymes including oxidative and peroxidative chlorophyll degrading enzymes (Martinoia *et al.*, 1982), galactolipase (Anderson *et al.*, 1974) and proteases (Heck *et al.*, 1981; Waters *et al.*, 1982; Dalling *et al.*, 1983). An increase in proteolytic activity occurs in attached leaves of *Hordeum vulgare* during senescence (Friedrich and Huffaker, 1980). The amount of proteases present in leaf tissue prior to the onset of senescence has been found to be sufficient to account for the breakdown of proteins during senescence (Waters *et al.*, 1982). Regulation of proteases involved in the degradation of Rubisco and other chloroplast components during senescence is unknown (Rosichan and Huffaker, 1984). However, regulation is thought to be due to *de novo* synthesis, activation and compartmentation.

Degradation of protein and chlorophyll, important features of chloroplast senescence, are coordinated (Thomas and Stoddart, 1975). Although photooxidation may cause chlorophyll degradation, peroxidases may also be involved in this process (Kato and Shimizu, 1985). Peroxidase is known to bleach chlorophyll in the presence of hydrogen-peroxide (H_2O_2) and certain phenolics such as 2,4-dichlorophenol (Matile, 1980; Huff, 1981; Martinoia *et al.*, 1982). The involvement of chlorophyllase in chlorophyll degradation has not been unequivocally demonstrated. In some species, the activity of the enzyme has been reported to decline during senescence. In some cases, increased activity has been associated with chlorophyll synthesis rather than its degradation (Gepstein, 1988).

Senescence is often accompanied by a decline in photosynthesis (Thomas and Huffaker, 1981). Because starch metabolism of assimilatory leaves is closely related to photosynthesis (Beck and Ziegler, 1989), a decrease in

plastid volume during leaf senescence is likely to favour export rather than import of metabolites (Mullet, 1988). During senescence, starch degradation is likely to be higher than synthesis, and thus possibly more sugar is transported from senescing tissues. Sucrose is the main transport carbohydrate in higher plants. The breakdown of starch to maltodextrins is thought to be caused by α-amylases, whereas degradation of the resultant maltodextrins is considered to be completed by hydrolases and/or phosphorylases (Steup *et al.*, 1983; Beck and Ziegler, 1989).

Carbohydrates are the primary components of the cell wall. In addition, together with phospholipids and proteins they make up the various membranes in the plant cell. During senescence, a progressive loss of membrane integrity occurs (Dhinsa *et al.*, 1981). Lipid degradation and the ensuing destabilization of the bilayer are features of membrane deterioration (Fobel *et al.*, 1987). However, the nature of the inducer(s) which start senescence in plant cells is not yet understood (Kato and Shimizu, 1985). Enhanced production of free radicals is one of many events associated with senescence (Lynch and Thompson, 1984; Thompson *et al.*, 1987). Lipoxygenase (LOX), an enzyme that is capable of forming activated oxygen and initiating lipid peroxidation, is associated with senescing membranes (Lynch and Thompson, 1984; Fobel *et al.*, 1987). As senescence progresses, linoleic and linolenic acids, which are substrates for lipoxygenase, are among the fatty acids released from membrane phospholipids. Free radicals generated during senescence are generally thought to be the agents responsible for bleaching of pigments during the LOX reaction (Vick and Zimmermann, 1987).

1.4 Objectives

Loss of chlorophyll has been routinely used to monitor the progress of leaf senescence (Garaizabal and Rodriguez, 1983; Matile *et al.*, 1987; Rodriguez *et al.*, 1987; Düggelin *et al.*, 1988b; Cuello *et al.*, 1989, 1990; Bortlik *et al.*, 1990; Schellenberg *et al.*, 1990), a process that is characterized by degradation of the photosynthetic apparatus. By comparison, little or no information is available concerning the fate of carotenoids during leaf senescence. This is surprising given that chlorophylls and carotenoids are both associated with the light harvesting complexes of PSI and PSII, and that carotenoids serve a photoprotective function in the

photosynthetic pigment bed. Furthermore, evidence is now almost conclusive that ABA is derived from carotenoid metabolism via the xanthophylls all-*trans*-violaxanthin and 9'-*cis*-neoxanthin. The current research programme therefore describes experiments carried out using attached senescing leaves of *Hordeum vulgare* to determine: (a) the possible interrelationship between chlorophyll loss and changes in carotenoid content and composition; (b) the relationship between changes in carotenoid composition and senescence-induced increases in ABA metabolism, and (c) whether such changes occur coincidentally with alterations in polypeptide pattern and increased activity of hydrolytic enzymes.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 CHEMICALS, SOLVENTS AND CHROMATOGRAPHIC MEDIA

2.1.1 Radiochemicals

$NaH^{14}C0_3$ ($1.91X10^{-3}$ TBq/mmol) and (R,S)-[G-^{3}H]-ABSCISIC ACID (specific activity, 4.26 TBq/mmol) marketed as DL-*cis, trans*- [G-^{3}H]-ABA, were obtainted from Amersham International, Amersham, UK.

2.1.2 General Chemicals

Abscisic acid methyl ester (ABAMe), sold as (±) *cis-trans* ABAMe; (±) *cis-trans* ABA; all-*trans*-β-carotene; linoleic acid; triethylamine; guaiacol; PBF-Percoll; glutathione; haemoglobin; acrylamide; D-ribulose 1,5-bisphosphate (RuBP); N-methyl-N′-nitro-N-nitrosoguanidine; hydrogen peroxide (H_20_2); bovine serum albumin (BSA); Coomassie Brilliant Blue G-250; ammonium persulfate; sodium dodecyl sulfate (SDS) and bromphenol blue were purchased from Sigma Chemical Company, St Louis, Mo, USA. 2-Mercaptoethanol; Tween 20; Polyethyleneglycol 6000; Tris (hydroxymethyl) aminomethane; L(+) ascorbic acid; Manganese chloride; Magnesium chloride and potassium hydroxide pellets were obtained from Merck Chemicals, Darmstadt, Germany. Ethylenediaminetetraacetic acid (EDTA); 2,6-di-tert-butyl-ρ-cresol (BHT) and potassium dihydrogen orthophosphate (KH_2PO_4) were obtained from B.D.H. Laboratory Chemicals Division, Poole, England. Glycine and sorbitol were purchased from Saarchem, Krugersdorp, South Africa. Triton X-100 was obtained from Aldrich Chemical Company, Inc., USA. Sodium hydroxide pellets were obtained from PAL Chemicals. 4-(2-hydroxyethyl)-1-piperazinethan-sulfonsäure (Hepes) was purchased from Boehringer Mannheim, Germany.

2.1.3 Solvents

Solvents used were HPLC grade. Ethyl acetate (carbonyl free) and methylene chloride were obtained from Baxter Healthcare Corporation, McGaw Park, Illinois, USA. Acetonitrile and methanol were purchased from Bio-lab laboratories Ltd, Jerusalem, Israel. Other solvents used were of analytical grade and were glass-redistilled at 64.7°C (methanol), 77°C (ethyl acetate) and 55.5 to 56.5°C (acetone).

2.1.4 Chromatographic media

Sep-Pak C_{18} cartridges were purchased from Waters Chromatography Division, Millipore Corporation, Milford, MA, USA. For thin layer chromatography (TLC), Kieselgel 60 GF_{254} was obtained from Merck, Darmstadt, Germany. Thin layer plates (0.25 mm thickness) of silica gel GF_{254} (Merck) were prepared by adding 60 mL of distilled water to 30 g of silica gel which was then shaken vigorously for 2 minutes and 5, 20 x 20 cm thin layer plates prepared using a DESAGA TLC spreader and the plates allowed to air dry overnight.

2.2 GENERAL TECHNIQUES

2.2.1 Preparation of ethereal diazomethane

Ethereal diazomethane was generated at room temperature without co-distillation, by the hydrolysis of N-methyl-N'-nitro-N-nitrosoguanidine with 5N NaOH in a Wheaton Diazomethane Generator (Pierce Chemical Company, Rockford, Illinois, USA) using the small scale procedure described by Fales *et al.* (1973). 133 mg N-methyl-N'-nitro-N-nitrosoguanidine and 0.5 mL distilled water, to dissipate any heat generated, were placed in the inside tube. 3 mL diethyl ether was placed in the outer tube and the two parts assembled and held together with a pinch-type clamp. The lower part of the apparatus was immersed in an ice-bath and 0.6 mL of 5N NaOH was injected through the teflon rubber septum, and the reaction allowed to proceed for 45 minutes to allow high yield of ethereal diazomethane.

2.2.2 Preparation of aqueous buffered carotene-linoleate solution

This solution, prepared immediately before use, contained 0.75 mg linoleic acid (*cis*-9,*cis*-12-Octadienoic acid), 0.01 mg β-carotene, 66 x 10^{-5} mL Tween 20 (Polyoxyethylene sorbitan monolaurate) and 0.50 mg EDTA per mL as described by Ben-Aziz *et al.* (1971). 1 mL aqueous linoleate (7.5% (w/v) linoleic acid in ethanol mixed with 0.3 mL 10% (v/v) Tween 20 in ethanol to which was added 5 mL 0.5% aqueous EDTA and the pH adjusted to 9.0 with 0.1N NaOH and the final volume made to 10 mL with distilled water) was mixed with 1 mL aqueous carotene (25 mg β-carotene and 0.9 mL Tween 20 dissolved in 25 mL chloroform. 1 mL of this solution was evaporated to dryness under nitrogen, and the residue dissolved in 10 mL 0.25% EDTA solution), and the volume adjusted to 10 mL with 0.05M potassium (K) phosphate buffer, pH 7.0.

2.2.3 Protein determination

The level of protein in cell-free extracts of *Hordeum vulgare* was determined spectrophotometrically using the Bradford dye-binding assay (Bradford, 1976). To 0.1 mL of protein solution or any dilution thereof, 5 mL of Bradfords Reagent (100 mg Coomasie Brilliant Blue G-250 dissolved in 50 mL 95% ethanol to which was added 100 mL 85% (w/v) phosphoric acid and the solution diluted to 1 L with distilled water) was added and the samples mixed thoroughly. Absorbance was read after 5 minutes and before 1 hour at 595 nm against a reagent blank using a Bausch and Lomb Spectronic 20 spectrophotometer. Protein concentration of the samples was determined from a standard curve for BSA.

2.3 PLANT MATERIAL

2.3.1 Growth of plants

Seeds of *Hordeum vulgare* L. cv. Dyan, obtained from SENSASKO, Malmesbury, South Africa, were imbibed in running tap-water for 3 hours and then sown in trays of moist vermiculite. These were maintained under continuous white light of 46 $\mu molm^{-2}s^{-1}$ (unless otherwise stated) at 25°C and watered daily. For studies on dark-mediated senescence, light-grown seedlings were transferred to darkness 7 days after sowing and

maintained under these conditions at 25°C. Dark-incubated seedlings were exposed only to brief periods of green light during watering and harvesting of leaf material.

2.3.2 Preparation of crude leaf extracts

Cell-free homogenates were prepared as described by Buchanan *et al.* (1979) and Godeh *et al.* (1981). All manipulations were carried out at 0 - 4°C. Leaf material was frozen in liquid nitrogen, pulverised and extracted in 3 mL 0.05M Tris-HCl buffer (pH 7.3). The homogenate was strained through 4 layers of muslin and centrifuged at 13 000 x g for 10 minutes (at 4°C). The resulting supernatant was used to measure total protein using the Bradford procedure (1976), or where stated, proteins were analysized using SDS-polyacrylamide gel electrophoresis.

2.3.3 Preparation of intact chloroplasts

Intact chloroplasts were prepared by employing a combination of differential and density-gradient centrifugation as described by Schuler and Zielinski (1989). All steps were performed at 0 - 4°C using cold reagents.

Leaf material was homogenized (1 g/10 mL) using a Waring blender in grinding (GR) medium (0.35M sorbitol, 0.05M Hepes/KOH, 2 x 10^{-3}M EDTA, 1 x 10^{-3}M $MgCl_2$, 1 x 10^{-3}M $MnCl_2$ and 1 g ascorbic acid) using two 2-second bursts. The homogenate was strained through 4 layers of muslin and centrifuged at 2 500 x g for 4 minutes at 4°C using Sorvall RC-5 Superspeed Refrigerated Centrifuge. The resulting crude chloroplast pellet was resuspended in 5 mL of GR medium, layered over a Percoll gradient (50% PBF-Percoll, 0.35M sorbitol, 0.05M Hepes/KOH, 1 x 10^{-3}M $MgCl_2$, 1 x 10^{-3}M $MnCl_2$, 2 x 10^{-3}M EDTA and 0.140 g ascorbate centrifuged at 40 000 x g for 40 minutes at 4°C) and centrifuged at 9 000 x g for 15 minutes at 4°C. The two green bands formed, the upper (stripped thylakoids) and lower (intact chloroplasts) were collected separately. Intact chloroplasts were resuspended in 10 mL of GR medium, the contents was mixed and centrifuged at 25 000 x g for 5 minutes at 4°C. The resulting purified chloroplast pellet was resuspended in 5 mL of GR medium. Intact chloroplasts were pelleted at 2 500 x g for 5 minutes at 4°C. Pellets were resuspended in

chloroplast lysis buffer (62.5 x 10^{-3}M Tris-HCl, pH 7.5 containing 2 x 10^{-3}M $MgCl_2$ and the final volume adjusted to 100 mL with distilled water) and the suspensions were held on ice for 15 minutes with occasional vortexing. Purified thylakoid membranes were collected by centrifuging lysed whole chloroplasts in an Eppendorf centrifuge for 5 minutes. The supernatant (stroma) and pellet (thylakoids) were collected separately.

2.4 ENZYME ASSAYS

For enzyme assays, conditions and the extraction procedure were as described in section 2.3.2, except that the homogenizing buffer was 0.05M K_2HPO_4/KH_2PO_4 (pH 7.0) containing 0.1% (v/v) Triton x-100. The strained homogenate was centrifuged either at 25 000 x g for 30 minutes (proteases), 2 000 x g for 10 minutes (α-amylase) or 23 000 x g for 15 minutes (peroxidase). The resulting supernatant was used to estimate enzyme activities and the protein content determined using the Bradford method (1976).

2.4.1 Proteases

Proteolytic activity was assayed as described by Drivdahl and Thimann (1977). Acid-denatured haemoglobin was used as substrate since it is readily soluble over a wide range of pH. The reaction mixture for acid protease contained 6.70 mL sodium acetate buffer (0.2M sodium acetate; 0.2M acetic acid, pH 4.2) and 1.4 mL 4% haemoglobin (dissolved in distilled water). For neutral protease, the reaction mixture consisted of 6.70 mL phosphate-citrate buffer (0.1M citric acid; 0.2M K_2HPO_4, pH 6.6 containing 0.01M glutathione) and 1.4 mL 4% haemoglobin. After the reaction mixtures were allowed to equilibrate at 50°C for 10 minutes, reactions were initiated by addition of 0.5 mL supernatant. Reactions were carried out at 50°C in a shaking reaction incubator for 90 minutes, and were terminated by the addition of 1.4 mL 40% trichloroacetic acid. Precipitated protein was removed by centrifugation at 12 000 x g for 10 minutes, and the level of aromatic amino acids in solution, released by proteolytic activity, was determined at 280 nm (using Shimadzu, UV-160A, UV-visible recording spectrophotometer). Protease activity was expressed as μg-L-tryptophan released per mL, determined from a standard curve for L-tryptophan (MERCK).

2.4.2 α-Amylase

α-Amylase activity was determined by a method modified from that described by Chrispeels and Varner (1967). 0.150 g potato starch dissolved in 100 mL of 0.01M $CaCl_2$ and 0.05M KH_2PO_4 was used as the substrate. The starch suspension was boiled for 1 minute, cooled and centrifuged at 10 000 rpm (using Beckman TJ-6 Tabletop centrifuge) for 15 minutes before being used. 0.5 mL of starch reagent was allowed to equilibrate at 25°C for 10 minutes. The reaction was initiated by addition of 0.1 mL supernatant, and stopped after 5 minutes by addition of 1 mL copper reagent (12 g Na_2C0_3, 6 g sodium potassium tartrate in 125 mL distilled water; 2 g $CuS0_4.5H_20$ in 20 mL distilled water; 8 g $NaHC0_3$; 90 g Na_2S0_4 in 250 mL distilled water; the mixture was boiled, cooled and made up to 500 mL with distilled water). The reaction mixture was heated to 100°C for 10 minutes, and cooled for 5 minutes in a cold water-bath. 1 mL of arseno-molybdate reagent (25 g ammonium molybdate in 450 mL distilled water; 21 mL concentrated H_2S0_4; 3 g sodium arsenate in 25 mL water) and 7.4 mL of distilled water were added, and the absorbance determined spectrophotometrically at 560 nm. α-Amylase activity was expressed as μg-Glucose released per mL, determined from a standard curve for glucose.

2.4.3 Lipoxygenase

Lipoxygenase (LOX) activity was assayed as described by Ben-Aziz *et al.* (1971). Crude homogenate (see Section 2.4) was used as a source of enzyme activity. Incubations for LOX activity contained 7.5 mL buffered carotene-linoleate, 2 mL distilled water and 0.5 mL supernatant. Reactions were initiated by addition of supernatant and allowed to proceed for 1 minute at 25°C before being terminated by the addition of BHT in ethanol (0.106 mg/1 mL) added to give a final concentration of 480 μM. The degree of bleaching was determined spectrophotometrically at 460 nm.

2.4.4 Peroxidase

Peroxidase activity was monitored as described by Kuroda *et al.* (1990). The assay mixture contained 3.4 x 10^{-3}M guaiacol, 0.9 x 10^{-3}M hydrogen peroxide and 0.05M sodium phosphate (pH 6.0). Reactions were initiated

by addition of 0.5 mL supernatant, incubated for 5 minutes at 25°C and stopped by adding 0.5 mL 5% (v/v) H_2SO_4. Enzyme activity was calculated using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ at 470 nm for tetraguaiacol.

2.4.5 Rubisco

The activity of Rubisco in intact chloroplasts was determined by ribulose 1,5-bisphosphate (RuBP)-dependent incorporation of [^{14}C] into 3-phosphoglyceric acid (PGA). The incubation procedure was as described by Friedrich and Huffaker (1980). 0.1 mL of chloroplast extract was incubated at 30°C for 10 minutes with 0.1 mL 44 x 10^{-3}M $MgCl_2$, 22 x 10^{-3}M $NaH^{14}CO_3$ (18.33 KBq) and 55 x 10^{-3}M Tris-SO_4 (pH 8.0). Reactions were initiated by addition of 0.02 mL 10 x 10^{-3}M RuBP and terminated after 2 minutes by addition of 0.05 mL 2N HCl. Levels of radioactivity in 0.05 mL aliquots from each assay mixture were determined using liquid scintillation spectrometry (see Section 2.6.5).

2.5 EXTRACTION OF PIGMENTS, ABA AND RELATED COMPOUNDS

In order to make the appropriate comparisons, levels of carotenoids, ABA and related compounds were measured in the same extracts. Leaf material was frozen in liquid nitrogen, pulverised and extracted in 100% acetone containing BHT (20 mg/L). The homogenate was filtered through Whatman No.1 filter paper under vacuum, and the residue washed with excess solvent. Filtrates were split into two equal volumes and processed separately for carotenoids and ABA. All manipulations were conducted in dim light to minimize isomerization of pigments, ABA and its catabolites.

2.5.1 Purification of extracts

2.5.1.1 Purification of plant pigments

Extracts were reduced to dryness *in vacuo* at 35°C, redissolved in 10 mL 70% methanol and applied to pre-rinsed Sep-Pak C_{18} cartridges (pre-rinsed with 5 mL methanol and 10 mL distilled H_2O). Pigments were eluted from the cartridges with 10 mL 100% acetone as described by Eskins and Dutton (1979). Acetone was

evaporated *in vacuo*, and the residue stored at -20°C under nitrogen. Prior to analysis, samples were resuspended in 1 mL methylene chloride and filtered through 0.2 μm Spinex™ disposable centrifugal filters.

2.5.1.2 Purification of ABA and its catabolites

To one half of the acetone extract, [^{3}H]-ABA (specific activity, 4.26 TBq/mmol) was added to correct for recovery and ABA methyl ester included as an internal standard. Extracts were dried *in vacuo*, resuspended in 5 mL 1% aqueous acetic acid and loaded onto pre-rinsed Sep-Pak C_{18} cartridges. ABA and related compounds were eluted from the cartridge with 7 mL methanol/1% aqueous acetic acid (65:35, v/v). Eluates were dried *in vacuo,* resuspended in a small volume of methanol/ethyl acetate (50:50, v/v) and chromatographed on thin layers of silica gel GF_{254} (MERCK) which were developed 2x to 15 cm using the solvent system toluene/ethyl acetate/acetic acid (50:30:4, v/v/v) containing BHT (20 mg/L). Plates were briefly scanned under HP-UVIS low pressure Hg-lamp (254 nm). ABA and related compounds were tentatively identified on the basis of their published R_f values (ABA, 0.62; PA, 0.50 and DPA, 0.22) as described by Tietz (1985) using the same solvent system. The zones of silica gel containing compounds of interest were scrapped off and eluted with water-saturated ethyl acetate in short glass columns plugged with glass wool. Water was removed by freezing and filtration. Filtrates were reduced to dryness *in vacuo* and stored at -20°C under nitrogen. For analysis, samples were resuspended in a known volume of methanol/water (50:50, v/v) and filtered through a 0.2 μm Spinex™ disposable centrifugal filters.

2.6 ANALYTICAL PROCEDURES

2.6.1 Spectrophotometric determination of Xanthophylls, Chlorophylls and Carotenes

Pigments were separated into 3 classes, namely, xanthophylls, chlorophylls and carotenes using Sep-Pak C_{18} cartridges as described by Eskins and Dutton (1979). Xanthophylls were eluted with 90% methanol, chlorophyll *a* and *b*, 100% methanol and carotenes, 100% acetone. The concentration of xanthophylls, chlorophyll *a* and *b* and carotenes in each eluate was determined spectrophotometrically as described by Lichtenthaler (1987) using a Shimadzu, UV-160A dual beam scanning spectrophotometer. Equations for

chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophylls (C_{a+b}) and total carotenoids (C_{x+c}) were recalculated for this instrument as recommended by Lichtenthaler (1987). This was achieved by preparing fresh chlorophyll *a* and *b* from the leaves of 7 day old, light-grown *Hordeum vulgare* seedlings. Chlorophylls were extracted in 100% acetone and separated on thin layers of silica gel using the solvent system benzene/ethyl acetate/ethanol (8:2:3, v/v/v) as described by Holden (1976). Chlorophyll *a* and chlorophyll *b* were eluted from the silica gel with ether in short glass columns plugged with glass wool. The ether fraction was reduced to dryness under nitrogen and the residue resuspended in the appropriate solvent. The purified chlorophylls were identified by scanning spectrophotometrically in 90% methanol, 100% methanol and 100% acetone. The wavelength position of chlorophyll *a* and *b* absorption maxima (Figure 2.1) in the red spectral region, and the relative absorbance at λ_{max} of the other chlorophyll (eg. absorbance of Chl *a* at λ_{max} Chl *b*), as well as that at 470 nm were determined for each solvent. The recalculated equations are shown in Table 2.1.

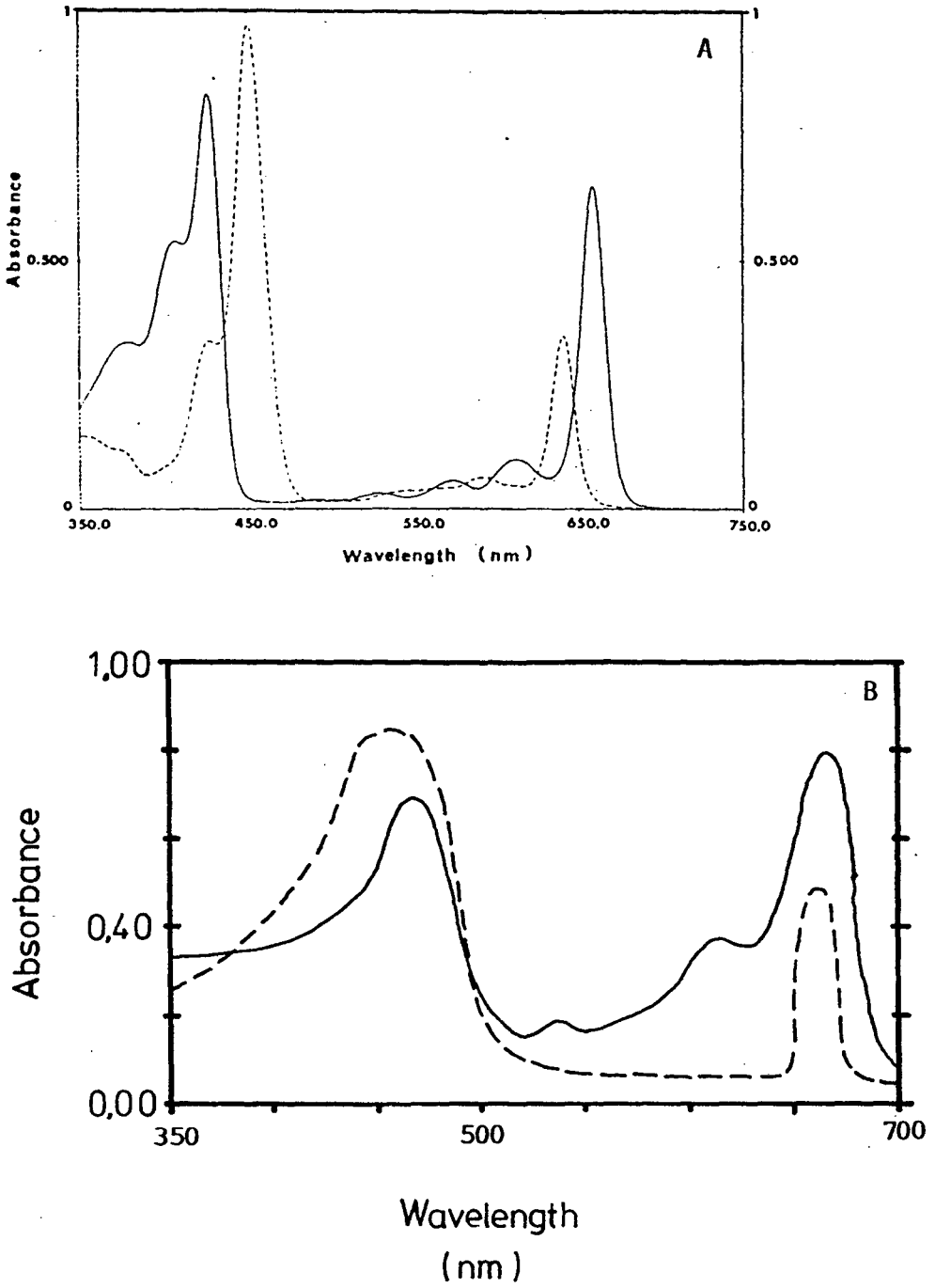

Figure 2.1 Absorption spectra of freshly isolated chlorophyll *a*(solid line) and chlorophyll *b*(broken line) in (A) diethyl ether, pure solvent (after Lichtenthaler, 1987) and (B) methanol, 100% (pure solvent).

Chlorophyll *a* and *b* were isolated from leaves of 7d old, light-grown *Hordeum vulgare* seedlings and separated by thin layer chromatography using the solvent system benzene/ethyl acetate/ethanol (8:2:3, v/v/v). The positions of the absorption maxima were determined using a Shimadzu, UV-160A, UV-visible recording spectrophotometer, which allows auto-absorption maxima of the pigments.

Table 2.1 Recalculated equations for the determination of chlorophyll *a* (C_a), chlorophyll *b* (C_b), total chlorophyll (C_{a+b}) and total carotenoid (C_{x+c}) content of pigment extracts.

Chlorophyll *a* and *b* were separated on thin layers of silica using the solvent system benzene/ethyl acetate/ethanol (8:2:3, v/v/v). Absorbance was read using Shimadzu, UV-160A, UV-visible recording spectrophotometer.

	90% methanol(v/v)	100% methanol, pure solvent	100% acetone pure solvent
C_a	$13.55A_{664.5} - 1.09A_{659}$	$13.55A_{665} - 1.05A_{659}$	$11.52A_{665} - 1.26A_{656.5}$
C_b	$29.73A_{659} - 23.85A_{664.5}$	$27.62A_{659} - 17.07A_{665}$	$20.63A_{656.5} - 11.49A_{665}$
C_{a+b}	$28.63A_{659} - 10.30A_{664.5}$	$26.12A_{659} - 3.52A_{665}$	$19.36A_{656.5} + 0.03A_{665}$
C_{x+c}	$\frac{1000A_{470} - 65.92C_a - 28.56C_b}{225}$	$\frac{1000A_{470} - 4.82C_a - 23.90C_b}{221}$	$\frac{1000A_{470} - 3.63C_a - 32.64C_b}{214}$

2.6.2 Quantification of pigments by reversed-phase HPLC

Aliquots of each sample were chromatographed on a Nucleosil 5 μm C_{18} (250 x 4.6 mm i.d.) column (Macherey-Nagel, Düren, West Germany) using either a linear gradient of 0-100% ethyl acetate in acetonitrile/water (9:1, v/v) over 25 minutes, or a linear gradient of 10-90% methylene chloride/methanol (6:4, v/v) in 85% methanol containing 0.1% (v/v) triethylamine over 45 minutes. In both systems, pigments were eluted at a flow rate of 0.8 mL/min. A typical chromatographic separation is shown in Figure 2.2. Peaks were detected at 410 nm using a LINEAR UVIS-200 variable wavelength detector and quantified by peak integration (Spectra-Physics SP 4290). β-Carotene was used as an external standard (Figure 2.3). The coefficient of β-carotene ($C_{\beta\text{-carotene}}$) was used to derive normalized coefficients ($C_{pigment}/C_{\beta\text{-carotene}}$) for each compound of interest as described by de las Rivas *et al.* (1989). The normalized coefficients used were, neoxanthin (1.71), violaxanthin (1.53), antheraxanthin (1.41), lutein (1.41), zeaxanthin (1.34), chlorophyll *a* (10.89), chlorophyll *b* (1.71) and β-carotene (1.00).

2.6.2.1 Identification of Xanthophylls

Xanthophylls were purified by Sep-Pak C_{18} cartridges and were eluted with 10 mL 90% methanol, the latter was evaporated *in vacuo* and the residue resuspended in 0.1 mL ethanol containing 1% triethylamine. Aliquots were fractionated by reversed-phase HPLC using the solvent system methylene chloride/methanol (6:4, v/v) in 85% methanol. The chromatographic system presented in this work gave a clear separation of six major peaks (Figure 2.4). Eluates corresponding to each peak in the chromatogram were collected and reduced to dryness under nitrogen. Peaks were tentatively identified by measuring their absorbance spectra in appropriate solvents (Davies, 1976; Val *et al.*, 1986), and by comparing the spectral characteristics (Table 2.2) with spectra published in the literature.

2.6.2.2 Xanthophyll Cycle Activity

Xanthophyll cycle activity was determined by calculating the percentage violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) of the V + A + Z pool. The epoxidation state (EPS) was determined from the expression (V + 0.5A)/(V + A + Z) as described by Thayer and Björkman (1990).

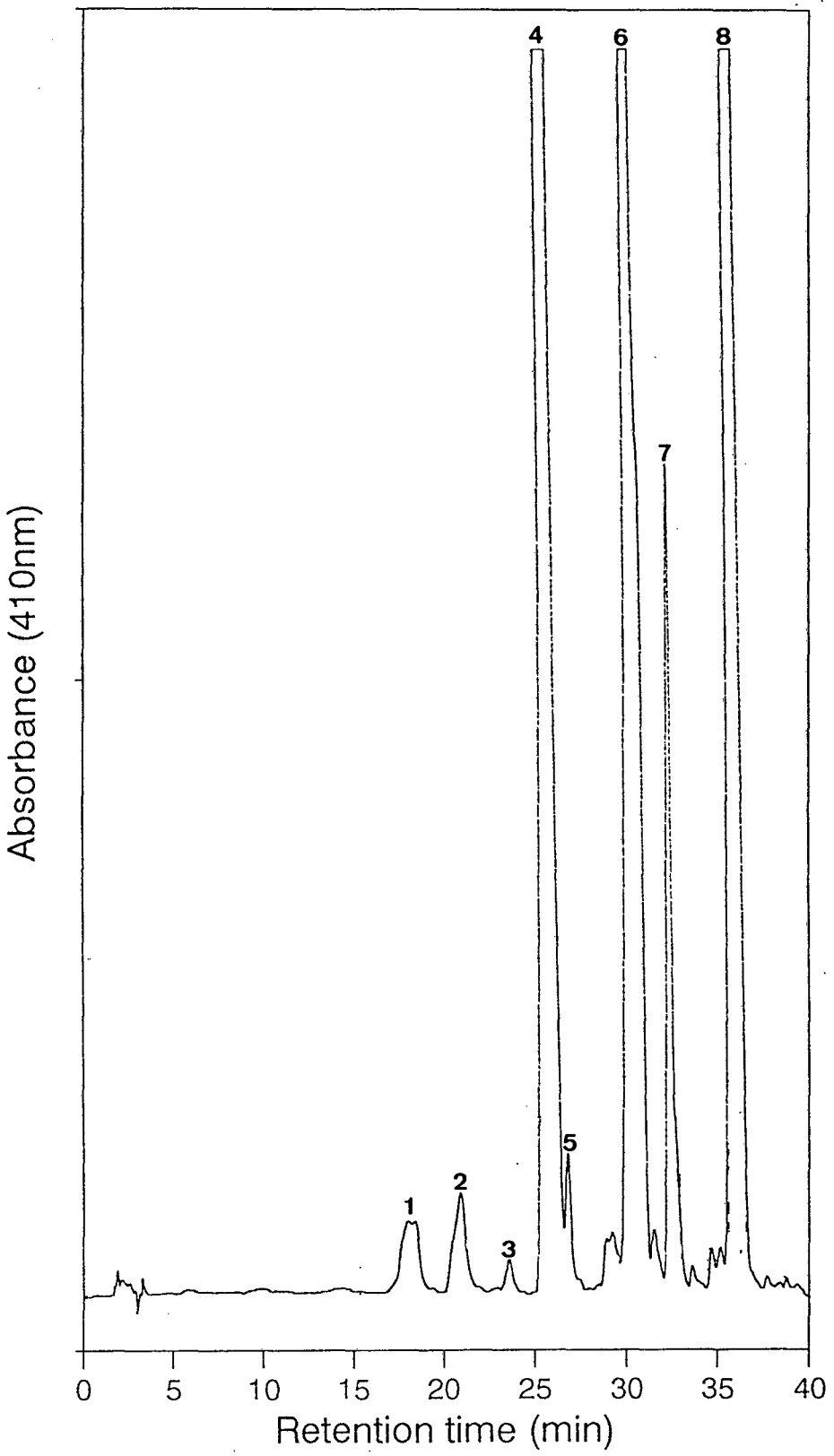

Figure 2.2 HPLC chromatogram of pigments extracted from leaves of 7d old, light-grown *Hordeum vulgare* seedlings.

The solvent system used was a linear gradient of 10-90% methylene chloride/methanol (6:4, v/v) in 85% methanol containing 0.1% (v/v) triethylamine. Peaks: 1, Neoxanthin; 2, all-*trans*-violaxanthin; 3, antheraxanthin; 4, lutein; 5, zeaxanthin; 6, chlorophyll *b*; 7, chlorophyll *a*; 8, β-carotene.

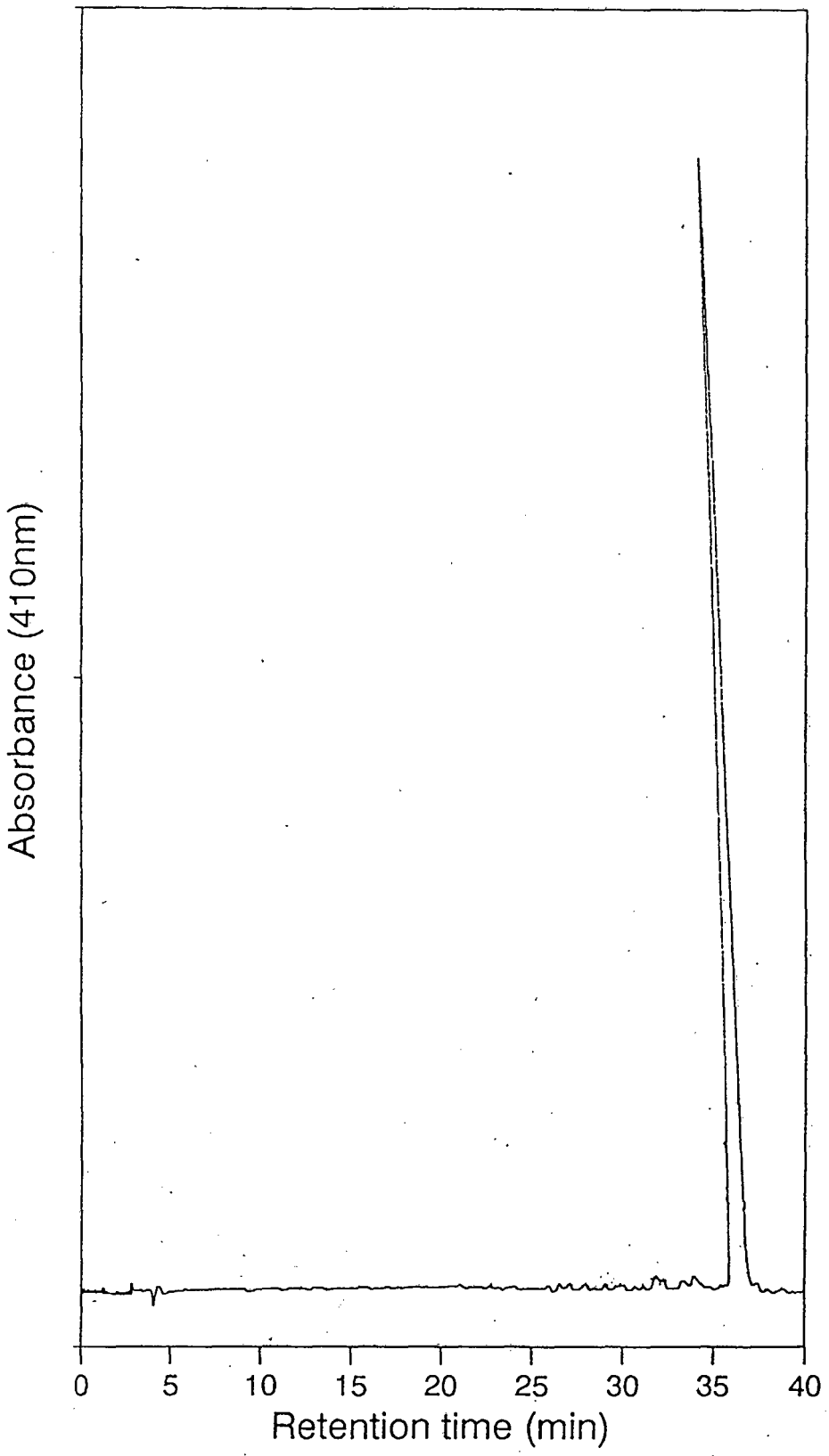

Figure 2.3 HPLC chromatogram of a β-carotene standard.

The solvent system used is as described in Figure 2.2.

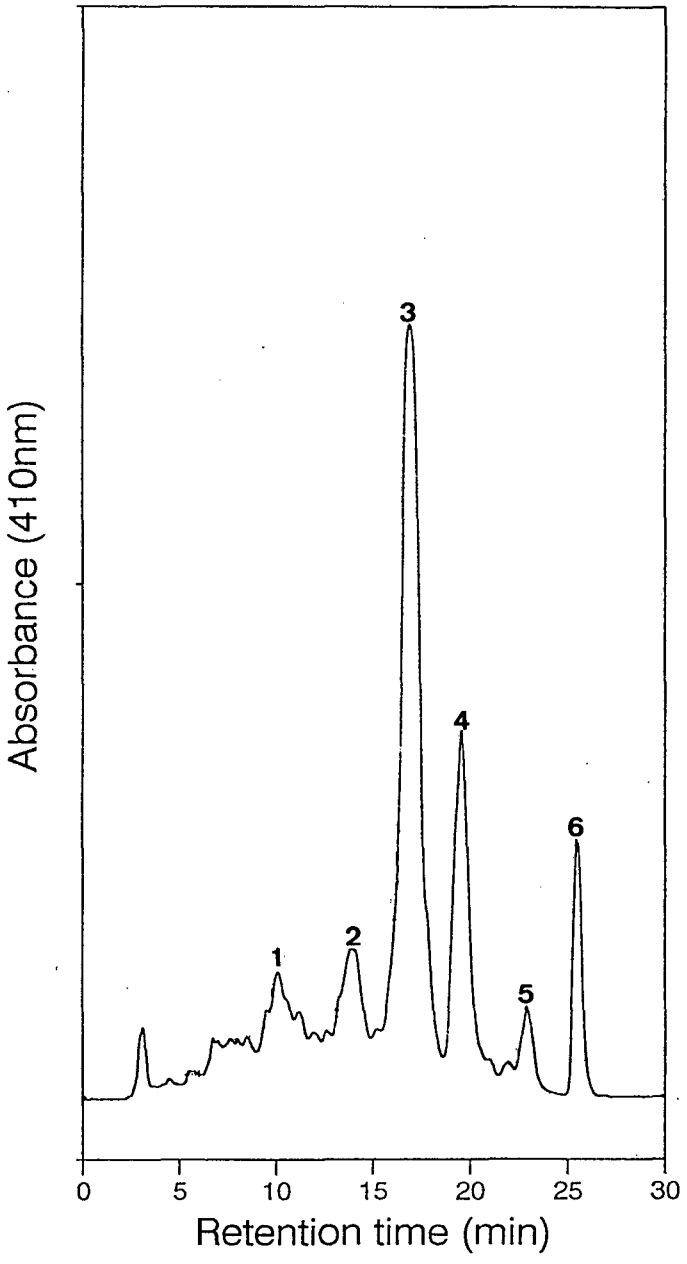

Figure 2.4 HPLC chromatogram of xanthophylls extracted from leaves of *Hordeum vulgare* seedlings. The solvent system used is as described in Figure 2.2. The identity of the pigments is given in Table 2.2.

Table 2.2 Absorbance maxima in hexane, ethanol, and ethanol plus HCl of extracted xanthophylls compared with published values.

Maximum wavelengths (λ_{max}) were determined using Shimadzu, UV-160A, UV-visible recording spectrophotometer.

Peak number	Pigment	Hexane		Ethanol		Ethanol + HCl		Ref.[c]
		Extr.[a] λ_{max}	Publ.[b] λ_{max}	Extr. λ_{max}	Publ. λ_{max}	Extr. λ_{max}	Publ. λ_{max} or shift	
1	Unidentified	351.5 400.5	None	398.0 411.5 421.0	None	386.5 399.5	None	
2	trans-Neo-xanthin	408.5 423.5	412 436	400 422.5 470	441 422 470	399 422 449	402 422 460	3;4
3	9'-*cis*-Neo-xanthin	429 463	436 465	412 442 463	412 438 464	423.5 453	13-16	2
4	Violaxan-thin	423.5 440 467	416 440 470	423.5 441 470	417 441 470	388 402 426	380 402 423	4
5	Antheraxan-thin	423.5 446 467	421 445 470	423.5 447	422 444/8	402 426.5	20-24	1;2
6	Lutein	445 473	445 473	445 474	445 470	444	NS	2;4

Abbreviations: [a]Extracted, [b]Published, NS: no shift was found
[c]References: 1, Davies, 1976; 2, Duckham *et al.*, 1991; 3, Parry *et al.*, 1990; 4, Val *et al.*, 1986.

2.6.3 Quantification of ABA and PA by HPLC

ABA and related compounds were analyzed by reversed-phase HPLC using a linear gradient of 0-100% methanol, containing 1% acetic acid throughout, and quantified by peak integration and calibration with reference compounds. Where specified, phaseic acid was similarly quantified in extracts using biosynthetically prepared PA (see Section 2.7.1) as a standard. Separation was achieved using a Bondaclone 10 μm C_{18} (150 x 3.9 mm i.d.) column (Phenomenex, Torrance, CA) at a flow rate of 1.0 mL/min over 45 minutes, and peaks were monitored at 254 nm with a LINEAR UVIS-200 variable wavelength detector.

2.6.4 Gas Chromatography

Following methylation, compounds for analysis by gas chromatography (GC) were routinely dissolved in a small volume of redistilled acetone. GC was performed on a Perkin-Elmer 8310 instrument fitted with an electron capture detector (^{63}Ni source). Compounds of interest were resolved on a capillary column (15 mm x 0.53 mm i.d.) of SPB-1 (SUPELCO, Bellefonte, CA, USA) programmed from 120°C at 5°C per minute with N_2 as carrier gas at a flow rate of 17 mL/min. Retention times for ABAMe (Figure 2.5) and PAMe (see Section 2.7) standards were 5.89 and 6.61 minutes, respectively.

2.6.5 Liquid scintillation spectrometry

The level of radioactivity in samples was determined using a Beckman LS 5801 scintillation spectrometer, programmed for automatic quench correction, following the addition of a small volume of methanol and 10 mL of cocktail (2,5-diphenyl oxazole pyrophosphate (PPO) in toluene, 5 g/L). Where required, radioactivity in aqueous phases was determined using Bray's scintillant prepared by dissolving naphthalene (30 g) and 2,5-diphenyl oxazole pyrophosphate, PPO (2 g) in 50 mL methanol, and the solution diluted to 500 mL in 1,4-dioxan (Bray, 1960).

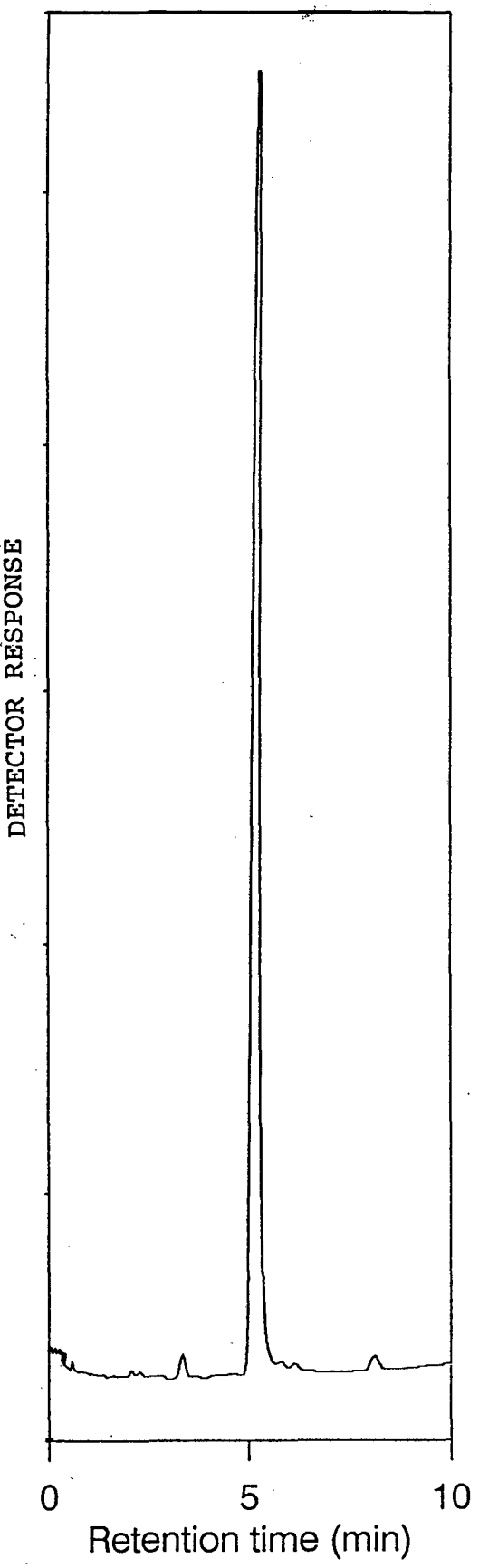

Figure 2.5 Gas chromatogram of the ABAMe standard.

GC-ECD was performed on a Perkin-Elmer 8310 instrument (EC source ^{63}Ni) using a capillary column of SPB-1 programmed from 120°C at 5°C per minute with N_2 as carrier at 17 mL/min.

2.6.6 Gas Chromatography and combined Gas Chromatography - Mass Spectrometry

Methylated samples for analysis by gas chromatography-flame ionization (GC-FID) were routinely dissolved in a small volume of acetone. GC was performed on a Hewlett-Packard 5890 instrument, using a thin film (0.33 μm)fused-silica capillary column (12 m x 0.2 mm i.d.) of HP-1 programmed from 160°C at 5°C per minute with He as carrier gas at a flow rate of 1.5-2 mL per minute. The retention times of ABAMe, t-ABAMe and PAMe were 9.32, 9.67 and 10.08 minutes, respectively.

Combined capillary gas chromatography - mass spectrometry (GC-MS) was carried out using a Hewlett-Packard 5890 instrument coupled to an HP 5988A MS system with GC conditions as described above. Electron impact (EI) mass spectra were recorded at 70eV and a source temperature of 250°C. Identification of compounds as ABAMe and PAMe was established from published spectra for these compounds (Neill and Horgan, 1987).

2.6.7 Electrophoresis

2.6.7.1 Sample preparation for SDS-Polyacrylamide gel electrophoresis

Samples were stored in either a low ionic strength buffer (0.10M Tris-HCl) in water or as a pellet, as recommended by Schuler and Zielinski (1989). Protein concentration of the samples was estimated by the Bradford assay (1976), and equal amounts of protein were loaded on the gels.

0.015 - 0.025 mL of the lysed chloroplast suspension and crude extract were mixed with equal amount of 2x sample loading buffer (0.12M Tris; 4% (w/v) SDS; 10% 2-mercaptoethanol; 20% (v/v) glycerol; 2 mg/mL bromphenol blue) in a microfuge tube, whilst thylakoid pellets were resuspended directly in 1x sample loading buffer. Since the concentration of stromal proteins was less (as determined by the Bradford assay), stromal proteins were concentrated as follows: 0.20 - 0.30 mL of protein was mixed with 0.02 mL 10% SDS and 1.0 mL acetone, and held at room temperature for 5 minutes. Proteins were precipitated by centrifuging in an Eppendorf for 2 minutes. The resulting pellets were dried under nitrogen and resuspended in 0.015 mL of 2% SDS and 1% 2-mercaptoethanol. Samples were dissolved in a water-bath set at 60°C, after which an equal amount of 2x loading buffer was added. All samples

were heated in a water-bath at a 100°C (tubes were tightly capped) for 1 minute to dissociate protein complexes, and were loaded immediately on the gel using a microliter syringe.

2.6.7.2 Molecular weight markers

The protein standards (molecular weight in Kilodalton) routinely included on the gels were bovine serum albumin (63.2); D-ribulose 1,5-bisphosphate carboxylase, spinach (45.2) and peroxidase, horse radish (36.1). As shown in Figure 2.6 (lane 1), the molecular weight markers phosphorylase B, rabbit muscle (97.4); albumin, bovine (66.0); glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36.0); carbonic anhydrase, bovine erythrocytes (29.0) and trypsin inhibitor, soybean (20.1) were used to determine among others, the molecular weight of the aforementioned protein standards (Lanes 2, 3 and 5).

2.6.7.3 Electrophoretic techniques

The separation of polypeptides by SDS-polyacrylamide gel electrophoresis was carried out with 12.5% acrylamide running gel and 4% acrylamide stacking gel, using the buffer system of Laemmli (1970). Electrophoresis was carried out at 60V and 25 mA for 20 hours. Gels were stained for 1 hour in 0.125% (w/v) Coomassie brilliant blue, 50% (v/v) methanol, 10% (v/v) glacial acetic acid and destained in 10% methanol, 7% acetic acid. Gels were preserved by compressing and drying onto filter paper under vacuum for 3 hours at 60°C. Gel scanning was performed using the UVP gel Documentation Software Package SW 2000.

2.7 PREPARATION OF STANDARD COMPOUNDS

2.7.1 Biosynthetic preparation of phaseic acid

Standard phaseic acid (PA) was prepared biosynthetically as described by Zeevaart amd Milborrow (1976), and Cowan and Railton (1987). (R,S)-ABA (10 mg) was fed to excised leaves (7 g fresh weight) in K phosphate buffer. Uptake of the substrate was achieved via the transpiration stream at 25°C under constant illumination (46 $\mu molm^{-2}S^{-1}$). Following a 6 hour incubation period, the leaf material was homogenized in ice-cold methanol/ethyl acetate (50:50, v/v). The homogenate was filtered and purified as described in Sections 2.5 and 2.5.1.2. The purity of PA was checked by reversed-phase HPLC (see Section 2.6.3) and

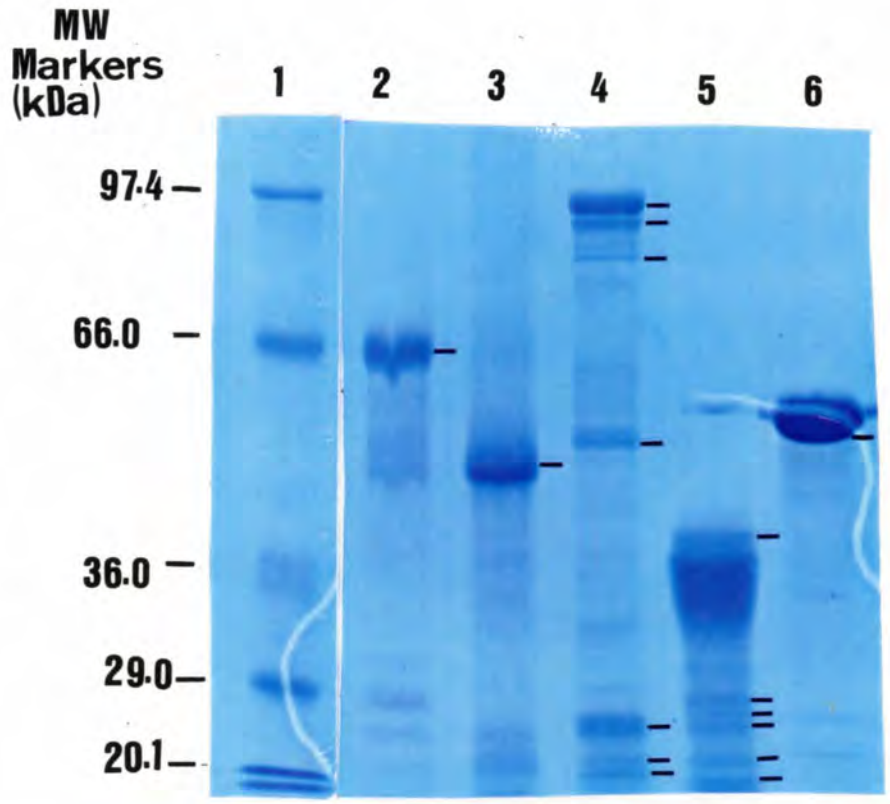

Figure 2.6 SDS-polyacrylamide gel electrophoresis of the molecular weight markers (lane 1) and protein standards (lane 2-6).

Wells were loaded with 15 μg (lane 1 and 3), 0.02 μg(lane 2), 20 μg(lane 4 and 6) and 25 μg(lane 5) of protein. After SDS-PAGE, proteins were stained for 1 hour in Coomassie blue G-250 and destained in methanol. Lane 2 and 3, bovine serum albumin and D-ribulose, 1,5-bisphosphate carboxylase, respectively. Lane 4, lipoxygenase and isoenzymes (93.6, 88.8, 81.5, 49.5, 25.1, 21.7 and 20.6); lane 5, peroxidase and isoenzymes (36.1, 29.8, 27.5, 24.3, 21.5 and 20.0) and lane 6, α-amylase (51.5).

confirmed by GC-ECD (see Section 2.6.4), and unequivocal characterization of PAMe was achieved by combined capillary GC-MS and the electron impact mass spectrum is shown in Figure 2.7.

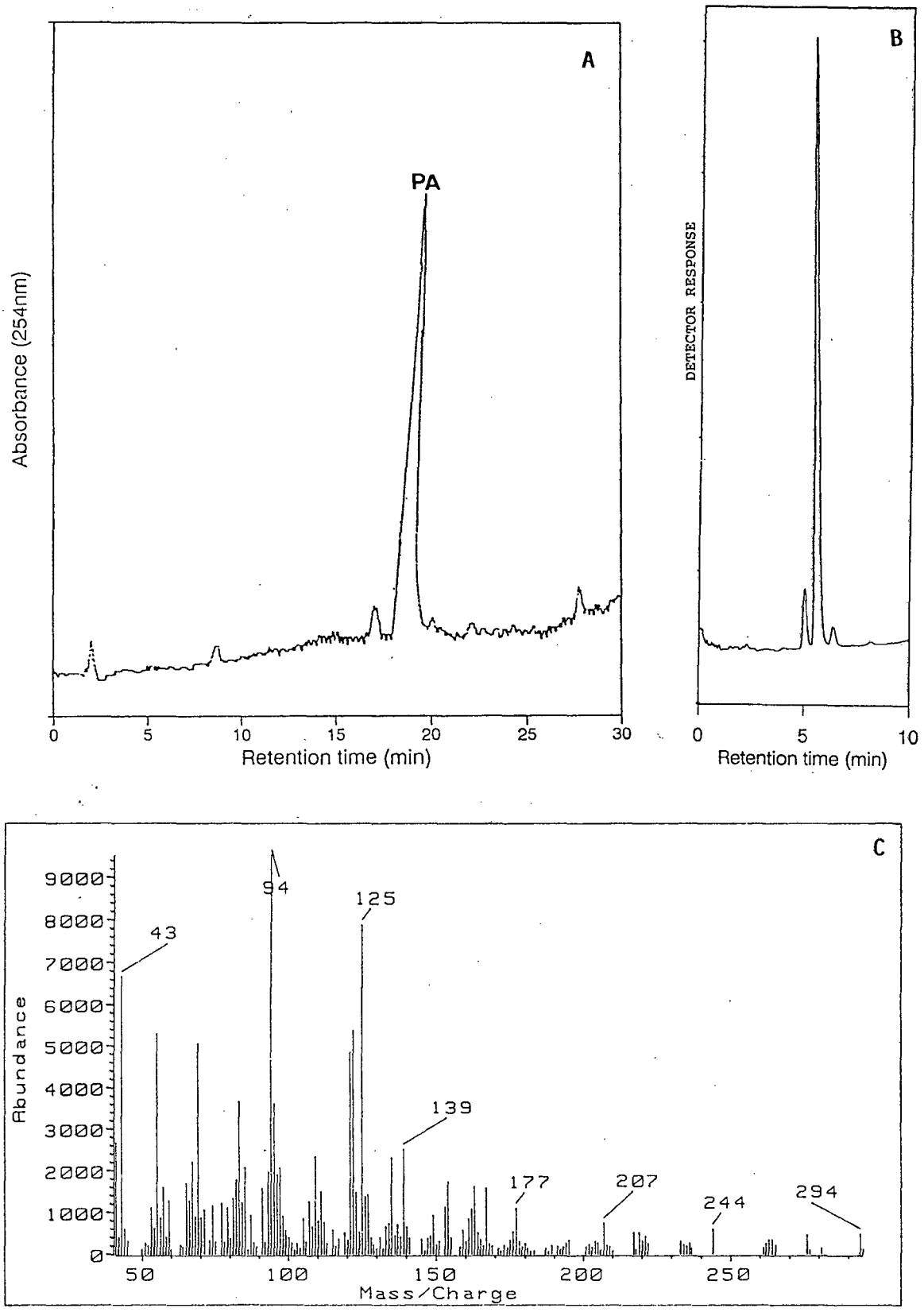

Figure 2.7 Phaseic acid. (A) HPLC trace of PA after fractionation. PA was analyzed as described in Section 2.6.3. (B) GC-ECD trace of PAMe standard. PAMe was chromatographed as described in Figure 2.5. (C) Electron impact mass spectrum of PAMe standard. EI mass spectrum was recorded at 70eV and a source temperature of 250°C (see Section 2.6.6).

CHAPTER THREE

SENESCENCE-INDUCED CHANGES IN CHLOROPHYLLS, CAROTENOIDS AND ABSCISIC ACID IN ATTACHED LEAVES OF *HORDEUM VULGARE*

3.1 INTRODUCTION

A characteristic of foliar senescence is the progressive loss of chlorophyll and yellowing of leaves, and an associated increase or decrease in the levels of certain plant growth regulators. With regard to the latter, it is now well established that levels of both ethylene and abscisic acid (ABA) increase during the course of leaf senescence (Beyer and Morgan, 1971; Paulin *et al.*, 1986; Noodén and Leopold, 1988; Sylvestre *et al.*, 1989). However, whether these growth regulators represent the trigger mechanism involved in initiating the process, or whether changes in levels of these compounds are a consequence of senescence, remains undecided and needs therefore to be elucidated.

3.1.1 Chlorophylls

Chlorophyll degradation is a visible and convenient indicator of leaf senescence (Amir-Shapira, *et al.*, 1986; Knee, 1991). Very little is known, however, about the biochemical events associated with the breakdown of chlorophyll in higher plants (Maunders *et al.*, 1983). Since chlorophyll is bound to membrane proteins (Malhotra *et al.*, 1982; Grumbach, 1984), the progressive loss of chlorophyll during senescence may involve disintegration of the photosynthetic membranes as well as actual breakdown of the pigment molecules (Amir-Shapira *et al.*, 1986). As the breakdown of chlorophyll requires cytoplasmic protein synthesis (Thomas, 1976), it is likely to be catalyzed by enzyme(s) which are newly synthesized upon the induction of foliar senescence (Matile *et al.*, 1992). Products of chlorophyll breakdown have been poorly characterized, even though derivatives of chlorophyll such as phaeophytin, chlorophyllide and phaeophorbide were identified as products of breakdown *in vivo* (Schellenberg *et al.*, 1990). However, a novel quest for the elucidation of chlorophyll breakdown is now emerging as several putative non-green catabolites have been discovered in

senescent leaves of *Festuca pratensis* and *Hordeum vulgare* (Düggelin *et al.*, 1988 a, b; Bortlik *et al.*, 1990). In their native form all of these catabolites are colourless (Bortlik *et al.*, 1990). One of these catabolites, RP 14, has been observed to accumulate during dark-induced senescence, and is completely sequestered in the vacuoles of mesophyll cells (Bortlik *et al.*, 1990).

Another group of catabolites, lipofuscin-like fluorescent compounds (FCs) have also been localized in vacuoles (Matile *et al.*, 1988; Düggelin *et al.*, 1988b). However, a small but significant pool of FC2 has been localized in the chloroplasts (Düggelin *et al.*, 1988b). The kinetics of accumulation during foliar senescence suggest that FCs are a pool of intermediary or even primary products (Düggelin *et al.*, 1988b; Bortlik *et al.*, 1990), whereas RP 14 represent a secondary or even final product of chlorophyll breakdown (Bortlik *et al.*, 1990). Thus, evidence now indicates that in senescing leaf tissue a catabolic pathway operates that starts with dephytylation to yield chlorophyllides and phaeophorbides (Amir-Shapira *et al.*, 1987). This is followed by an oxygen-dependent step, during which the tetrapyrrole ring probably opens (Thomas *et al.*, 1989) to yield non-green products (Schellenberg *et al.*, 1990). Porphyrins and particularly chlorophylls are photodynamic compounds which in the presence of oxygen and light give rise to the occurrence of singlet oxygen (Matile *et al.*, 1989). Within the complexes of thylakoids, the photodynamic reactions are quenched by the carotenoids and the potential damaging action of singlet oxygen is prevented (Demmig-Adams, 1990; Thayer and Björkman, 1990).

3.1.2 Carotenoids

Carotenoid content and composition has been extensively investigated in the green tissues of higher plants (Frosch and Mohr, 1980; Grumbach, 1981, 1984; Malhotra *et al.*, 1982; Oelmüller and Mohr, 1985; Barry *et al.*, 1991). According to these authors, carotenogenesis is enhanced by light and by the availability of chlorophyll. However, Ben-Amotz and Avron (1983) observed that the accumulation of β-carotene coincides with chlorophyll depletion in the alga, *Dunaliella bardawil*. Furthermore, Grumbach (1984) demonstrated the enhancing effect of far-red light on the accumulation of acyclic carotenoids and β-carotene. Because

darkness enhanced the loss of chlorophyll, a characteristic feature of senescence, it is likely that senescence may cause the accumulation of some carotenoids. So far, no detailed information exists on the carotenoid content and composition of senescing tissues. As a result, our knowledge of carotenoid composition in senescing tissues is still limited. Interestingly, evidence has apparently been obtained which suggest that carotenoids are major precursors of abscisic acid (ABA) in higher plants (Li and Walton, 1990; Parry *et al.*, 1990; Duckham *et al.*, 1991; Parry and Horgan, 1991a, b; Rock and Zeevaart, 1991; Rock *et al.*, 1992).

3.1.3 Abscisic Acid

Thimann (1987) has provided evidence in support of a role for ABA in leaf senescence. Several studies have recently examined the effect of ABA on senescence in leaves of *H. vulgare* (Zhi-Yi *et al.*, 1988; Frommhold, 1989; Cuello *et al.*, 1990). Although there is substantial evidence to implicate ABA as a promoter of foliar senescence in *H. vulgare*, little is known about its role in the regulation of this process (Noodén, 1988b). Most evidence available suggests that ABA is derived from a carotenoid (Parry *et al.*, 1990; Parry and Horgan, 1991a) and some of these carotenoids constitute components of the xanthophyll cycle in plants. Thus, evidence exists to show that zeaxanthin is a precursor of ABA (Duckham *et al.*, 1991; Rock and Zeevaart, 1991). As shown in Figure 3.1, zeaxanthin is converted to all-*trans*-violaxanthin via antheraxanthin (Bilger *et al.*,1989; Demmig-Adams, 1990) and all-*trans*-violaxanthin is a precursor of ABA in plants (Li and Walton, 1990). The conversion of all-*trans*-violaxanthin to 9′-*cis*-neoxanthin, the immediate precursor of xanthoxin, has also been demonstrated (Parry and Horgan, 1991b). Thus, there is ample evidence linking the metabolism of carotenoids to the synthesis of ABA and two recent reports have also shown that an increase in β-carotene synthesis occurs concurrent with rapid ABA production in cultures of *Cercospora rosicola* (Norman, 1991) and *Dunaliella salina* (Cowan and Rose, 1991).

3.1.4 Carotenoid - Abscisic Acid Interrelationship

Central to the process of foliar senescence is the degradation of the photosynthetic apparatus. Because senescence is a co-ordinated developmental process, regulated uncoupling and/or partial repair of the

photosynthetic reaction centres might be required. Repair of reaction centres seems to involve carotenoids (Rau, 1988) which either quench chlorophyll triplet excited states, thereby preventing singlet oxygen production, or quench singlet oxygen directly. In either case, potentially harmful excited states are dissipated harmlessly as heat (Thayer and Björkman, 1990). Demmig-Adams *et al.* (1987) proposed that zeaxanthin dissipates excess energy in the antenna chlorophyll, and recent evidence suggests that increased xanthophyll cycle (Figure 3.2) turnover regulates energy dissipation in the pigment bed (Demmig-Adams, 1990; Thayer and Björkman, 1990; Adams and Demmig-Adams, 1992). It is therefore possible that changes in endogenous ABA levels might result, either directly or indirectly, from the operation of the xanthophyll cycle.

3.1.5 Objectives

In view of the lack of detailed information concerning changes in levels of individual carotenoids during senescence, and because ABA is now regarded as an apocarotenoid, changes in the chlorophyll content, carotenoid content and composition and levels of ABA and associated catabolites were monitored in senescing, intact leaves of *H. vulgare*. These studies were carried out to elucidate the possible relationship between ABA production and carotenoid metabolism during foliar senescence.

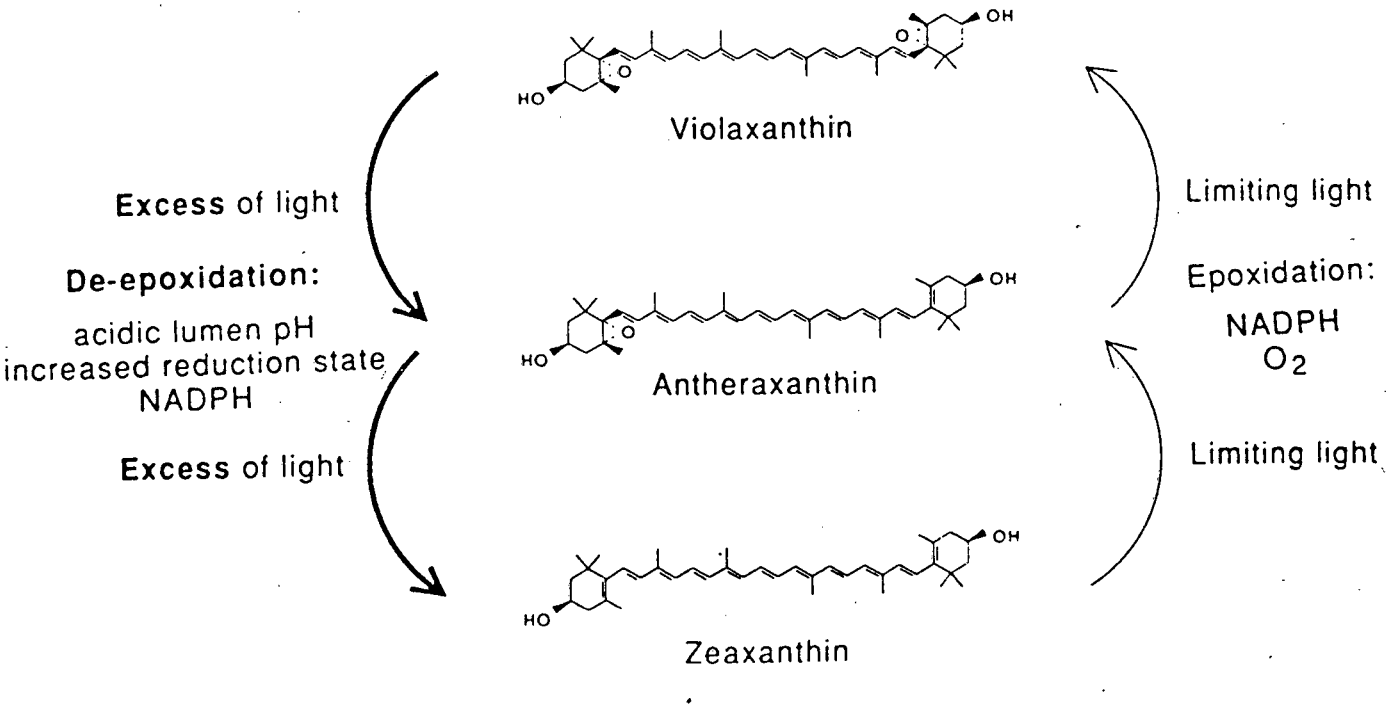

Figure 3.1 The xanthophyll cycle

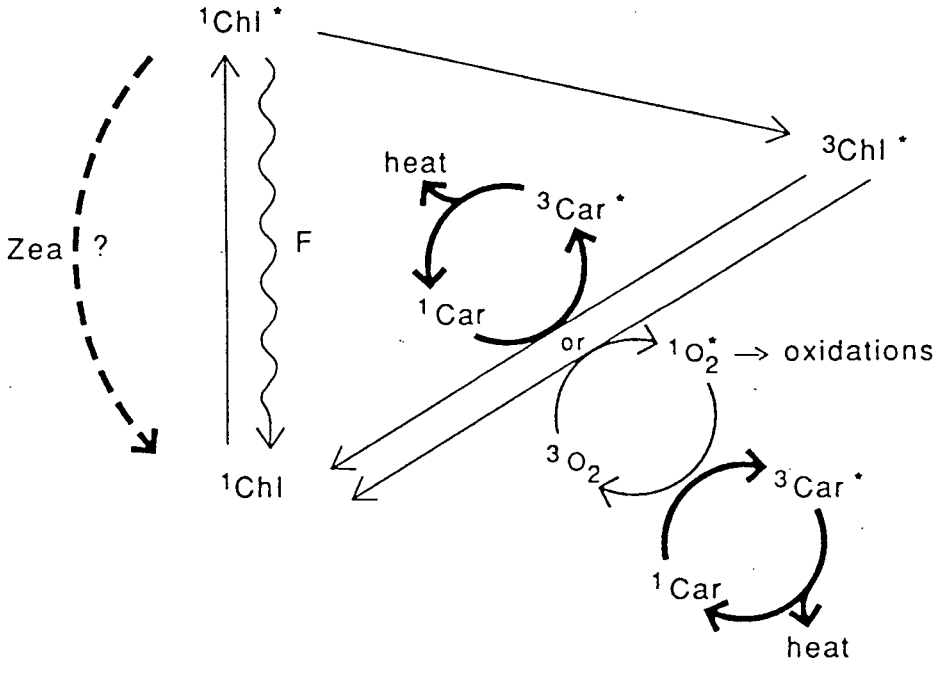

Figure 3.2 Schematic diagram of possible sites for an interaction of carotenoids with excited states of chlorophyll or oxygen.

3.2 RESULTS

3.2.1 Spectrophotometric determination of changes in total chlorophyll and carotenoid content

The degree of senescence in intact leaves of *H. vulgare* was determined by monitoring the levels of chlorophyll in leaves of dark and light-incubated seedlings. Darkness induced rapid chlorophyll loss (Figure 3.3). In leaves from the light-incubated plants, the loss of chlorophyll was sustained for the first 2 days, after which chlorophyll was gradually degraded. Calculation of the rate of loss of chlorophyll (Figure 3.4) revealed that the rate of loss was high during the first 2 days of dark-incubation, after which it declined. This observation seems to suggest that chlorophyll was rapidly lost during the first 2 days after transfer of plants to total darkness. In light-incubated plants, there was an increase in the rate of chlorophyll loss between days 2 and 4, after which it remained fairly constant throughout the incubation period. The low rate of chlorophyll loss in the light-incubated plants suggests that the degradation of chlorophyll was retarded in the light, which is consistent with the findings of Cuello *et al.* (1990).

Chlorophyll *a/b* ratio is a good indicator of the rate of either greening or degreening (Barry *et al.*, 1991). As shown in Figure 3.5, chlorophyll *a/b* ratio increased in both treatments but was higher in the dark-incubated plants. Analysis of changes in levels of chlorophyll *a* and chlorophyll *b* revealed that chlorophyll *a* was always higher in both treatments (Figure 3.6). With the increase in incubation time, there was a decline in chlorophyll *a* and chlorophyll *b* content of both the light and dark incubated leaves. An inverse relationship between chlorophyll $(a+b)$ concentration and chlorophyll *a/b* ratio was observed for both treatments (Figure 3.7). This observation is consistent with the findings of Walmsely (1991) for leaves of *H. vulgare*. Therefore, an increase in the chlorophyll *a/b* ratio with time suggests that chlorophyll *b* was preferentially broken down during the senescence process.

During the course of leaf senescence, chlorophylls have been reported to be degraded faster than carotenoids, thus resulting in leaf yellowing (Woolhouse, 1984; Siffel *et al.*, 1991). Figure 3.8 illustrates the effect of

dark-incubation on the carotenoid content of the leaves. Total carotenoid content of light-incubated leaves gradually declined. By contrast, a rapid loss of carotenoids occurred during the initial stages of dark-incubation. This decline was followed by a rise in the level of carotenoid, after which the level remained constant. In an attempt to determine a relationship between the loss of chlorophyll and carotenoid, the chlorophyll $(a+b)$:carotenoid $(x+c)$ ratio was calculated for both treatments. As shown in Figure 3.9, the chlorophyll $(a+b)$:carotenoid $(x+c)$ ratio decreased only slightly with the progression of senescence in light. An increase in the ratio during the initial stages of incubation in the light may be attributed to the high chlorophyll content. Nevertheless, the data indicate that chlorophyll was degraded faster than carotenoid, particularly in the dark-incubated leaves.

The loss or apparent accumulation of carotenes and xanthophylls proceeded somewhat differently during the incubation period. As shown in Figure 3.10, the carotene content decreased in the dark, whereas it remained fairly constant in the light. A decrease in the level of carotenes in the dark was followed by a rise to the initial level. The xanthophylls decreased gradually during the initial stages of light-incubation, and the rate of decrease was accelerated with the increase in incubation time. In the dark-incubated plants, xanthophylls just like carotenes, were rapidly degraded. The rapid loss of xanthophylls was followed by a transient increase in their level, after which the level remained fairly constant throughout the incubation period.

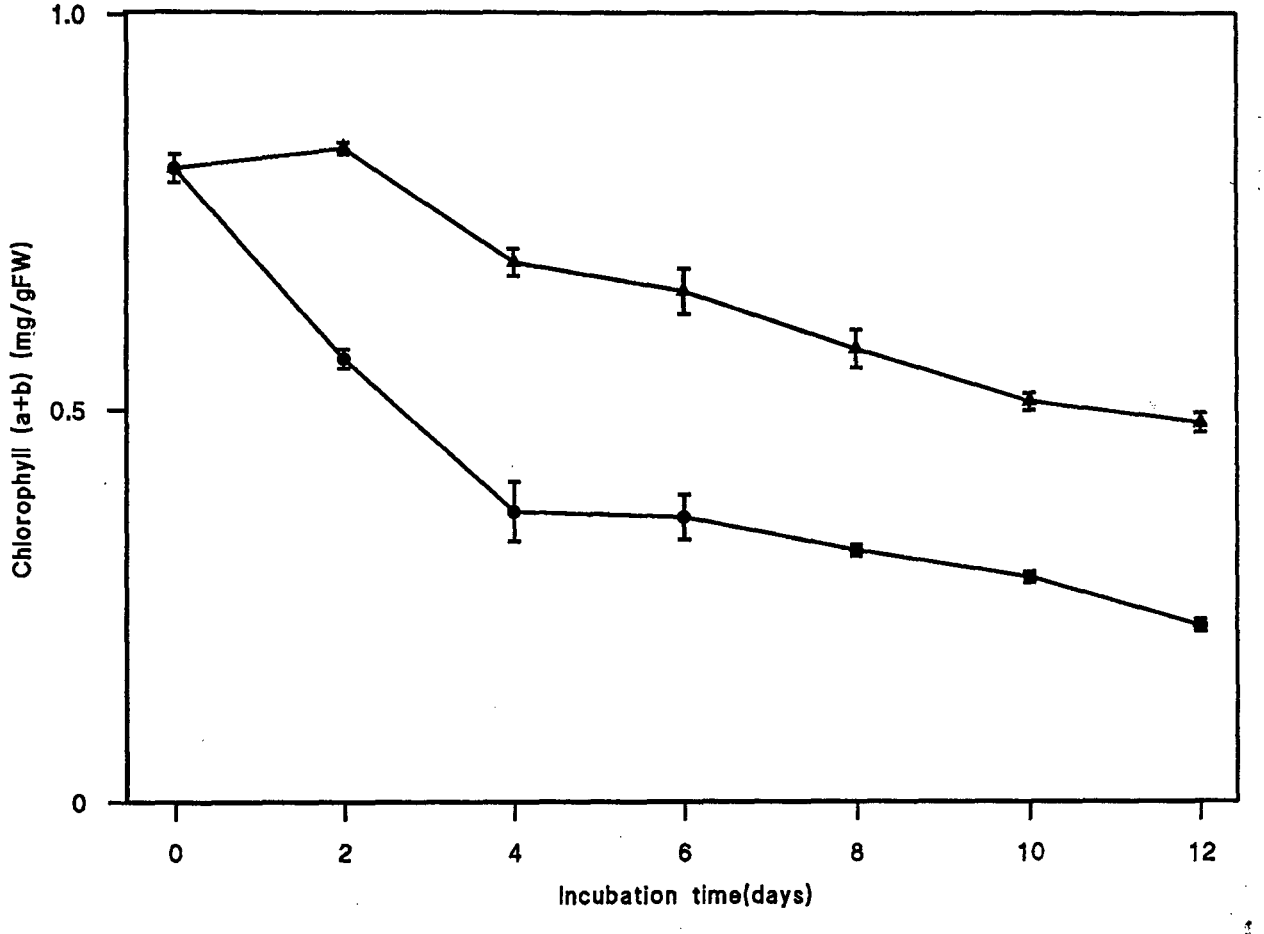

Figure 3.3 Chlorophyll ($a+b$) content of attached, senescing leaves of *Hordeum vulgare.*

7d old light-grown seedlings were incubated in either continuous light (▲) or total darkness (o) at 25°C. Leaf material was prepared as described in Sections 2.5 and 2.6.1. Data represent the mean of 3 replicates ±SE.

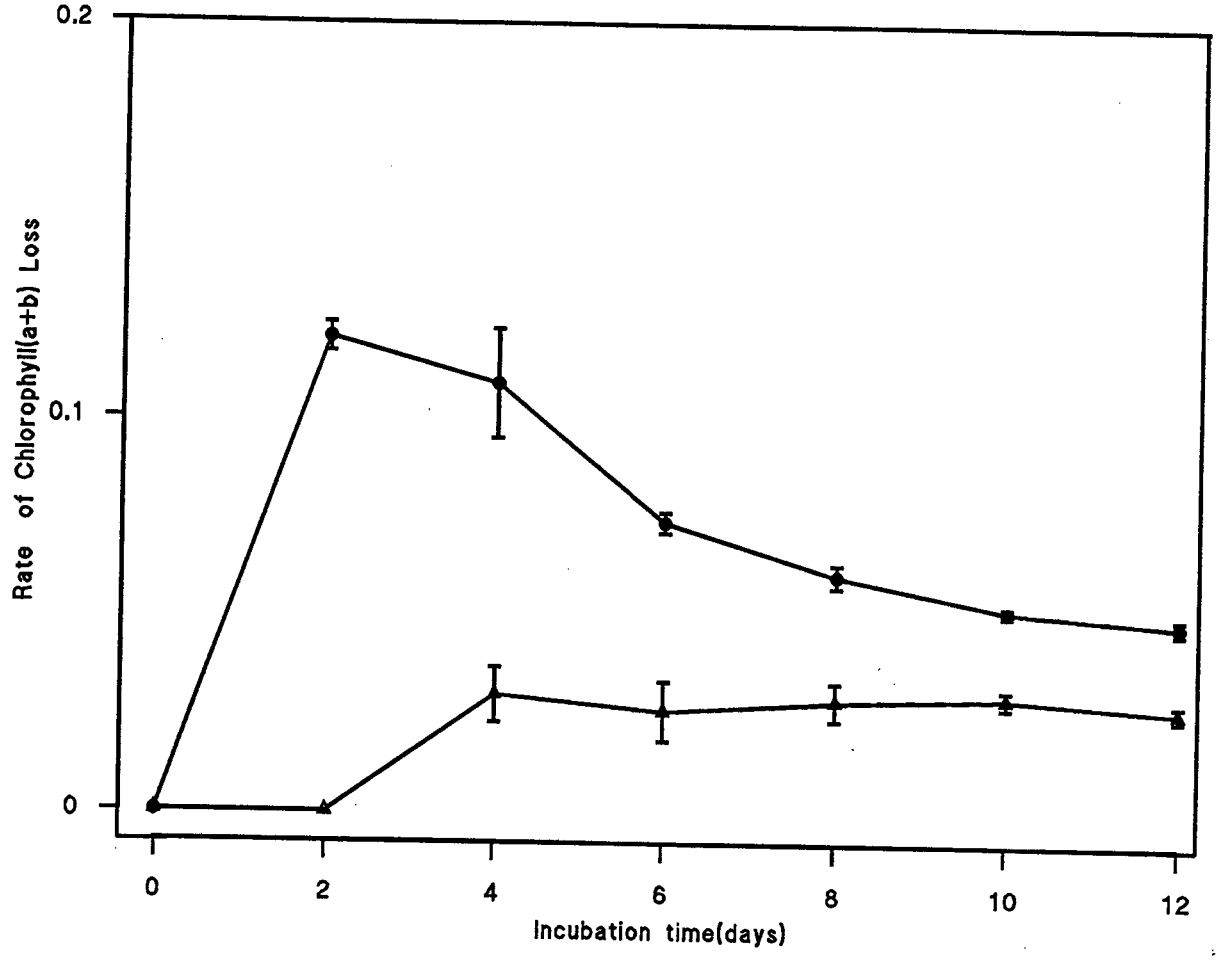

Figure 3.4 The rate of chlorophyll (a+b) loss from attached, senescing leaves of *Hordeum vulgare.*

Rate of chlorophyll ($a+b$) loss was calculated for each time point in Figure 3.3 by subtracting the initial concentration from the final concentration divided by the time taken to reach the final concentration. Values were plotted against the end time point of each time interval. Data are expressed as the mean of 3 replicates ±SE.

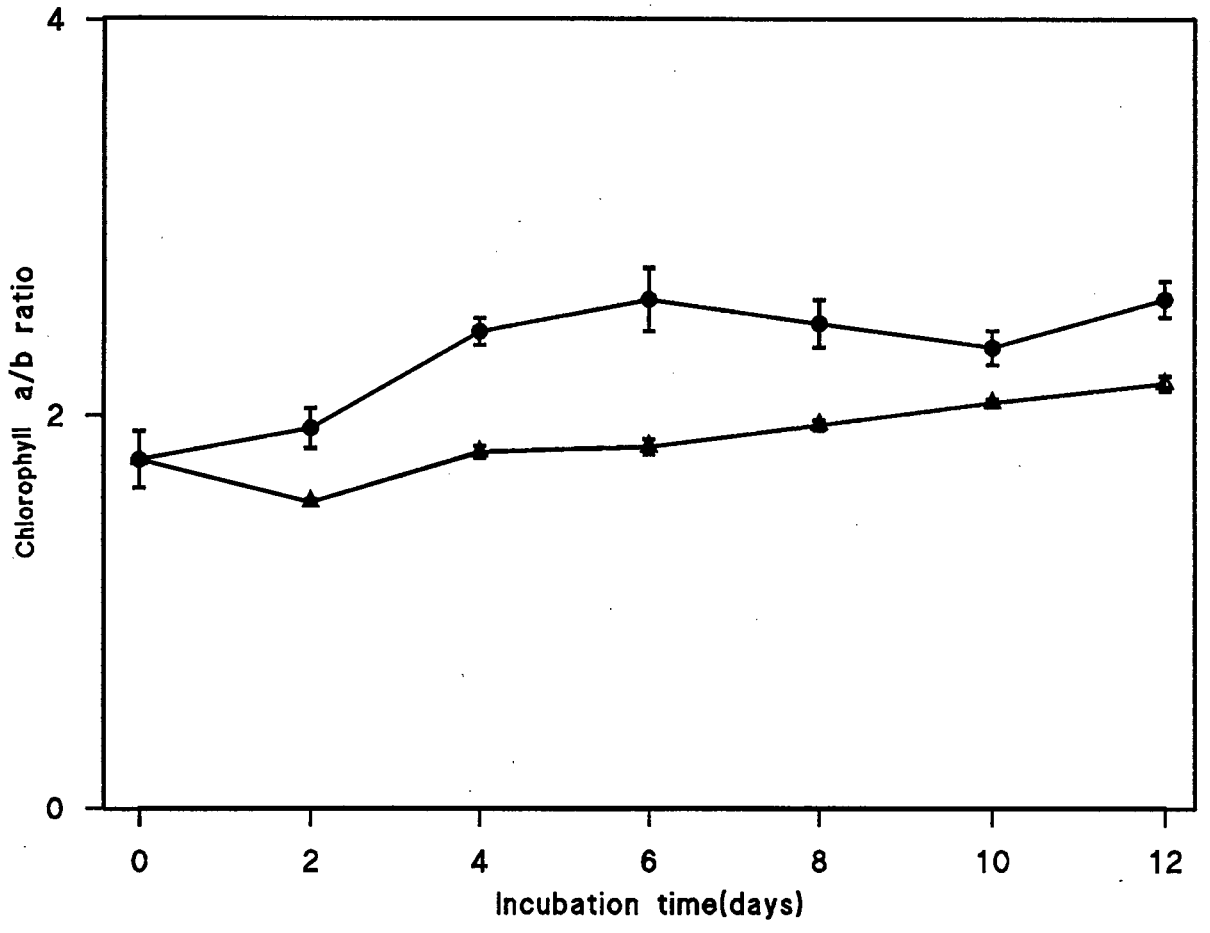

Figure 3.5 Kinetics of changes in the chlorophyll *a/b* ratio from attached, senescing leaves of *Hordeum vulgare*.

Seedlings were incubated in either continuous light (▵) or total darkness (o) at 25°C. The chlorophyll *a/b* ratio was calculated from values in Figure 3.3. Data are expressed as the mean of 3 replicates ±SE.

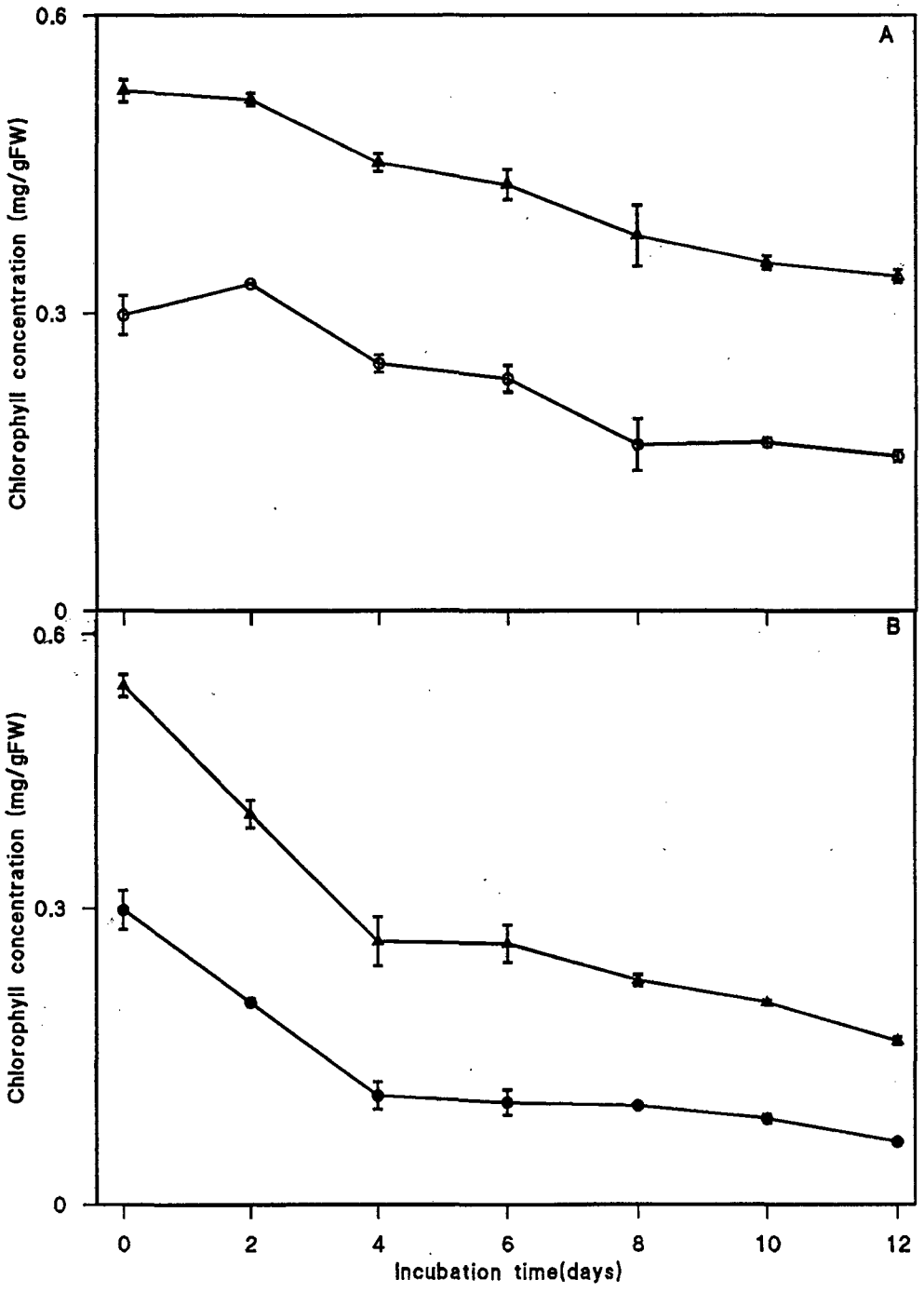

Figure 3.6 Changes in the content of chlorophyll *a* and chlorophyll *b* in attached, senescing leaves of *Hordeum vulgare.*

7d old light-grown seedlings were incubated in either continuous light (A) or total darkness (B) at 25°C. Chlorophyll *a* (▲) and chlorophyll *b* (o). Data represent the mean of 3 replicates ±SE.

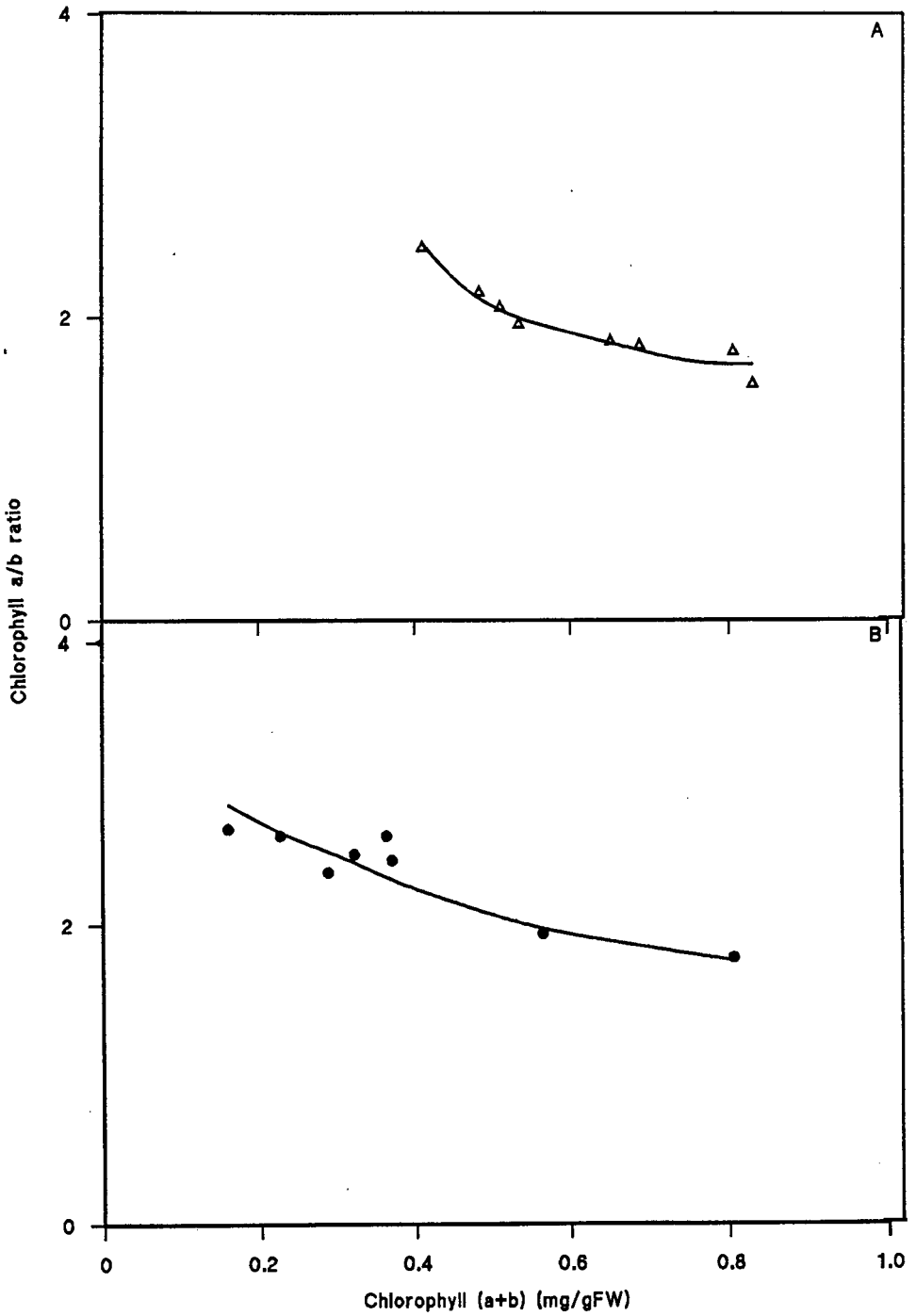

Figure 3.7 Relationship between chlorophyll $(a+b)$ concentration and chlorophyll a/b ratio in the attached, senescing leaves of *Hordeum vulgare.*
The relationship was determined for both the light (A) and dark (B) incubated seedlings. Each point is the mean of 3 replicates.

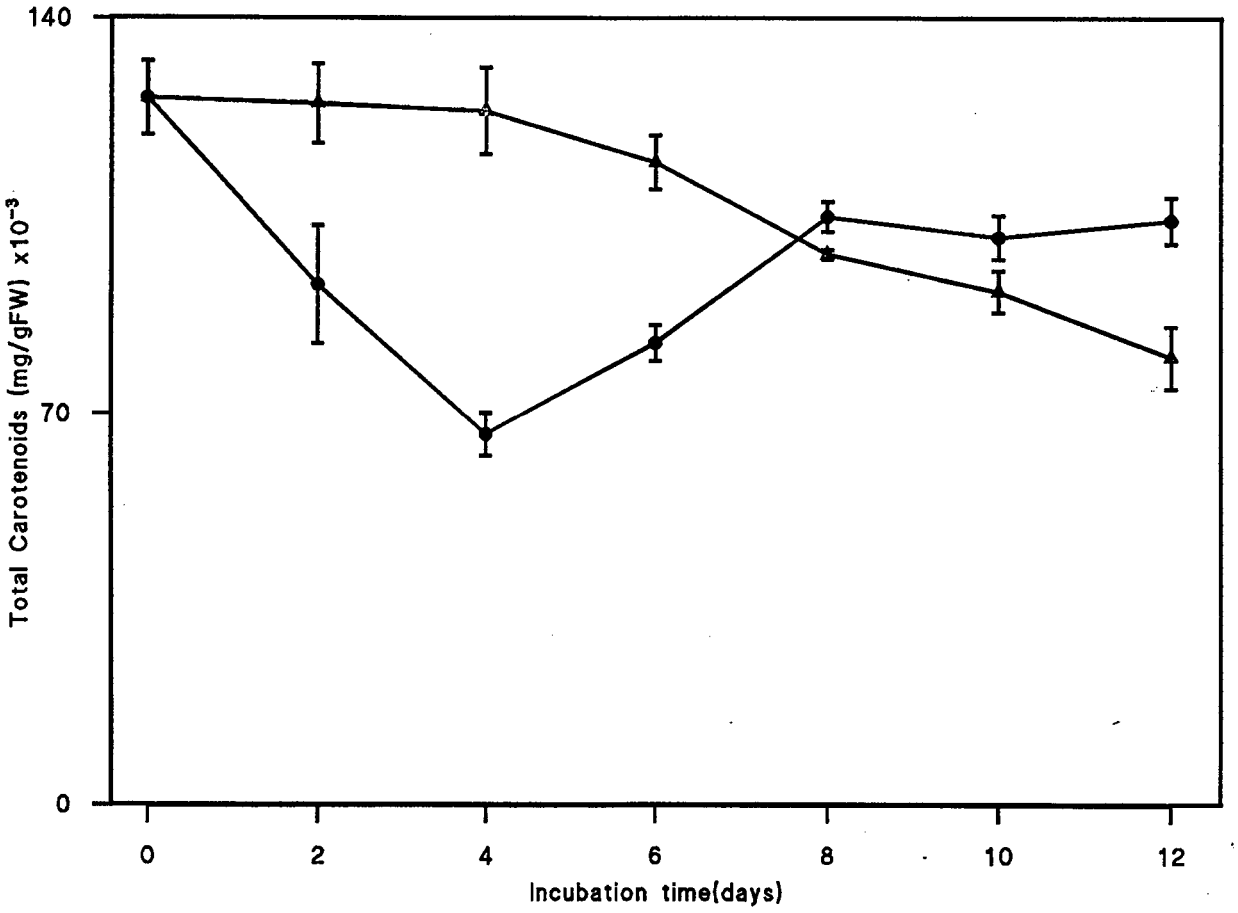

Figure 3.8 Total carotenoid content of attached, senescing leaves of *Hordeum vulgare.*

Total carotenoid content was calculated for both the light (▲) and dark (o) incubated plants from the values of xanthophylls and carotenes. Data represent the mean of 3 replicates ±SE.

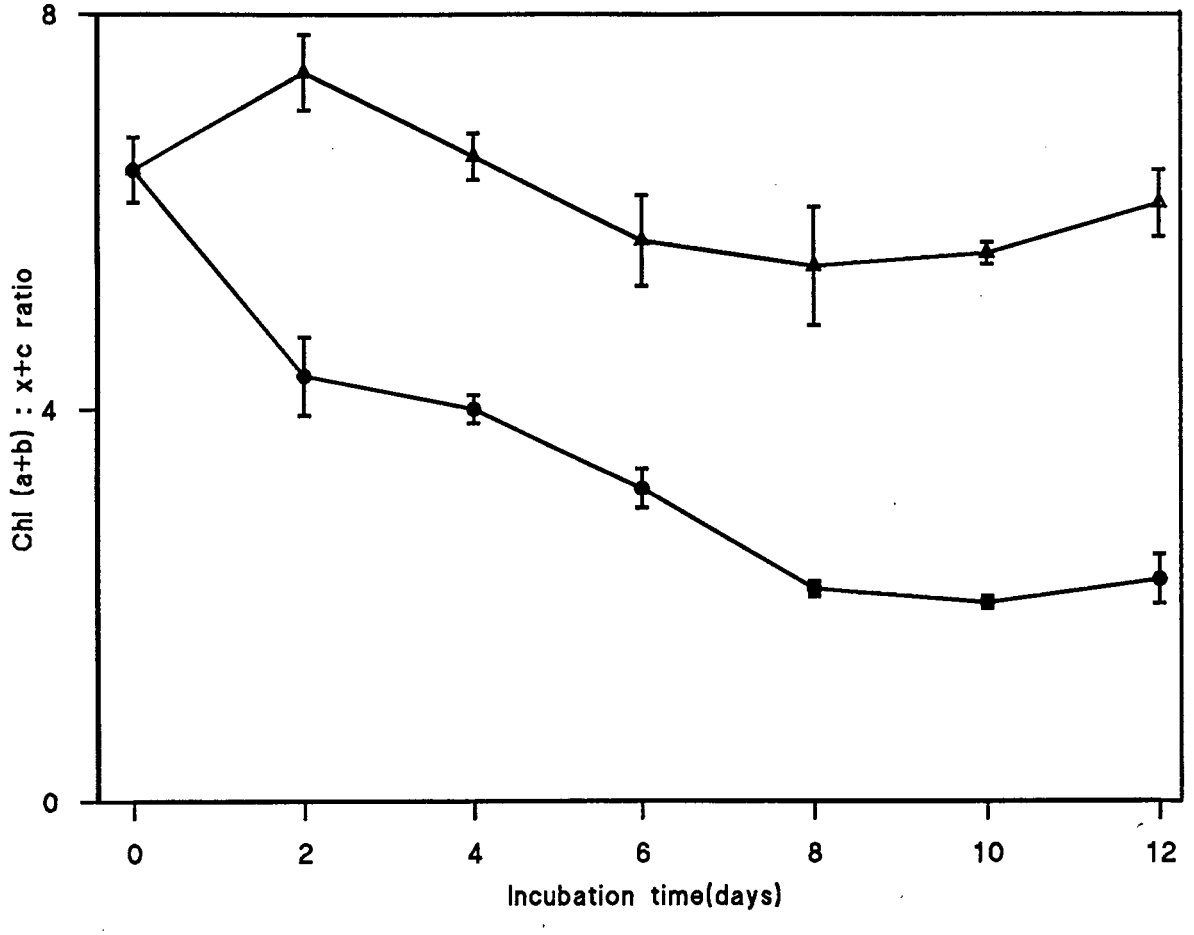

Figure 3.9 Kinetics of the chlorophyll ($a+b$):total carotenoid ($x+c$) ratio from attached, senescing leaves of *Hordeum vulgare.*

The ratio was calculated for both the light (▵) and dark (o) treatments by dividing the chlorophyll ($a+b$) concentration with that of total carotenoid at each time point. Data represent the mean of 3 replicates ±SE.

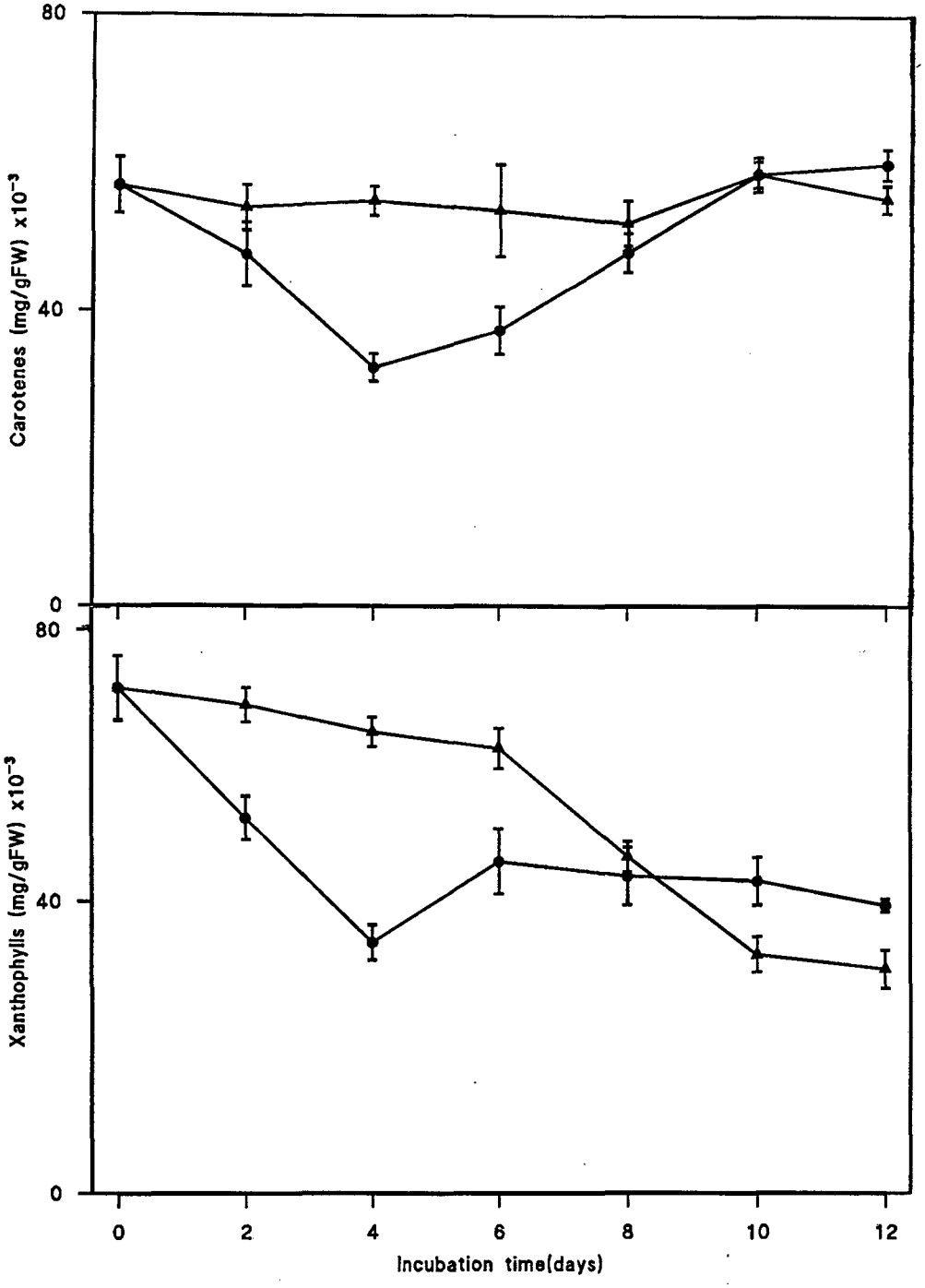

Figure 3.10 The carotene and xanthophyll content of attached, senescing leaves of *Hordeum vulgare.*

Leaf material was prepared as described in Sections 2.5 and 2.6.1. Light (▲) or dark (o) incubated. Data represent the mean of 3 replicates ±SE.

3.2.2 Determination of changes in carotenoid composition

Figure 3.11 shows the composition of carotenoids in the leaves of *H. vulgare* as determined by HPLC. Changes in levels of β-carotene, lutein, zeaxanthin, violaxanthin and antheraxanthin in senescing leaves of *H. vulgare* seedlings, incubated in either continuous light or total darkness, are presented in Figures 3.12 and 3.13. The results show that as foliar senescence progressed in light-incubated leaves, levels of β-carotene (Figure 3.12A) increased gradually and that this increase was associated with a decline in levels of zeaxanthin, antheraxanthin and violaxanthin (Figure 3.13A).

By comparison, the gradual increase in β-carotene levels from leaves of dark-incubated seedlings (Figure 3.12B) occurred concomitant with a marked decline in levels of zeaxanthin, antheraxanthin and violaxanthin (Figure 3.13B). Interestingly, in leaves from the light-incubated seedlings, levels of zeaxanthin remained greater than levels of both antheraxanthin and violaxanthin throughout the incubation. Conversely, in leaves from dark-incubated seedlings, levels of violaxanthin and antheraxanthin were greater than zeaxanthin later in the incubation. This observation suggested possible alterations in xanthophyll cycle activity due to an apparent increase in carbon flux via zeaxanthin to antheraxanthin and violaxanthin.

In order to elucidate the abovementioned aspect more fully, the time course of epoxidation of zeaxanthin to antheraxanthin and violaxanthin was calculated for both the light and dark treatments, and the results are presented in Figure 3.14. When expressed as percentage of the xanthophyll cycle pool (V+A+Z), the zeaxanthin component increased and was associated with decreased violaxanthin while percentage antheraxanthin remained fairly constant in senescing leaves from the light-incubated seedlings (Figure 3.14A), suggesting de-epoxidation of violaxanthin.

Calculation of the epoxidation state (EPS) of the xanthophyll cycle revealed a decline in the EPS as senescence progressed (Figure 3.14C), thus confirming that violaxanthin was de-epoxidized. In leaves from dark-incubated seedlings, the percentage zeaxanthin of V+A+Z declined and at the same time the EPS increased (Figure

3.14D). This observation indicated that in senescing leaves from dark-incubated seedlings, epoxidation state of the xanthophyll cycle favoured synthesis of all-*trans*-violaxanthin, a proposed precursor to ABA in higher plants. Although levels of violaxanthin declined substantially during the early stages of dark-induced senescence (Figure 3.13B), percentage violaxanthin of the V+A+Z did not change significantly during the course of incubation in darkness (Figure 3.14B), suggesting that violaxanthin may be further metabolized, possibly to ABA.

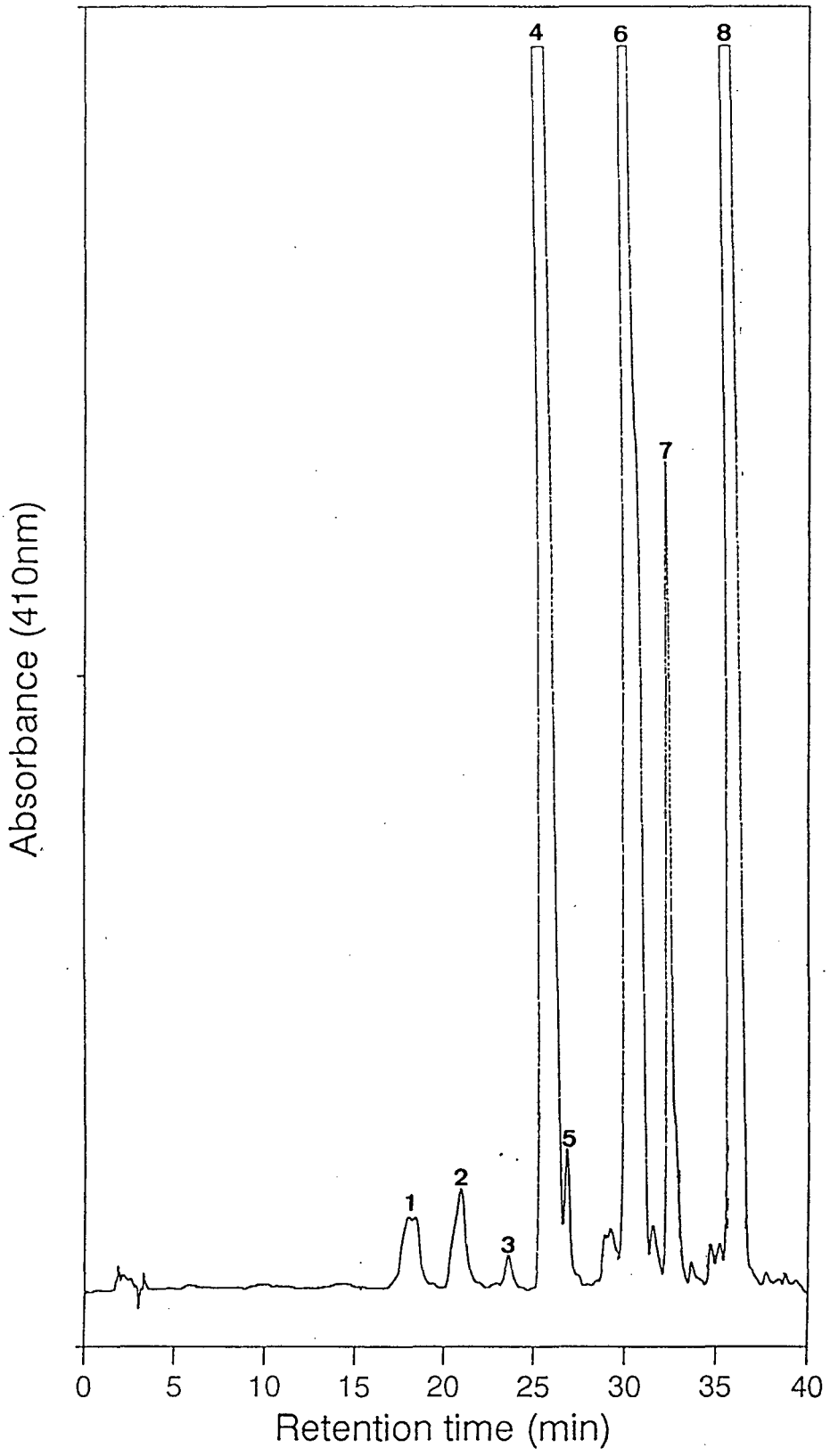

Figure 3.11 HPLC chromatogram of pigments extracted from leaves of 7d old, light-grown *Hordeum vulgare* seedlings.

Peaks: 1, Neoxanthin; 2, all-*trans*-violaxanthin; 3, antheraxanthin; 4, lutein; 5, zeaxanthin; 6, chlorophyll *b*; 7, chlorophyll *a*; 8, β-carotene.

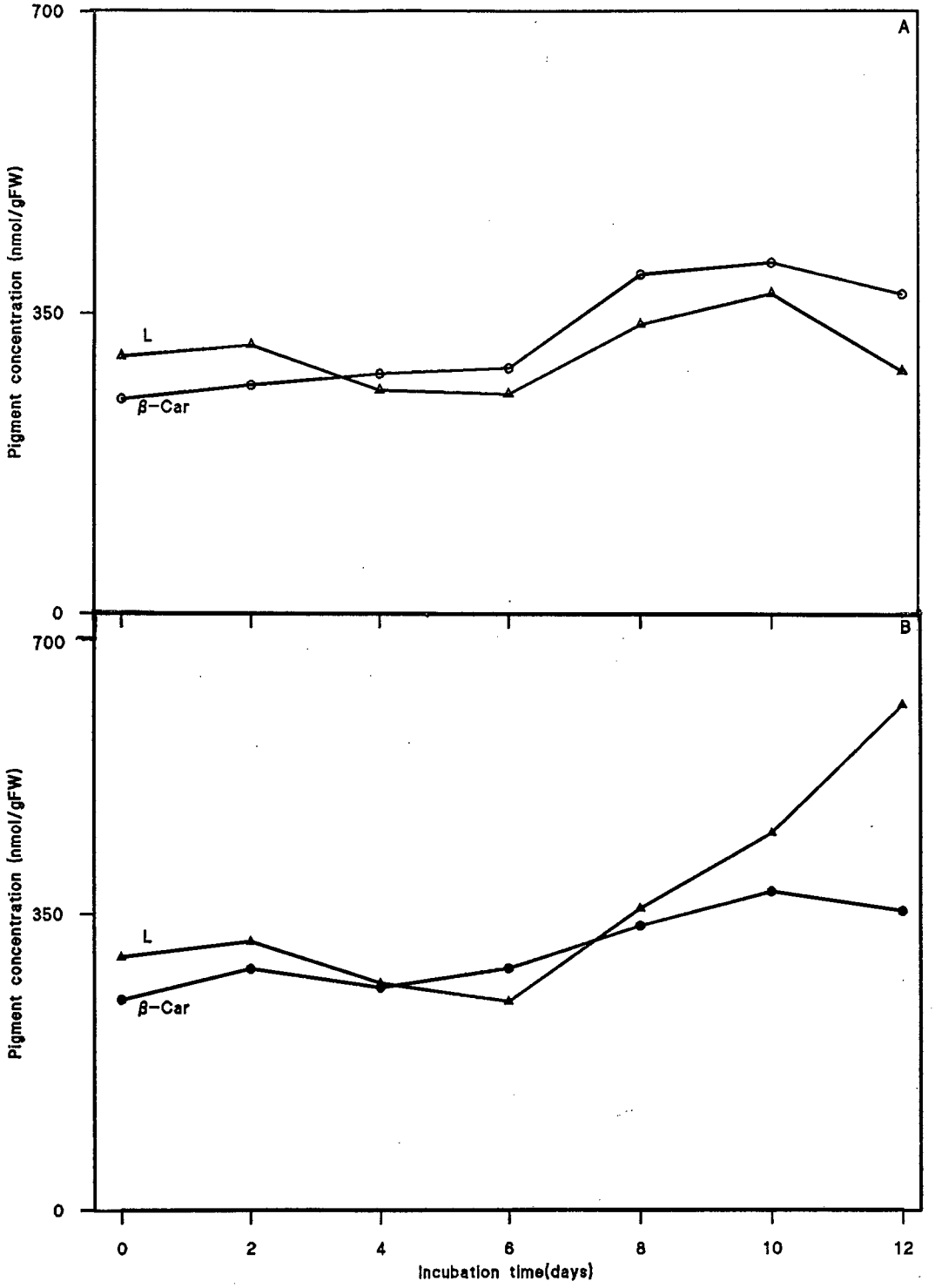

Figure 3.12 Changes in levels of lutein and β-carotene in attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown seedlings were incubated in either continuous light (A) or total darkness (B) at 25°C. Extracts were prepared as described in Sections 2.5 and 2.5.1.1.

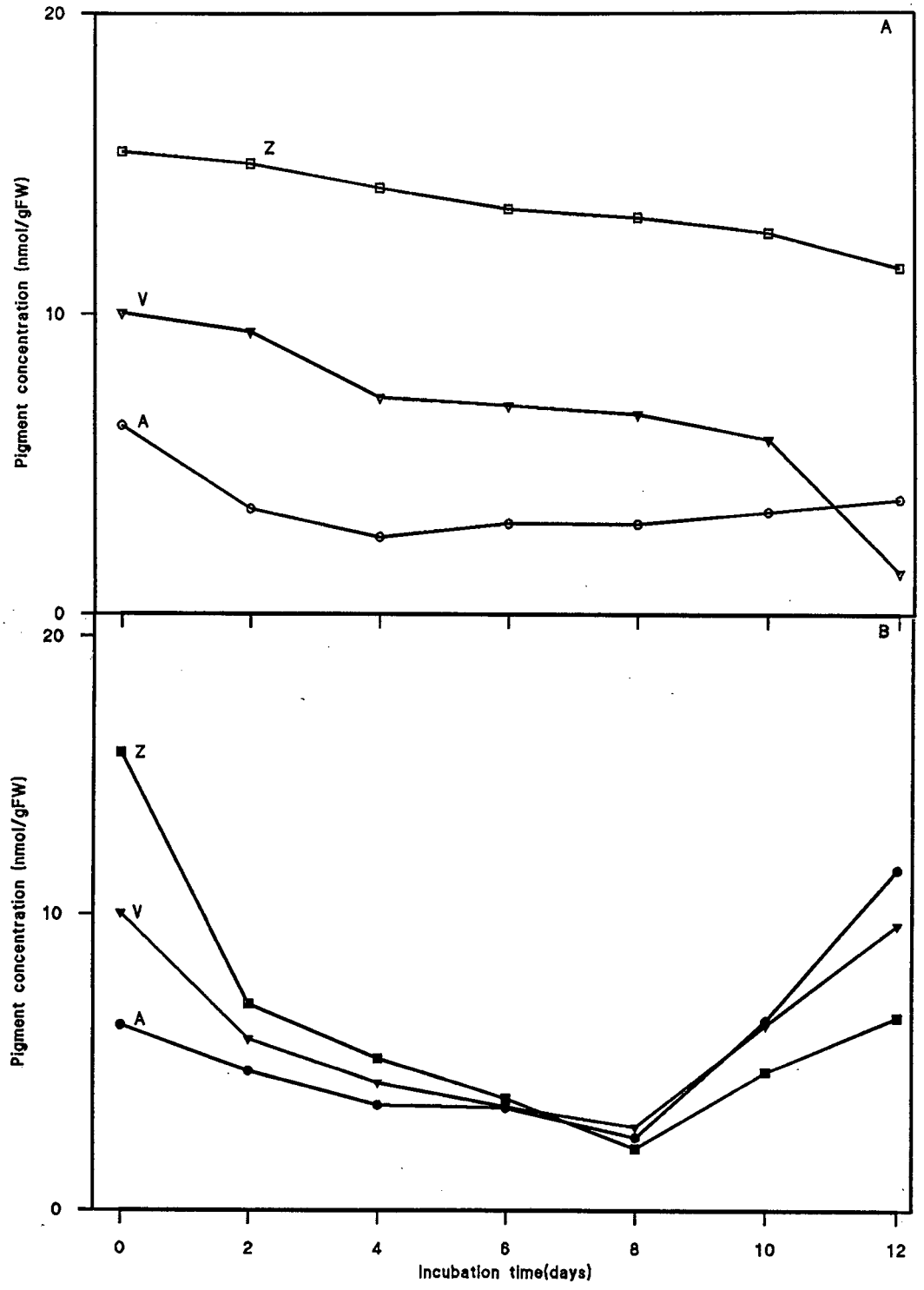

Figure 3.13 Changes in levels of zeaxanthin (Z), antheraxanthin (A) and violaxanthin (V) in attached, senescing leaves of *Hordeum vulgare*.

A, light-incubated; B, dark-incubated. Extraction and analysis of pigments were as described in Sections 2.5 and 2.5.1.1.

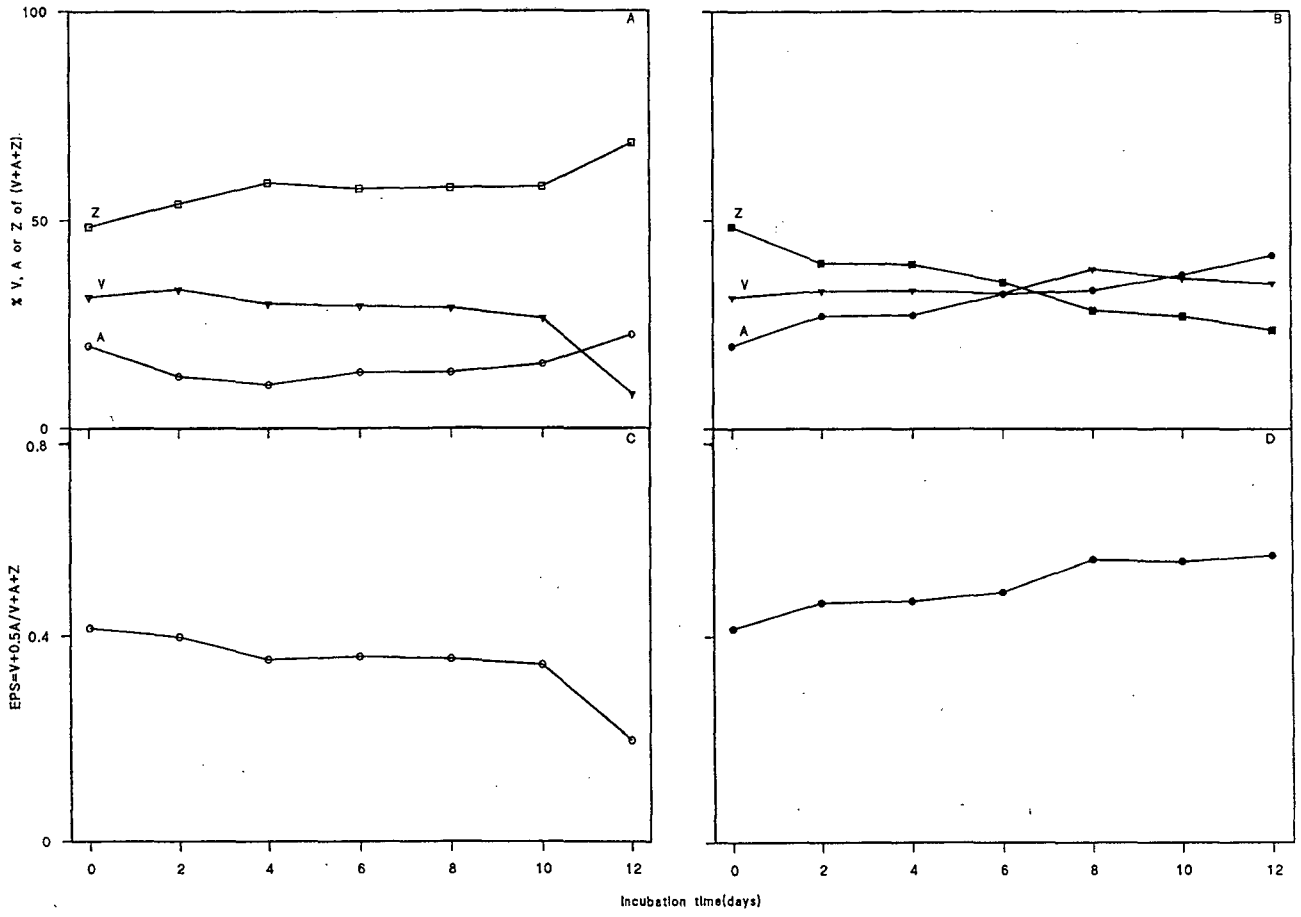

Figure 3.14 Time course of epoxidation of zeaxanthin (Z) to antheraxanthin (A) and violaxanthin (V) in senescing, attached leaves of *Hordeum vulgare*.

Results were calculated as described in Section 2.6.2.2 from the values in Figure 3.13. A and C, light-incubated; B and D, dark-incubated.

3.2.3 Changes in abscisic acid metabolism

In an attempt to correlate xanthophyll and ABA metabolism, the endogenous levels of both ABA and PA were monitored in senescing leaves from light and dark-incubated seedlings. Prior to kinetic studies, ABA and PA were unequivocally characterised by combined GC-MS as described in Section 2.6.6, and the electron impact mass spectra of the respective methyl ester derivatives are shown in Figure 3.15.

The results presented in Figure 3.16 show that levels of both ABA and PA increased only slightly as senescence progressed in attached leaves of light-incubated seedlings. In leaves from dark-incubated seedlings, ABA levels increased sharply, reaching a maximum 4 days after exposure to total darkness. Interestingly, maximum levels of ABA coincided with highly reduced levels of the carotenes and xanthophylls in the dark (Figure 3.10), suggesting a possible relationship between ABA synthesis and carotenoid metabolism. Furthermore, the decline in ABA levels after day 4 was accompanied by a rise in levels of PA, suggesting enhanced metabolism of ABA to PA in the dark-incubated plants.

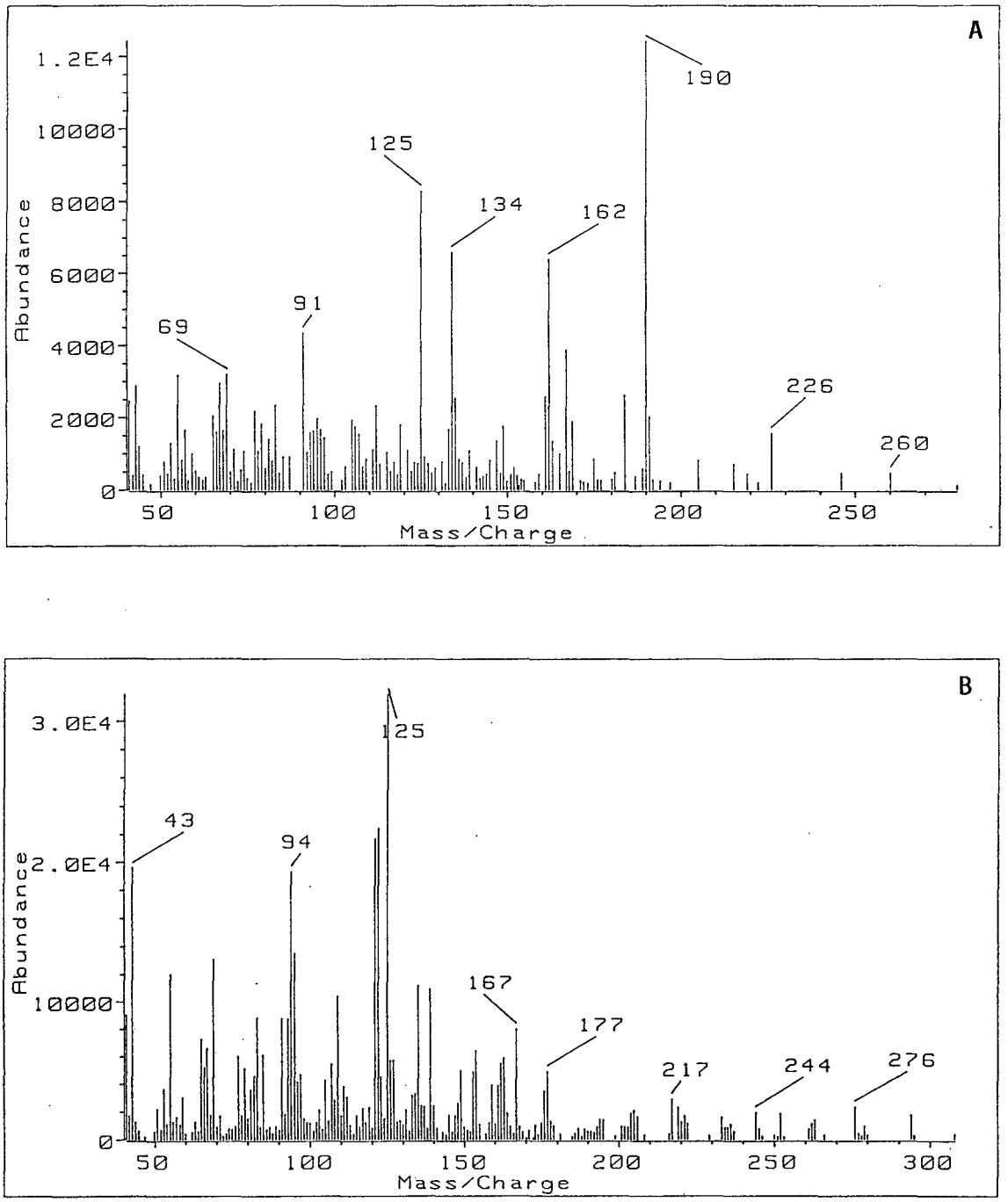

Figure 3.15 Electron impact mass spectra of ABAMe (A) and PAMe (B) prepared from attached, senescing leaves of *Hordeum vulgare*.

EI mass spectra were recorded at 70eV and a source temperature of 250°C (for details, see Section 2.6.6).

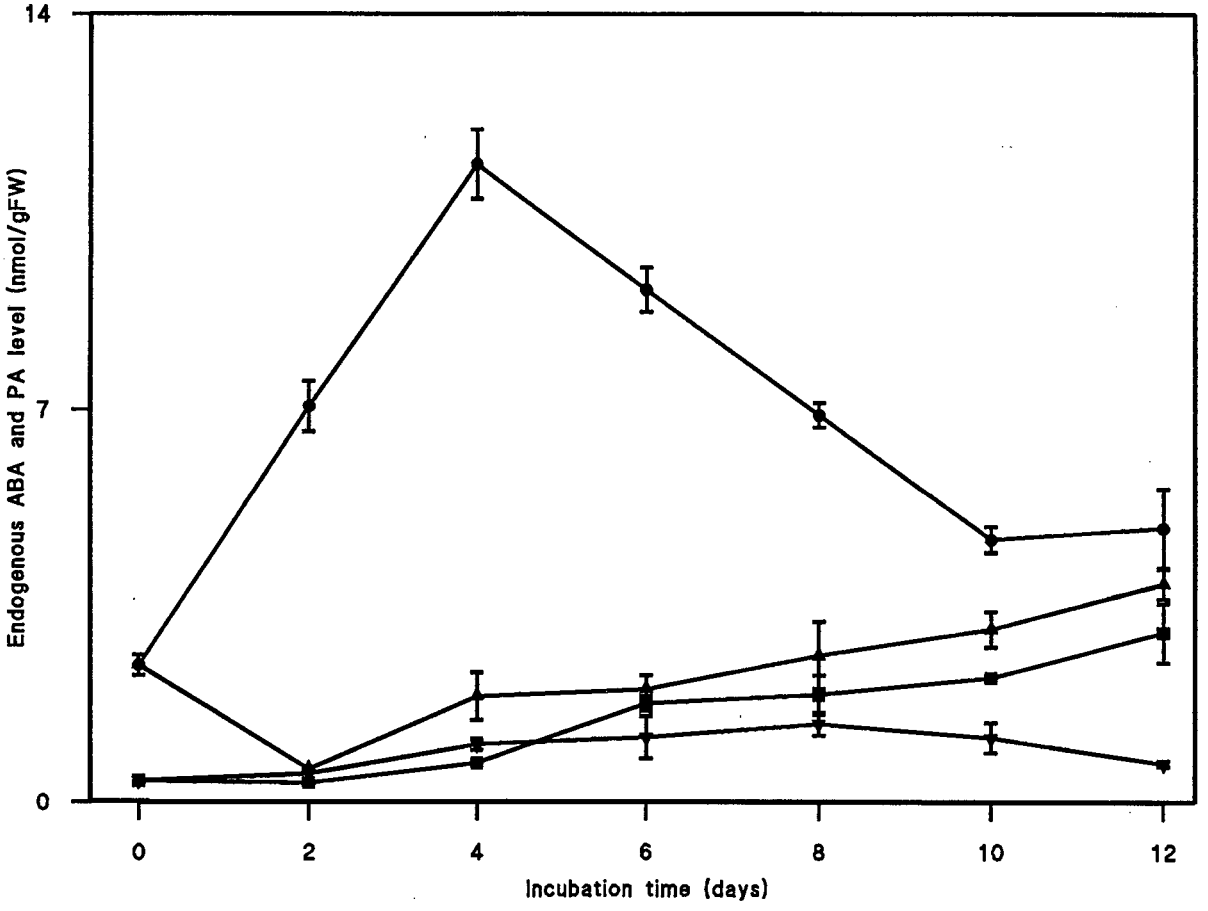

Figure 3.16 Changes in ABA and PA levels in attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown seedlings were incubated in either continuous light or total darkness at 25°C. Levels of ABA and carotenoids were measured in the same acetone extracts as described in Section 2.5. ABA and PA were purified and quantified as described in Sections 2.5.1.2 and 2.6.3. ABA in light (▲) or dark (o), and PA in light (▼) or dark (□).

3.2.4 Stoichiometry of the ABA, carotenoid interrelationship

The results presented in Table 3.1 show the kinetics of reduction in levels of violaxanthin and 9'-*cis*-neoxanthin and accumulation of ABA and PA in senescing leaves from dark-incubated seedlings. After exposure to darkness, 9'-*cis*-neoxanthin levels were reduced and then remained constant throughout the incubation period, while levels of violaxanthin decreased up to day 8. The data show an apparent 1:1 relationship on a molar basis between loss of violaxanthin and 9'-*cis*-neoxanthin and accumulation of ABA and PA during the first 6 days of incubation. Lack of stoichiometric relationship from days 8 to 10 of incubation may be due to factors such as reduced pool of ABA precursors, increased rate of ABA catabolism and/or export of ABA and related compounds from senescing tissues.

The apparent stoichiometric relationship after 12 days exposure to darkness is anomalous since levels of violaxanthin, antheraxanthin and zeaxanthin accumulate during the later stages of dark-induced senescence (Figure 3.13B), suggesting inhibition of oxidative cleavage of carotenoid precursors. Because the amount of ABA ultimately produced is equal to the amount of xanthophylls which disappear (Li and Walton, 1990), it is possible that either a normal intermediate or a compound which can be reconverted to a normal intermediate accumulates during senescence. However, no such compound was detected in the present investigation. Nevertheless, results from the present study are consistent with the findings of Li and Walton (1990).

Table 3.1 Kinetics of Reduction in Violaxanthin and 9'-*cis*-Neoxanthin and Accumulation of ABA and PA in leaves of light-grown *Hordeum vulgare* Seedlings Exposed to Darkness

Seedlings were grown for 7 days under continuous white light at 25°C, and then transferred to total darkness. At intervals, levels of violaxanthin, 9'-*cis*-neoxanthin, ABA and PA were determined in the same acetone extracts as described in Section 2.5. Results are differences between initial and final values, and are the mean of 3 replicates ±SE. Initial values were violaxanthin, 10.1 ± 0.9; 9'-*cis*-neoxanthin, 5.9 ± 0.4; ABA, 2.5 ± 0.3; PA, 0.2 ± 0.09, all nmol/gFW.

Time	V	9'-*cis*-Nx	V+9'-*cis*-Nx	ABA	PA	ABA + PA
d		decrease nmol/gFW			increase nmol/gFW	
2	4.3±0.7	1.7±0.1	6.0±0.8	4.5±0.1	0.6±0.1	5.1±0.2
4	5.7±0.7	4.9±0.3	10.6±1.0	10.6±1.0	0.9±0.1	11.5±1.1
6	6.6±0.5	4.9±0.2	11.5±0.7	8.8±0.6	1.6±0.6	10.4±0.8
8	7.2±0.6	4.8±0.3	11.4±0.9	4.9±0.2	1.8±0.2	6.7±0.4
10	3.8±0.6	4.9±0.3	8.7±0.9	2.2±0.2	2.3±0.3	4.5±0.4
12	0.4±0.0	4.3±0.2	4.7±0.2	2.3±0.1	3.0±0.2	5.3±0.3

CHAPTER FOUR

SENESCENCE-INDUCED CHANGES IN POLYPEPTIDE PATTERNS AND ACTIVITY OF HYDROLYTIC ENZYMES

4.1 INTRODUCTION

Senescence consists of a complex temporal sequence of events, and is characterized by a decline in chlorophyll and protein content, photosynthetic capability and by reduction of carbohydrate content, leading finally to disintegration of the chloroplast and to cell death (Boschetti *et al.*, 1990). A decline in photosynthetic capability can be attributed in part to curtailed synthesis and rapid degradation of Calvin cycle enzymes, and a consequent decline in the dark reactions of photosynthesis (Roberts *et al.*, 1987). Levels of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in particular are known to decrease dramatically during the early stages of leaf senescence (Vera *et al.*, 1990).

In addition to protein breakdown, studies have revealed the synthesis of new proteins during the course of foliar senescence, suggesting the involvement of *cis*- and *trans*-acting elements. However, in spite of the available information regarding senescence, no information on the nature of these elements involved in the initiation of gene expression during senescence of leaves has been forthcoming. Nevertheless, it has been shown that chloroplasts isolated from attached senescent *Hordeum vulgare* leaves, synthesize several proteins that are different from those synthesized in young leaves (Garcia *et al.*, 1983; Martin and Sabater, 1989; Vera *et al.*, 1990; Cuello *et al.*, 1991; Guéra and Sabater, 1992; Thomas *et al.*, 1992). Although the functional role of the polypeptides synthesized by chloroplasts during senescence is unknown (Martin and Sabater, 1989), Thomas and Stoddart (1982) have suggested that some of these polypeptides synthesized '*de novo*' might be representative of precursors of hydrolytic enzymes which are known to increase during senescence.

Evidence has been provided for genes induced by darkness. Kawakami and Watanable (1988) reported that mRNA for cytosolic glutamine synthetase was one of the dark-inducible mRNAs which increased during the late stages of dark-induced senescence. A group of senescence-related cDNAs has been cloned from a library constructed with mRNA from ripening *Lycopersicon esculentum* (Davies and Grierson, 1989). The level of mRNAs corresponding to these cDNA clones were controlled by ethylene produced by the tissue. Azumi and Watanable (1991) reported the expression of a gene in senescing *Raphanus sativus* cotyledons shortly after exposure of the cotyledons to darkness. The expression of dark inducible genes was modulated not only by light but also by physiological stimuli such as ethylene and cytokinin. From these reports, it seems that light and plant growth regulators may contribute to the co-ordination of senescence at the transcription and/or translational level.

The occurrence of two isoperoxidases that increased during ethylene-induced senescence has been reported for *Cucumis sativa* cotyledons (Abeles *et al.*, 1988). Roberts and Kolattukudy (1989) also demonstrated the induction of a highly anionic protein in calli of *Lycopersicon esculetum* and *Solanum tuberosum.* This protein had similar mobility to that of purified anionic peroxidase and it was therefore suggested that this ABA-induced protein is the highly anionic peroxidase. The induction of anionic peroxidase by ABA involved transcriptional activation of the anionic peroxidase gene rather than a post-translational activation of a pre-existing protein (Roberts and Kolattukudy, 1989). Thus, the expression of peroxidase genes appears to be regulated by plant growth regulators.

Lipid peroxidation is a characteristic feature of senescing membranes (Dhinsa *et al.*, 1981; Fobel *et al.*, 1987). Although the physiological role of LOX in plants remains unclear, increase in LOX activity has been associated with senescing membranes (Lynch and Thompson, 1984; Fobel *et al.*, 1987; Siedow, 1991). The bleaching of carotenoid solutions by LOX has been demonstrated (Firn and Friend, 1972; Parry and Horgan, 1991a). Incubation of LOX and violaxanthin produced xanthoxin (Xan) and *trans*-Xan, although in low yields. Xanthoxin is readily converted to ABA by plant tissues (Parry *et al.*, 1988). Hildebrand (1989) speculated that

LOX may be involved in an alternative pathway for ABA synthesis from the carotenoid violaxanthin. Indeed, Creelman *et al.* (1992) have recently reported the involvement of a LOX-like enzyme in the biosynthesis of ABA. It is therefore likely that LOX may be involved in carotenoid cleavage in senescing tissues.

Accumulation of ABA is a characteristic response of plants exposed to stress. In addition, extractable activity of a number of hydrolytic enzymes also increases. In this regard, the response of plants to stress is similar to the responses observed during the course of senescence. It has been shown that water stress enhances the expression of an α-amylase gene in leaves of *H. vulgare* (Jacobsen *et al.*, 1986). Tucker and Grierson (1987) have also demonstrated an increase in α-amylase activity during ripening. Thus, it is not unreasonable to expect that activity of α-amylase in *H. vulgare* leaves might be altered by the onset of senescence.

Leaf senescence and proteolysis have been investigated in several tissues (Kang and Titus, 1989). Rubisco is the most abundant protein substrate degraded during senescence (Kasemir *et al.*, 1988). According to Kervinen *et al.* (1990), changes in protease activities in senescing tissues could regulate the degradation of protein. Therefore, the major regulatory mechanisms involved in proteolysis of senescing leaves may include synthesis of proteolytic enzymes and change in enzyme activity (Miller and Huffaker, 1985; Dalling, 1987; Veierskov and Thimann, 1988).

4.2 OBJECTIVE

Leaf senescence is associated with changes in the polypeptide pattern, particularly that of the chloroplast (Guéra *et al.*, 1989; Vera *et al.*, 1990; Thomas *et al.*, 1992) and as mentioned above some of these polypeptides might be representative of hydrolytic enzymes. The present investigation involved (a) demonstrating senescence-induced changes in the pattern of polypeptides in leaves of *H. vulgare* and (b) attempting to relate these change to changes in hydrolytic enzyme activity.

4.3 RESULTS

A characteristic response shown by senescing organs is loss of total extractable protein (for review, see Peoples and Dalling, 1988). A change in protein content not only implies increased degradation but also an increase in protein turnover. Additionally, evidence is now accumulating that several novel proteins are synthesized during the course of leaf senescence. In order to determine changes in protein complement during the course of leaf senescence in *H. vulgare*, extractable protein was measured, differential changes in polypeptide patterns were examined and alterations in the activity of several hydrolytic enzymes investigated.

4.3.1 Senescence-induced changes in total extractable protein

Although foliar senescence is associated with loss of total protein (Peoples and Dalling, 1988), recent evidence suggests that several novel polypeptides are synthesized in senescing chloroplast (Guéra and Sabater, 1992; Thomas *et al.*, 1992). The results presented in Figure 4.1 show that loss of total leaf protein was retarded in light, whereas it continued rapidly in dark-incubated seedlings. In isolated chloroplasts, purified thylakoid and stromal fractions there was an increase in protein content during the first 4 days of dark-incubation, after which it declined. In the light, there was a gradual increase in protein content and it decreased in the thylakoid after 4 days of incubation. The data tend to suggest that transfer of seedlings into darkness results in a transient increase in the level of chloroplastic proteins which may result from alterations in polypeptide pattern and/or synthesis of new enzymes.

4.3.2 Senescence-induced change in polypeptide patterns

4.3.2.1 Percoll-purified chloroplasts

The viability of the chloroplasts in senescing intact leaves of *H. vulgare* was tested by assaying for the marker enzyme, ribulose-1,5- bisphosphate carboxylase/oxygenase (Rubisco). Carboxylase activity was determined in intact chloroplasts by the incorporation of [^{14C}] into the acid-stable product, 3-phosphoglyceric acid (PGA). As shown in Figure 4.2, there was no significant difference in the incorporation of radioactivity into PGA between the light- and dark-incubated plants, suggesting that the chloroplast was functional in both treatments.

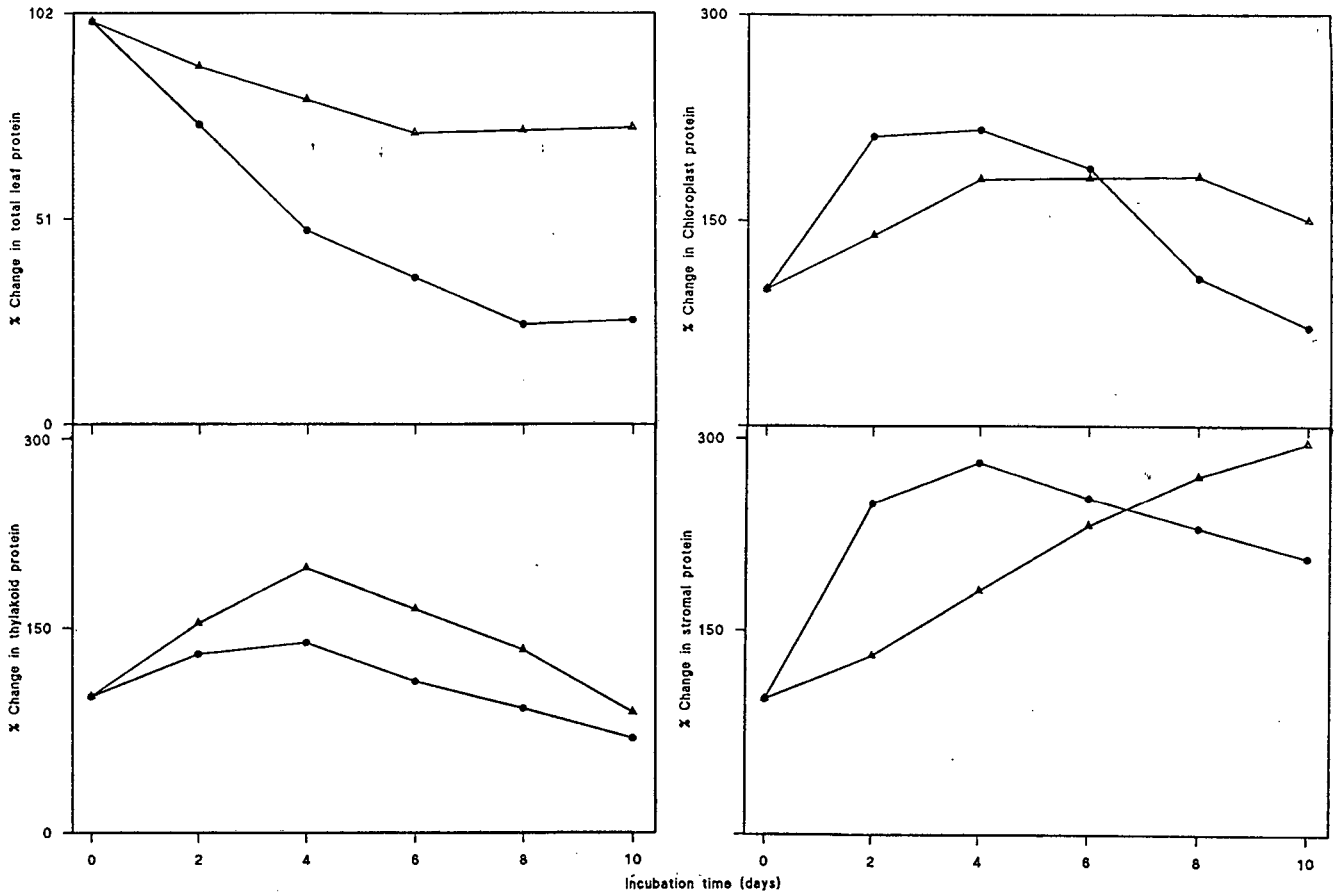

Figure 4.1 Percentage change in the protein content of attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown (140 $\mu molm^{-2}s^{-1}$ for chloroplasts preparation) seedlings were incubated in either continuous light (▵) or total darkness (o) at 25°C. Crude extract and chloroplasts were prepared as described in Sections 2.3.2 and 2.3.3, respectively. Protein content was measured using the Bradford method. Percentage change was calculated by subtracting initial concentration from the final one and dividing by initial concentration. Initial protein concentrations were total leaf protein, 15.65 mg/gFW; chloroplast, 258.0 μg; thylakoid, 465.0 μg; stroma, 25.0 μg.

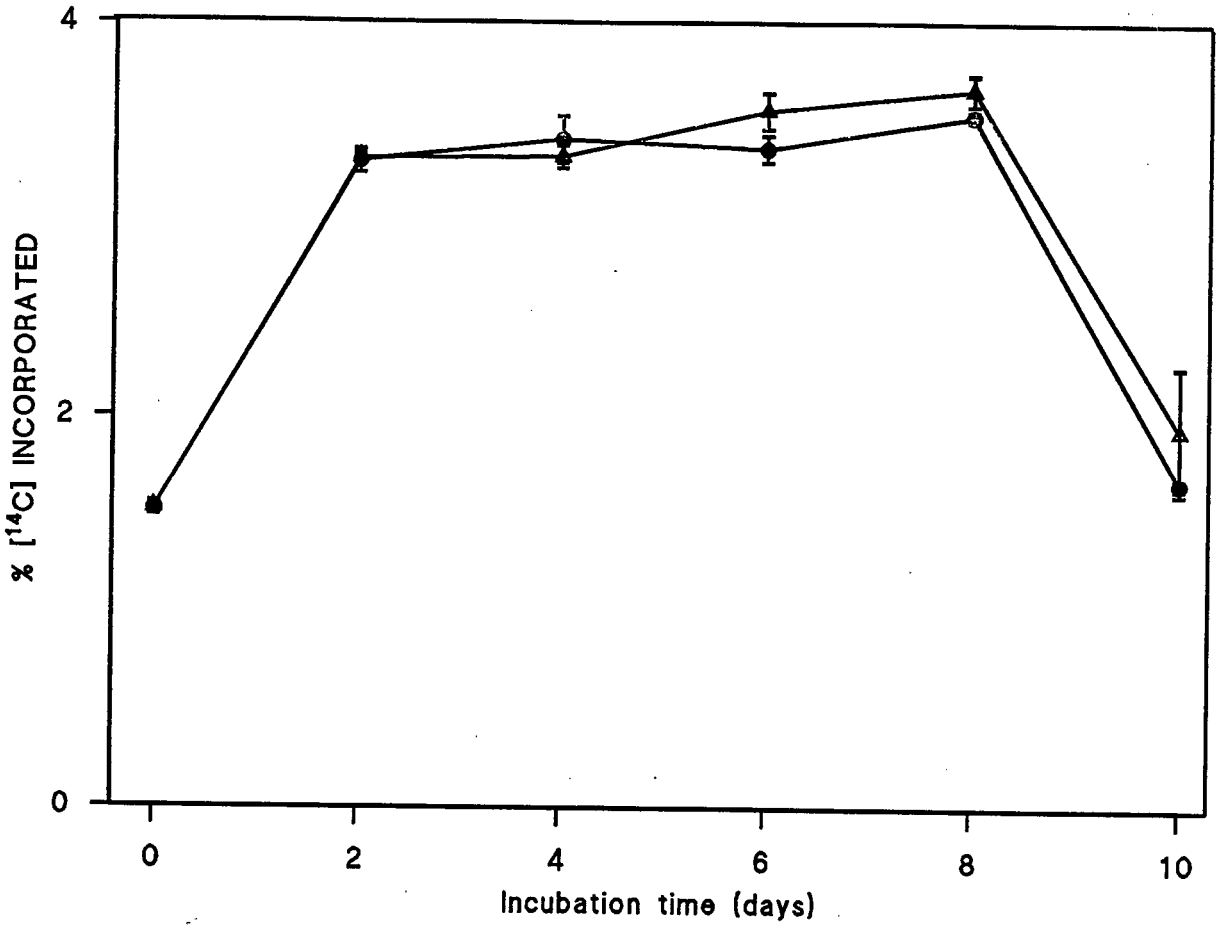

Figure 4.2 Time-course of Rubisco activities from intact chloroplast of *Hordeum vulgare*.

7d old light-grown (140 $\mu molm^{-2}s^{-1}$)seedlings were incubated in either continuous light (▲) or total darkness (o). Chloroplasts were prepared and incubated as described in Sections 2.3.3 and 2.4.5. Rubisco activity was determined by measuring the rate of incorporation of [^{14}C] into acid-stable reaction products. Data represents the mean of 3 replicates ±SE.

Results presented in Figure 4.3 show that the polypeptide pattern present in chloroplasts (as detected by Coomassie staining) were very similar in leaves from light- and dark-incubated plants. The major polypeptide bands were 47.9, 24.3, 21.9 and 20.9 kDa. Some faint bands with molecular masses 29.8, 27.5 and 26.0 kDa were resolved. A 47.9 kDa protein was very close in its mobility to a 48 kDa polypeptide reported by Martin and Sabater (1989) in isolated intact chloroplast from senescent leaves of *H. vulgare*. In addition, the 26.0 and 27.5 kDa proteins co-migrated with polypeptides identified by Grebanier *et al.* (1979) as subunits of light-harvesting complex in the chloroplast of *Zea mays*. With the exception of a 20.9 kDa protein, the intensity of the major polypeptide bands decreased with the progression of senescence. However, decline in intensity of the bands was more pronounced in leaves from the dark-incubated plants. Intensity of the minor polypeptide bands was greatly reduced in dark-incubated plants.

4.3.2.2 Thylakoid membranes

As shown in Figure 4.4, SDS-PAGE resolved more clearly 7 polypeptide bands in the thylakoids prepared from senescing leaves of *H. vulgare*. The most prominent polypeptide bands have molecular masses of 51.2, 25.3, 22.5 and 20.5 kDa. Minor polypeptide bands with molecular masses 57.9, 29.9 and 28.7 kDa were resolved. The 51.2 kDa polypeptide was very close in its mobility to the 51 kDa PSII protein reported for *Spirodela oligorrhiza* by Callahan *et al.* (1989). Intensity of the 51.2 kDa band (lane 7 and 10), and of some bands with lower molecular masses in the region of 25.3 (lane 10) and 22.5 kDa (lane 10) slightly decreased with the progression of senescence. A 57.9 kDa polypeptide became very faint in the dark (lane 7-10). The appearance of a new polypeptide band with an apparent molecular mass of 28.7 kDa (lane 8- 10) was observed, and that correlated with the observed alterations in the 29.9 kDa band. It therefore remains possible that the new polypeptide may be a degradation product of the 29.9 kDa protein. Since equal amounts of protein were represented in each lane, these results tend to suggest that little changes did occur in the thylakoid polypeptide bands during senescence.

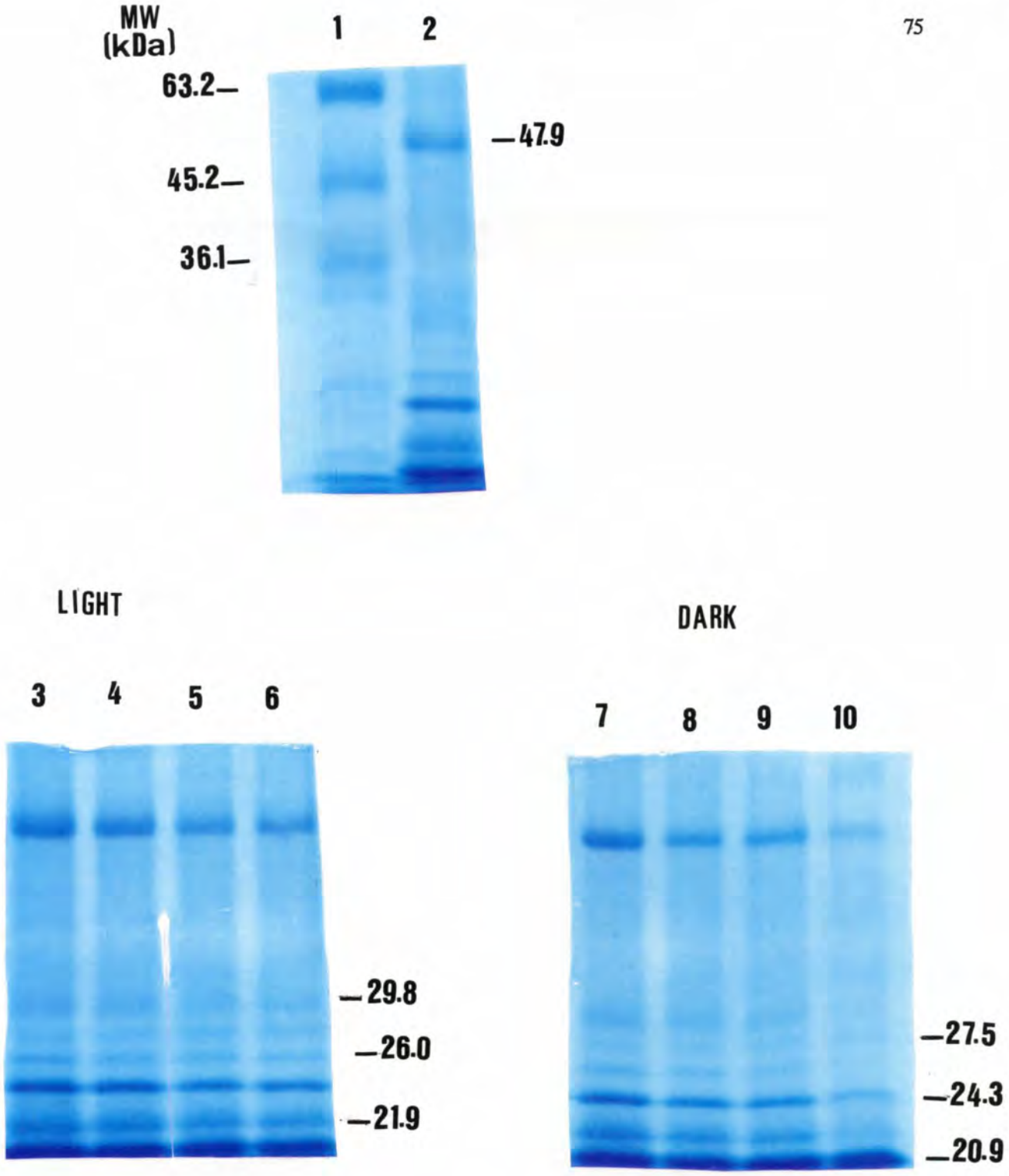

Figure 4.3 SDS-polyacrylamide gel electrophoresis of the lysed chloroplasts prepared from attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown (140 $\mu molm^{-2}s^{-1}$)seedlings were incubated in either continuous light or total darkness at 25°C. Chloroplasts were prepared as described in Section 2.3.3. Gel lanes were loaded with 5 μg of chloroplast protein (lane 2-10). Molecular weight markers BSA, Rubisco and peroxidase (lane 1) were loaded as described in Section 2.6.7.1.

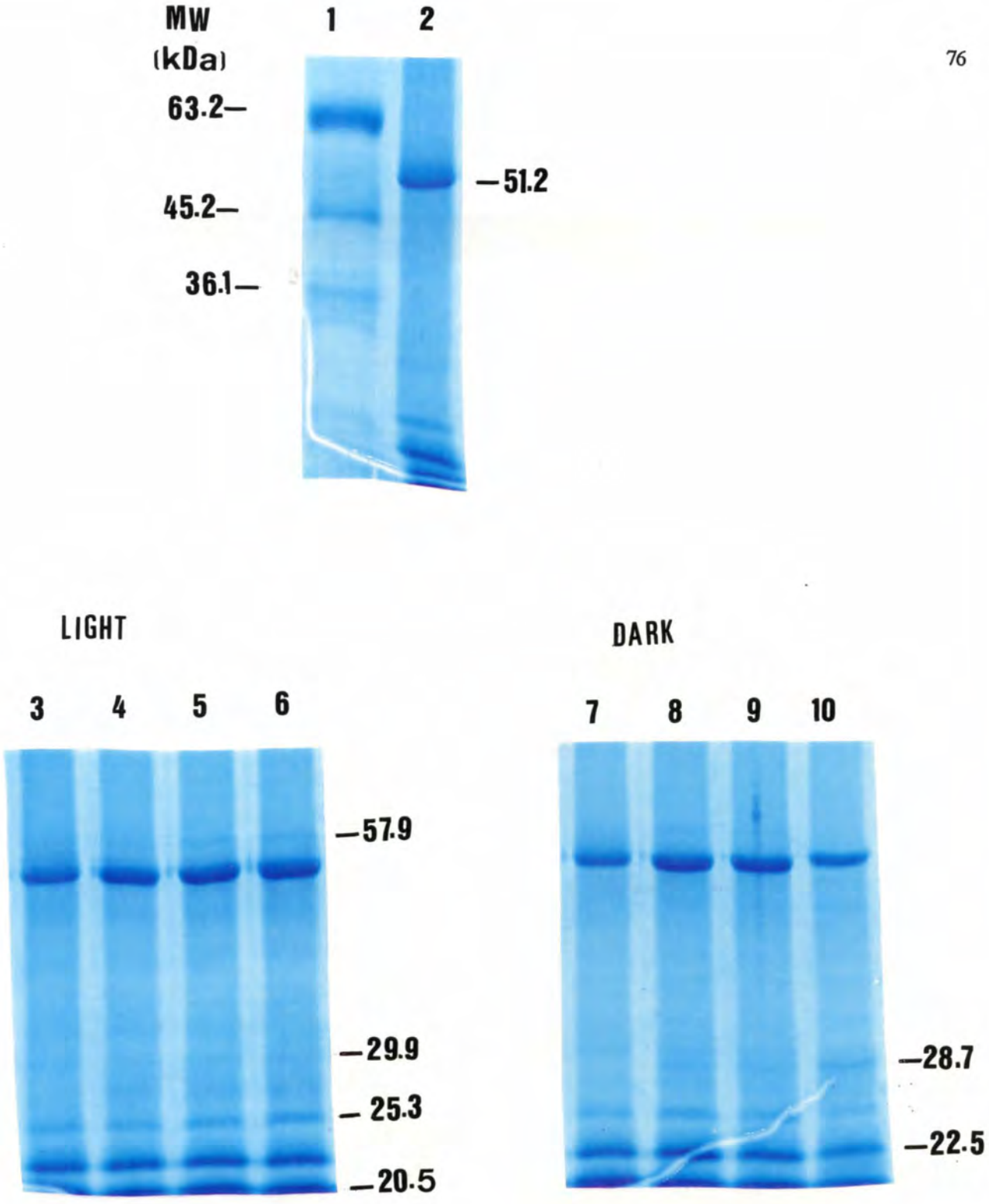

Figure 4.4 SDS-polyacrylamide gel electrophoresis of the thylakoids prepared from attached, senescing leaves of *Hordeum vulgare*.

Thylakoids were prepared as described for the chloroplast in Section 2.3.3. Gel lanes were loaded with 10 μg of thylakoid protein (lane 2-10).

4.3.2.3 Stromal fractions

As shown in Figure 4.5, the major polypeptide bands have the molecular masses of 65.1, 45.2 and 21.5 kDa. A 65.1 kDa polypeptide was very close in its mobility to a 65 kDa protein reported by Guéra *et al.* (1989) in the stroma of *H. vulgare*. A 45.2 kDa polypeptide co- migrated with authentic Rubisco used as a molecular weight marker. As shown in Figure 4.6 analysis of a 45.2 kDa band revealed a decline in its intensity with the progression of senescence. This decline was more rapid in leaves from the dark-incubated plants. The intensity of a 21.5 kDa band declined in both the light (lane 5-6) and dark-incubated plants. A new polypeptide with a molecular mass 20.7 kDa was observed in the dark-incubated plants (lane 7, 9 and 10). Intensities of minor polypeptide bands with molecular masses 50.1, 26.7 and 23.9 kDa were drastically reduced in both the light- and dark-treatments.

4.3.2.4 Whole tissue homogenates

Results presented in Figure 4.7 show that the most prominent polypeptide bands had molecular masses of 43.5, 40.8, 38.0, 27.8 and 21.7 kDa. Some very faint band with a molecular mass 57.7 kDa was resolved. Intensities of the 40.8, 38.0 and 35.5 kDa bands decreased with the progression of senescence in both the light- and dark-incubated plants. A 43.5 kDa band had its intensity enhanced in the light (lane 3-6) and reduced in the dark (lane 8-10). Intensity of a 27.8 kDa band (lane 7-10) was reduced, whereas the intensity of a 21.7 kDa band remained unchanged throughout the incubation period.

4.3.3 Senescence-induced change in activity of hydrolytic enzymes

The breakdown of protein in senescing leaves is associated with an increase in protein-hydrolyzing enzymes (Veierskov and Thimann, 1988). Because loss of total leaf protein was rapid in the dark (see Figure 4.1A), it is not unreasonable to expect high proteolytic activity in leaves from dark-incubated plants.

Results presented in Figure 4.8 revealed an increase in the activity of both acid and neutral proteases in leaves from dark-incubated plants. Although the activity of both enzymes increased, neutral protease activity was

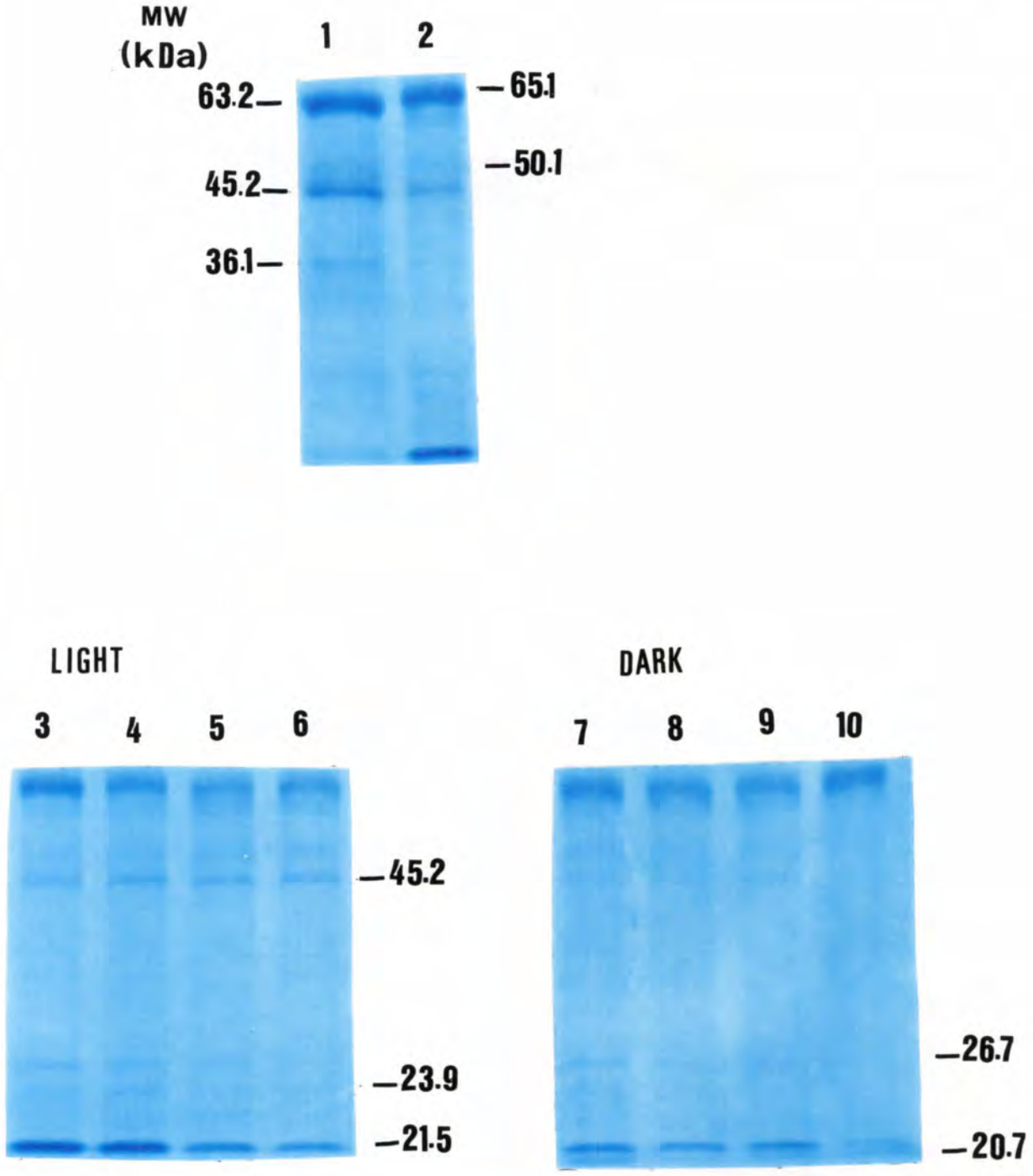

Figure 4.5 SDS-polyacrylamide gel electrophoresis of the stroma prepared from attached, senescing leaves of *Hordeum vulgare*.

Stroma was prepared as described for the chloroplasts in Section 2.3.3. Samples were concentrated as described in Section 2.6.7. Gel lanes were loaded with 10 μg of stromal protein (lane 2-10).

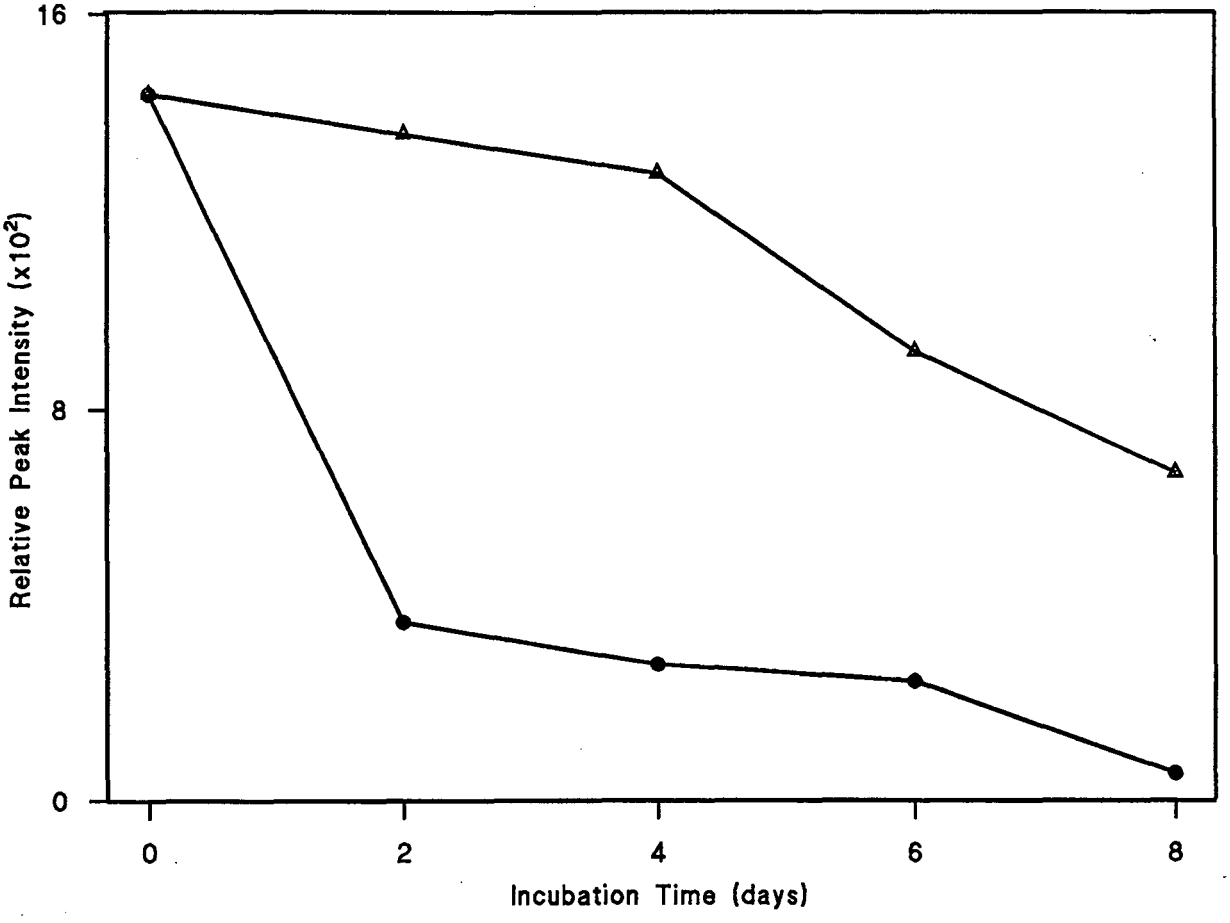

Figure 4.6 Changes in relative peak intensity of a 45.2 kDa stromal protein band in light (▲) and darkness (●).

Relative peak areas were determined from scans of the bands obtained using the UVP gel Documentation Software Package SW 2000.

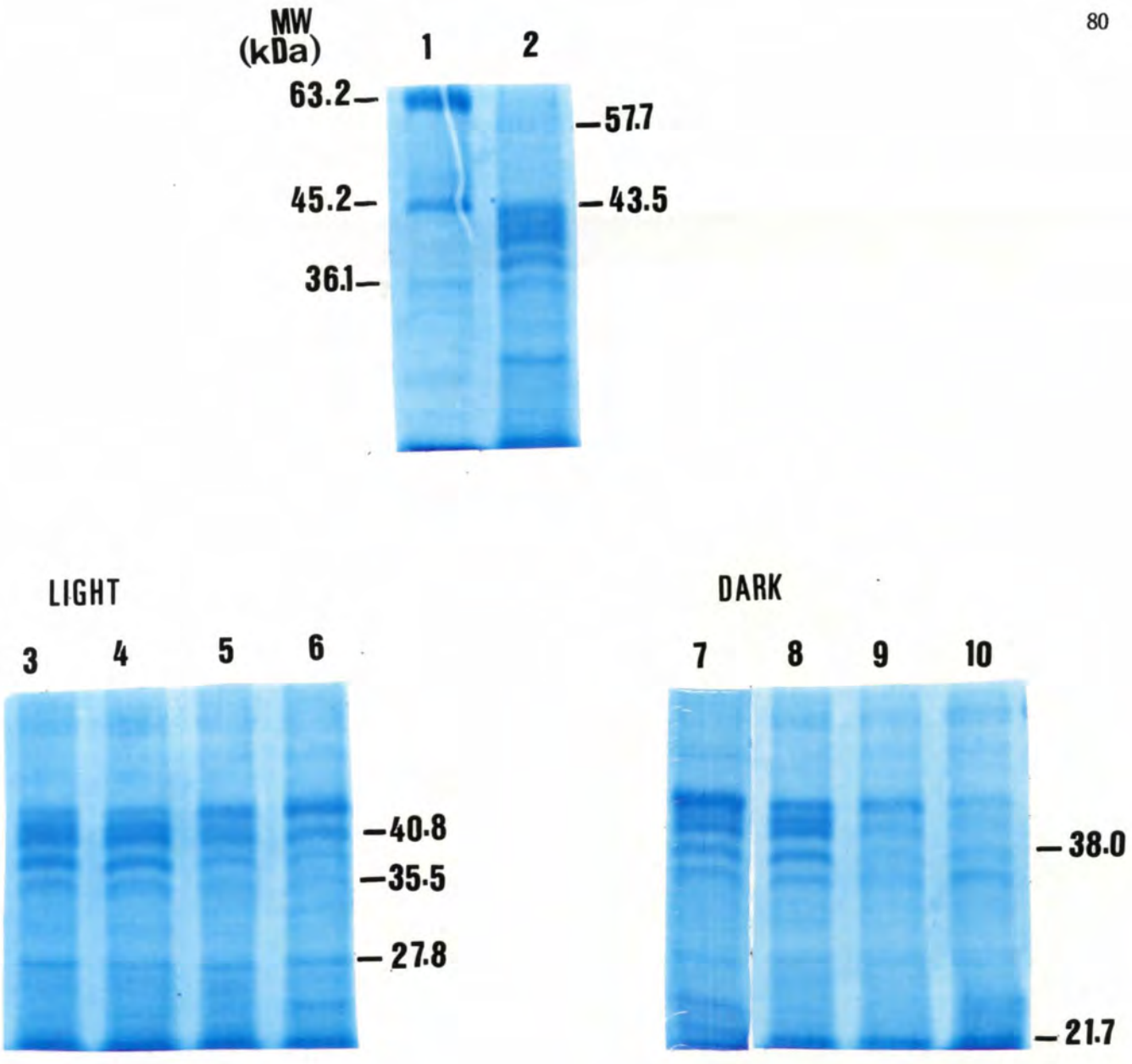

Figure 4.7 SDS-polyacrylamide gel electrophoresis of the crude extracts prepared from attached, senescing leaves of *Hordeum vulgare*.

Conditions of plant incubation were as described in Figure 4.3. Samples were prepared as described in Sections 2.3.2 and 2.6.7.1. Gel lanes were loaded with 15 μg of protein (lane 2-10).

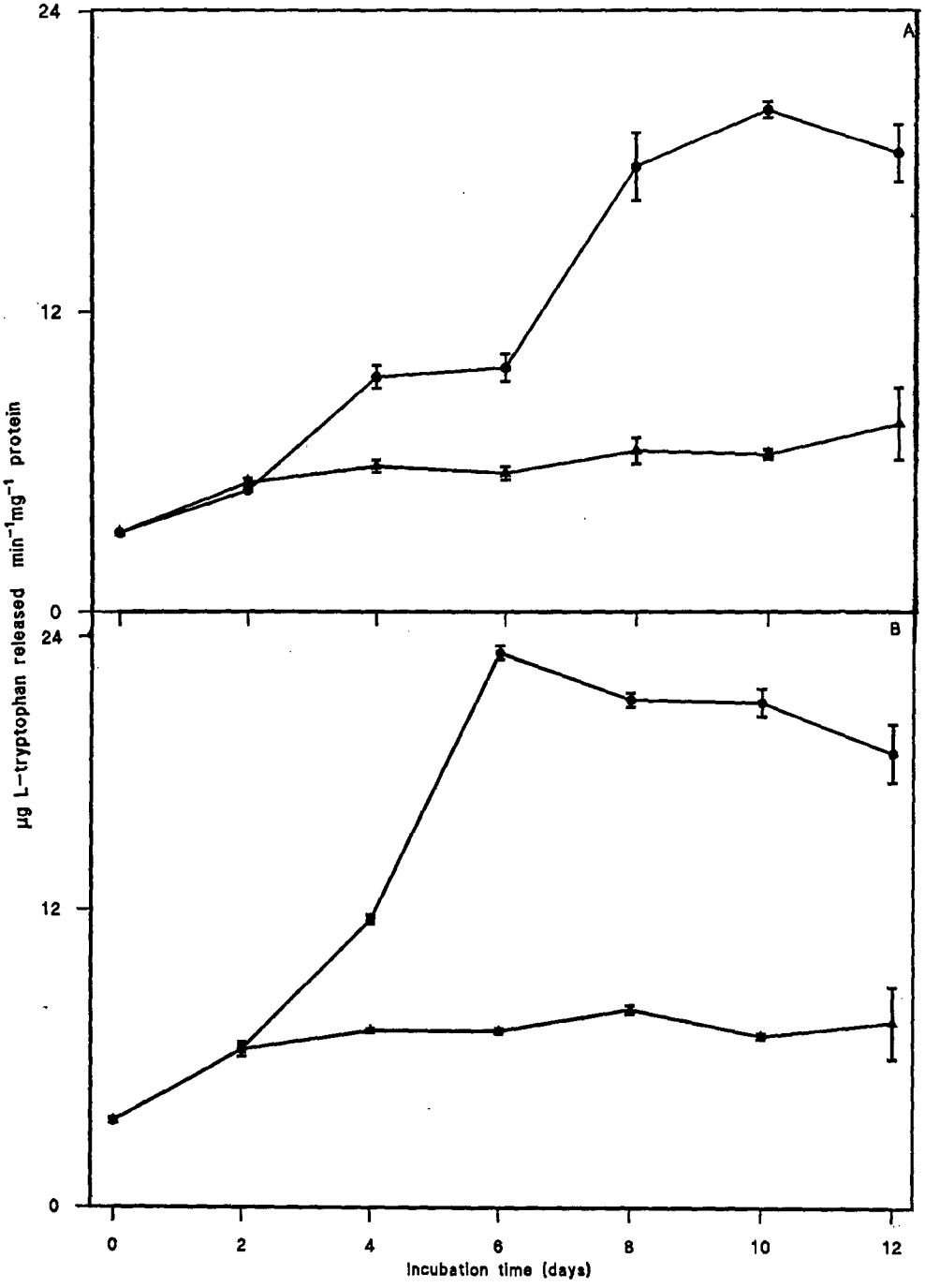

Figure 4.8 Kinetics of the changes in level of activities of acid (A) and neutral (B) proteases in attached, senescing leaves of *Hordeum vulgare.*

7d old light-grown seedlings were incubated in either continuous light (▵) or total darkness (o) at 25°C. Preparation of the leaf material and incubation of samples were as described in Sections 2.4 and 2.4.1. Data represent the mean of 3 replicates ±SE.

apparently higher than that of acid protease. Maximum levels of neutral protease activity were reached on day 6, after which there was a gradual decline in the activity. In leaves from the light-incubated plants, there was a slight increase in the activity of both enzymes during the first 2 days of incubation. Thereafter, the activity of both enzymes remained fairly constant throughout the incubation period. A marked increase in the activity of acid and neutral proteases coincided with the loss of total leaf protein in dark-incubated plants, suggesting the involvement of these enzymes in the breakdown of protein during senescence. Therefore, the results of the present study are consistent with the findings of Martin and Thimann (1972), Peterson and Huffaker (1975) and Veierskov and Thimann (1988).

The loss of chloroplast constituents, for example chlorophyll, requires proteases to penetrate the chloroplast envelope, thus making chloroplast constituents accessible to hydrolytic attack (Veierskov and Thimann, 1988). Kuroda *et al.* (1990) have reported that increased peroxidase activity is correlated to the loss of chlorophyll in leaves of *H. vulgare*. The results presented in Figure 4.9 show an initial gradual increase in peroxidase activity in both the light- and dark-treatments. After 6 days of incubation, a rapid increase in peroxidase activity was observed in leaves from the dark-incubated plants. Therefore, enhanced peroxidase activity was preceded by the loss of chlorophyll (see Section 3.2.1, Figure 3.3), suggesting that peroxidase is at least not involved in initiating the breakdown of chlorophyll. Abeles and Dunn (1989) reported similar findings for *Cucumis sativus* cotyledons.

Lipoxygenase (LOX), in the presence of certain fatty acids is known to cause carotenoid cleavage (Ben-Aziz *et al.*, 1971; Firn and Friend, 1972). To determine whether increased violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) arose late in the senescence process (see Section 3.2.2, Figure 3.13B) due to alterations in carotenoid cleavage, LOX activity was monitored as senescence progressed in both the light- and dark-incubated plants. The results presented in Figure 4.10 show that activity of LOX increased more rapidly in leaves from dark-incubated plants. Maximum levels of enzyme activity coincided with reduced levels of Z,

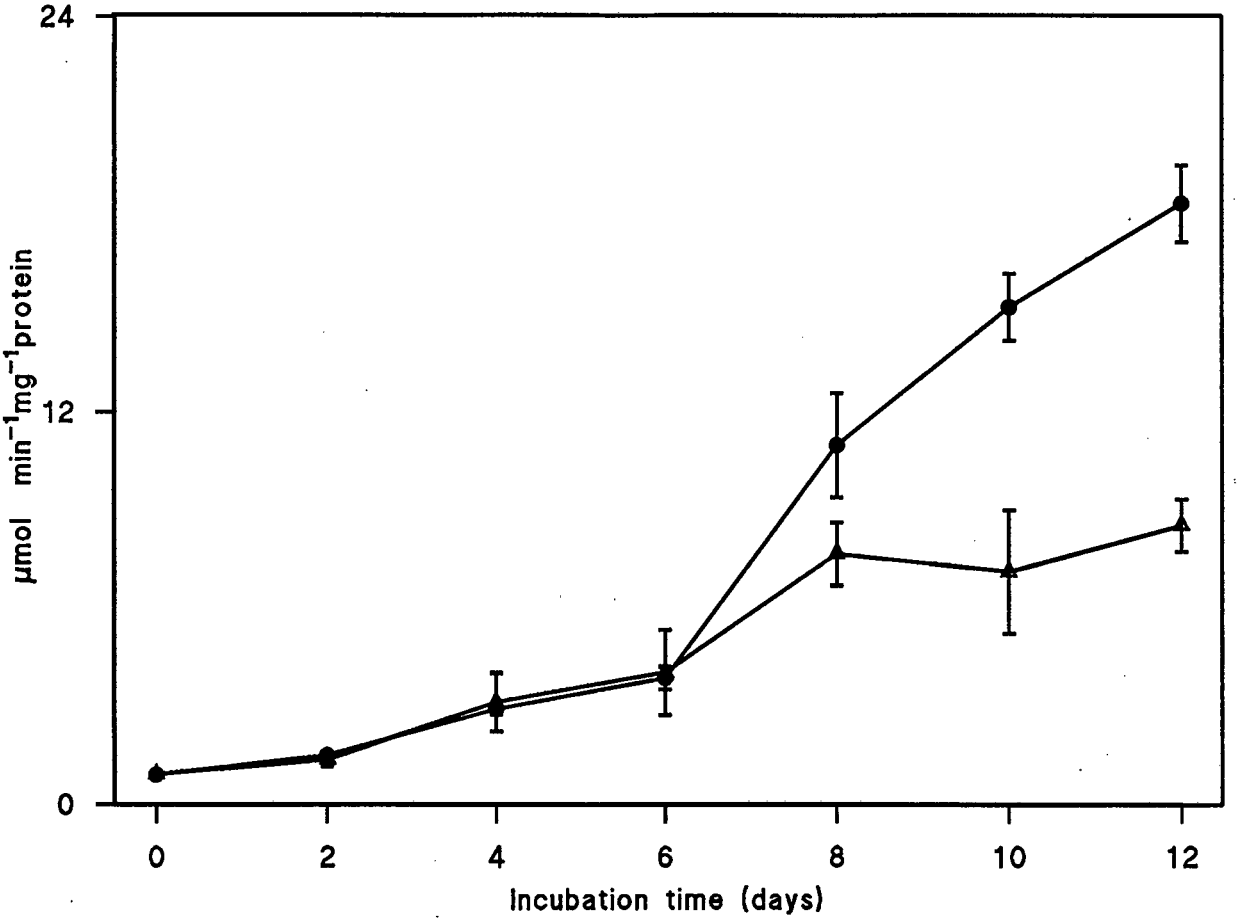

Figure 4.9 Kinetics of the change in levels of activity of peroxidase in attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown seedlings were incubated in either continuous light (▲) or total darkness (o) at 25°C. Leaf material was prepared and incubated as described in Sections 2.4 and 2.4.4. Data represent the mean of 3 replicates ±SE.

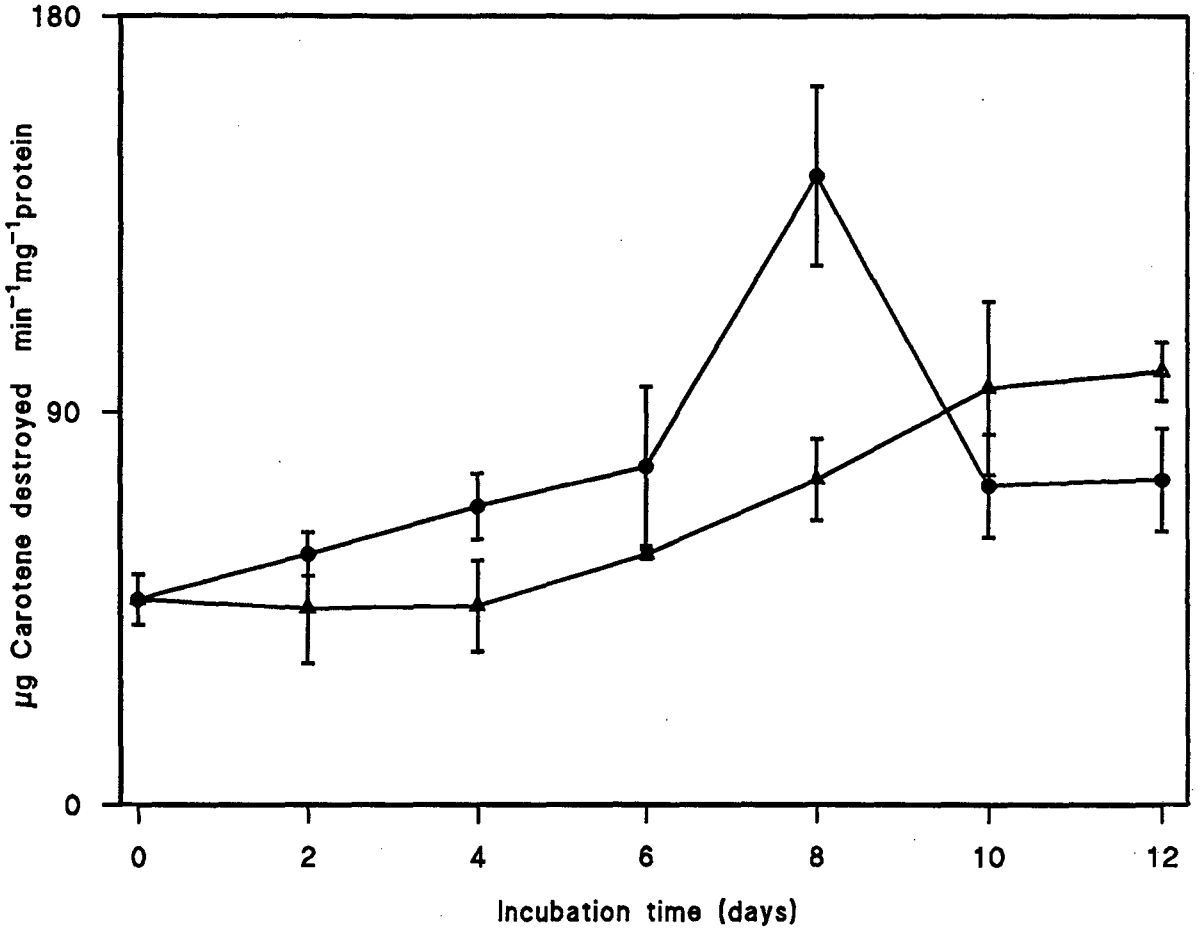

Figure 4.10 Kinetics of the change in levels of activity of lipoxygenase in attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown seedlings were incubated in either continuous light (▲) or total darkness (o) at 25°C. Leaf material was prepared and incubated as described in Sections 2.4 and 2.4.3. Data represent the mean of 3 replicates ±SE.

A and V at day 8 from dark-incubated seedlings (see Section 3.2.2, Figure 3.13B). Interestingly, a decline in LOX activity after day 8 (Figure 4.10) was associated with an increase in levels of V, A and Z (see Section 3.2.2, Figure 3.13B), suggesting inhibition of oxidative cleavage for these xanthophylls. These data suggest a positive interrelationship between enhanced LOX activity and the increased metabolism of xanthophylls.

Export rather than import of metabolites is favoured during foliar senescence (Mullet, 1988). It is therefore not unreasonable to assume that degradation of starch into sucrose transport takes place in senescing tissues. Degradation of starch is thought to be carried out by α-amylases (Beck and Ziegler, 1989). The results presented in Figure 4.11 show a marked increase in α-amylase activity in leaves from the dark-incubated plants. Since dark-incubation is known to induce senescence rapidly, a high activity of α-amylase in the dark could be indicative of the fact that starch was actively hydrolyzed in senescing tissues.

4.3.4 Interrelationship between polypeptide patterns and hydrolytic enzymes

Protein standards of peroxidase, lipoxygenase and α-amylase were electrophorized with markers (see Section 2.6.7.2, Figure 2.6) and the molecular weights of these enzymes were determined. An attempt was made to correlate the molecular weights of the abovementioned hydrolytic enzymes with that of the polypeptides from crude extract and chloroplast preparations. Peroxidase isoenzymes co-migrated with polypeptide bands of molecular weights (in kDa) 24.3, 27.5 and 29.8 in the chloroplast, 21.5 in the stroma and 29.9 in the thylakoids. A 21.8 kDa isoenzyme of LOX was very close in its mobility to a chloroplastic band of 21.9. Similarly, isoenzymes of LOX with molecular weights 20.6 and 25.1 were very close in their mobility to the 20.5 and 25.3 kDa polypeptides found in the thylakoids. A 43.5 kDa polypeptide from the crude extract (see Figure 4.7) co-migrated with a 43.5 kDa α-amylase protein purified from shoots and cotyledons of *Pisum sativum* seedlings (Beers and Duke, 1990). Although co- migration does not provide substantial evidence for the identity of polypeptides, it appears that α-amylase is an extra-chloroplastic enzyme, whereas peroxidase and LOX are chloroplastic enzymes. Peroxidases appear to exist in two forms, the soluble and membrane-bound types. LOX appears to be exclusively a membrane-bound enzyme.

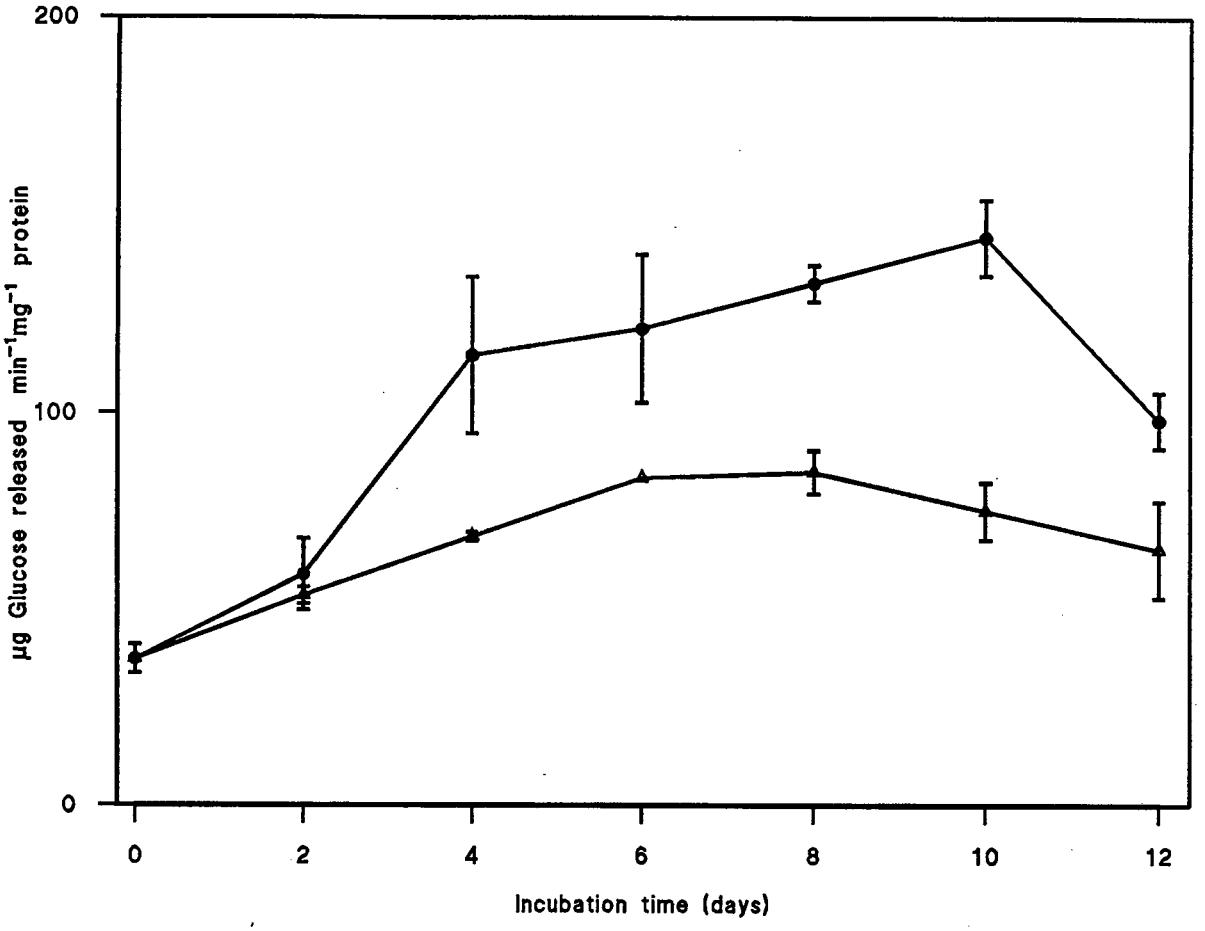

Figure 4.11 Kinetics of the change in levels of activity of α-amylase in attached, senescing leaves of *Hordeum vulgare*.

7d old light-grown seedlings were incubated in either continuous light (▲) or total darkness (o) at 25°C. The homogenate was prepared and incubated as described in Sections 2.4 and 2.4.2. Data represent the mean of 3 replicates ±SE.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

The terms senescence and aging are often misused. Because the biochemical nature of senescence and aging is not precisely known, it is premature to define these processes more exactly or to draw a fine line between them (Noodén, 1988a). Nonetheless, senescence represents endogenously controlled degradative processes leading to death and it occurs in correspondence with maturation of plants and plant organs. Aging encompasses passive or nonregulated degenerative processes driven primarily by exogenous factors. Aging does not in itself cause death but may decrease resistance to stress and increase the probability of death.

Senescence is genetically controlled but can also be environmentally induced, allowing the plant to adapt to changing environmental conditions (Huffaker, 1990). Leaf senescence is often induced by dark treatment. A problem associated with dark-induced senescence however, is that since many plant genes require light for expression (Nelson *et al.*, 1984), the disappearance of specific proteins during incubation can be a function of darkness rather than senescence (Thomas and Hilditch, 1987). Since normal plant developmental stages include maturation and aging followed by senescence, dark-incubation may therefore induce premature senescence.

The results in Chapter 3 show that in leaves of *H. vulgare* chlorophyll loss was retarded in light whereas it continued rapidly in darkness. Similar findings have been reported by Cuello *et al.* (1990). Although the metabolism of chlorophyll is regulated by light, apparently via phytochrome, the molecular mechanism of phytochrome action in delaying leaf senescence is not known. However, control by phytochrome of mRNA synthesis and mediation of both nuclear and plastid gene expression (Tobin and Silverthorne, 1985; 1987) and its regulatory action on the stability of thylakoid membrane proteins has been suggested (Okada *et al.*, 1992).

In thylakoids, formation of light-harvesting chlorophyll *a/b* proteins (LHCP) declines during senescence (Roberts *et al.*, 1987). Dephytylation of chlorophyll *in vivo* yields chlorophyllide, still associated with the light harvesting complex (Thomas *et al.*, 1989). It appears therefore that a key reaction of the catabolic pathway is the release of chlorophyll from its apoproteins (Schellenberg *et al.*, 1990). Increases in chlorophyll *a/b* ratio (Chapter 3) reflect preferential destruction of chlorophyll *b* and thylakoid instability (Walmsley and Adamson, 1989; Walmsley, 1991).

The breakdown of chlorophyll is associated with increases in the corresponding hydrolytic enzymes, probably by stimulation of transcription of structural genes (Sabater and Rodriguez, 1978). Peroxidase has been proposed to be responsible for chlorophyll breakdown during senescence. In the present study, enhanced peroxidase activity was preceded by loss of chlorophyll, suggesting that peroxidase was not responsible for initiating the breakdown of chlorophyll in senescing leaves of *H. vulgare*. Similar findings were reported by Abeles and Dunn (1989) for senescing *Cucumis sativus* cotyledons. A possible explanation is that the vacuolar localization of peroxidases (Matile, 1980) may prevent contact with chlorophyll in young, non-senescing tissues (Mäder and Walter, 1986). As senescence progresses, membrane lipid peroxidation may cause membrane leakiness (Dhindsa *et al.*, 1981), thus making interaction between the vacuole and chloroplasts possible (Peoples *et al.*, 1980; Wittenbach *et al.*, 1982). However, absolute reliance on the vacuolar enzyme is inconsistent with the view that chloroplasts may already contain the necessary enzymes to either partly or entirely achieve their own senescence (Wardley *et al.*, 1984).

During the course of leaf senescence, chlorophylls are degraded faster than carotenoids which results in leaf yellowing (Siffel *et al.*, 1991). Chlorophyll to carotenoid ratio decreased with the progression of senescence (Chapter 3), suggesting that chlorophyll was broken down faster than carotenoids. Therefore, during the course of foliar senescence, carotenoids are probably more stable than chlorophylls. Senescence is a co-ordinated developmental process; therefore regulated uncoupling and/or partial repair of the photosynthetic reaction centres might be required. Carotenoids are essential components of the photosynthetic apparatus and there

is increasing evidence for their involvement in maintaining functional PSII (Markgraf and Oelmüller, 1991). Furthermore β-carotene functions in the reaction centres of PSI and PSII to protect chlorophyll from photo-oxidation and also serve as a light-absorbing pigment. Lutein and neoxanthin function as accessory pigments in photosynthetic light absorption, whereas zeaxanthin may function to dissipate excess energy from the pigment bed (Siefermann-Harms, 1985; 1987), thus protecting the photosynthetic reaction centres from photooxidative damage (Demmig-Adams, 1990).

Enhanced production of free radicals is one of many events associated with senescence (Lynch and Thompson, 1984). Free radicals have been implicated in membrane deterioration and loss of important physiological functions in senescing tissues (McRae and Thompson, 1983). Carotenoids, in particular xanthophylls, are proposed to function as free-radical scavengers (Demmig-Adams, 1990; Thayer and Björkman, 1990). Therefore, the onset of senescence is likely to result in alteration of carotenoid metabolism. Recent evidence supports a precursor role for carotenoids in the synthesis of ABA (Li and Walton, 1990; Duckham *et al.*, 1991; Parry and Horgan, 1991a, b; Rock and Zeevaart, 1991; Rock *et al.*, 1991; 1992). Zeaxanthin (Z), antheraxanthin (A) and violaxanthin (V) constitute the xanthophyll cycle which functions under conditions of stress to dissipate excess energy trapped within the photosynthetic pigment bed (Demmig-Adams, 1990). Since both Z and V are precursors of ABA (Li and Walton, 1987; 1990; Duckham *et al.*, 1991; Rock and Zeevaart, 1991), it follows that any change in xanthophyll cycle activity should be manifested as changes in levels of ABA and *vice versa*. Results from the present investigation show that enhanced levels of ABA, induced by senescence, coincided with changes in xanthophyll cycle activity and were characterized by increased epoxidation of Z to A and V (Afitlhile *et al.*, 1993). Furthermore, senescence-induced increases in ABA occurred at the expense of V and 9′-*cis*-neoxanthin, and the apparent stoichiometry suggests that on a molar basis a 1:1 relationship exists. Therefore, these results indicate that ABA accumulated during foliar senescence in *H. vulgare* seedlings is derived from a carotenoid.

The immediate precursor of ABA has been identified as ABA-aldehyde (Sindhu and Walton, 1987; Taylor *et al.*, 1988; Duckham *et al.*, 1989), and most work is consistent with xanthoxin (Xan) being an intermediate between the xanthophyll(s) and ABA-aldehyde (Sindhu and Walton, 1987; Parry *et al.*, 1988). Evidence has also been presented which implicates 9′-*cis*-neoxanthin as the immediate pre-cleavage carotenoid precursor of ABA (Parry *et al.*, 1990; Parry and Horgan, 1991a, b). In addition, it has been proposed that cleavage of 9′-*cis*-neoxanthin and violaxanthin to yield xanthoxin is the rate limiting step in ABA biosynthesis. Although xanthoxin was isolated from incubations of V and LOX (Firn and Friend, 1972; Parry and Horgan, 1991a), more recent attempts to demonstrate a role for LOX in cleavage of 9′-*cis*-neoxanthin have been unsuccessful (Parry and Horgan, 1991a). In addition, since senescence-induced increases in ABA in *H. vulgare* leaves preceded enhanced levels of LOX activity, it seems unlikely that this enzyme is involved in cleavage of the carotenoid precursor in ABA synthesis. Nevertheless, results from the present study indicate a positive interrelationship between enhanced LOX activity and increased metabolism of xanthophylls (Afitlhile *et al.*, 1993).

LOX and products of LOX-catalyzed reactions generate free radicals during senescence (Hildebrand, 1989; Siedow, 1991), which can be quenched by carotenoids in the xanthophyll cycle (Rau, 1988; Demmig- Adams, 1990) and possibly result in increased epoxidation of Z to V. It is therefore likely that increased LOX activity may contribute to increased ABA indirectly by promoting synthesis of V, whereas cleavage of 9′-*cis*-neoxanthin to liberate xanthoxin may be carried out by a specific dioxygenase. Although, recent evidence suggests that a nonheme oxygenase with LOX-like activity is involved in ABA biosynthesis (Creelman *et al.*, 1992). However, the mechanism of this reaction system remains unknown. Activity of LOX was always lower in leaves from light-incubated seedlings. Although several factors may account for low LOX activity, Cohen *et al.* (1984) obtained evidence to suggest that chlorophyll could inhibit LOX activity in light-grown leaves. One of the outstanding differences between dark- and light-incubated seedlings was the higher level of chlorophyll in the light-grown plants (Chapter 3). It is possible that inhibition of LOX activity in light could be due to the presence of chlorophyll. However, since the function of LOX is dependent on the presence of linoleic acid or a similar compound having the *cis*, *cis*-1,4-pentadiene structure (Ben-Aziz *et al.*, 1971; Vick and

Zimmermann, 1987), activity of LOX may also be limited by the availability of free fatty acids. Therefore, degradation of membrane lipids and de-esterification of fatty acids may be regulatory factors in the functioning of LOX (Chéour *et al.*, 1992).

Although ABA and PA accumulated in light-incubated leaves, calculation of the epoxidation state indicated that de-epoxidation of the xanthophyll cycle was apparently being favoured. Thus V, the proposed substrate for ABA biosynthesis, was preferentially converted to Z, which might explain the low levels of ABA and PA present in light-incubated leaves. Alternatively, increased conjugation of ABA similar to that observed by Weiler (1980) could have contributed to the deviation in stoichiometry. In dark-incubated leaves, ABA levels eventually declined as senescence progressed. This decline may be attributed to enhanced catabolism or export of ABA from the senescing tissue. Alternatively, enhanced ABA catabolism coupled with reduced flux of carbon into carotenoids may have contributed to overall lower levels of ABA. This is particularly so given that loss of ABA was mirrored by an increase in levels of Z, A and V. Higher V levels might suggest the potential for de-epoxidation. It is therefore proposed that both ABA and xanthophyll cycle responses are tightly regulated, and that the magnitude of the ABA response is dependent on the size and epoxidation state of the xanthophyll cycle (Afitlhile *et al.*, 1993).

ABA is thought to accelerate senescence by inducing stomatal closure which is apparently achieved by promoting synthesis of one or more proteins (Park and Thimann, 1990). Since ABA has been implicated to act either at the transcriptional and/or translational level, it is likely that ABA may regulate the synthesis of proteins which are involved in stimulating senescence and these may be representative of hydrolytic enzymes. In the present investigation, maximum levels of ABA preceded enhanced peroxidase activity in the dark, suggesting that ABA could be involved in the expression of peroxidase gene(s). ABA is known to induce the expression of an anionic peroxidase in potato callus (Roberts and Kolattukudy, 1989). Recent evidence suggests that ascorbate peroxidase is the key enzyme in the scavenging of H_20_2 in chloroplasts (Asada, 1992; Cakmak and Marschner, 1992; Chen and Asada, 1992; Miyake and Asada, 1992). H_20_2 is one of the molecular species

of active oxygen if not immediately scavenged, it produces the highly reactive hydroxyl radical and singlet oxygen which have destructive effects on the functional and structural integrity of chloroplasts (Asada, 1992; Cakmak and Marschner, 1992). Increases in peroxidase activity may therefore be associated with retardation rather than promotion of the senescence process. Thus, it is likely that ABA may contribute to senescence by suppressing the expression of peroxidase gene(s).

Extractable activity of a number of hydrolytic enzymes increases during senescence (Sabater and Rodriguez, 1978; Veierskov and Thimann, 1988) and stress (Todd, 1972). In this regard, the response of plants to stress is similar to responses during the course of senescence. Jacobsen *et al.* (1986) demonstrated that water stress enhanced the expression of an α-amylase gene in leaves of *H. vulgare*. This apparently involved changes in the pattern of protein synthesis involving relative increases and decreases in polypeptide synthesis. In the present investigation, a polypeptide band co-migrating with authentic α-amylase was only found in crude preparations indicating an extrachloroplastic location. Several studies have reported similar findings (Jacobsen *et al.*, 1986; Kakefuda *et al.*, 1986; Beers and Duke, 1990). Therefore, the localization of α-amylase outside the chloroplast provides little insight into its role as a starch degrading enzyme. Jacobsen *et al.* (1986) suggested that there is regulated movement of α-amylase into and out of chloroplasts. In the present study, there was a positive correlation between enhanced activities of proteases and α-amylases in the dark-incubated leaves. This observation might imply that α-amylases are ensured direct access to stored starch by the action of concomitantly produced proteases which dissolve complex membrane proteins. Therefore, starch degradation may indicate cooperative action of hydrolytic enzymes.

Loss of total leaf protein was accompanied by a concomitant increase in the activity of both acid and neutral proteases. At least in *H. vulgare* leaves the activity of neutral protease was the first to develop and it gradually decreased with increasing activity of acid protease. Although tentative, this observation suggests that neutral proteases initiate hydrolysis of proteins, a process which is then completed by acid proteases.

Isolated chloroplasts, purified thylakoid and stromal fractions showed an increase in protein level for at least 4 days in darkness. Although this may be interpreted as suggesting that new proteins were being synthesized, the increase could also have been due to the need for protease, presumably in the cytosol to penetrate the chloroplast envelope. In light-incubated leaves, retarded loss in total leaf protein could imply that light enables resynthesis to keep pace with protein breakdown. However, this explanation is unsatisfactory because the activities of both neutral and acid proteases were low in light. Veierskov and Thimann (1988) proposed that in the light proteases do not have access to substrates because these enzymes are contained within the vacuole (Matile, 1978). This implies that proteolysis, typical of senescence, may only take place after the permeability of one or more membranes has increased to allow enzymes to pass through (Noodén and Leopold, 1988). Thus, the triggering action of senescence may be a change in permeability of the tonoplast, and later perhaps of the chloroplast membrane. Ethylene biosynthesis has been linked to membrane transport processes (for review, see Yang and Hoffman, 1984). Therefore, change in membrane permeability might be a consequence of ethylene production, this is particularly so given that the location of ethylene-forming enzyme is thought to be either in the plasmalemma or tonoplast, or both (Rhodes, 1987). It is therefore not unreasonable to assume that leaf senescence depends directly on membrane modification. In green tissue where light delays senescence, this would mean that the action of light is to sustain the membrane structure. Veierskov and Thimann (1988) suggested that the tonoplast is unstable but in the presence of light (or any senescence-delaying factor) its integrity is continually being reinstated. Thus, the nucleus is constantly stimulated to produce mRNAs that code for tonoplast protein.

The synthesis of specific proteins encoded from nuclear and chloroplastic DNA is also required for senescence (Yu and Kao, 1981; Cuello *et al.*, 1984). Chloroplast isolated from attached senescent *H. vulgare* leaves synthesize several proteins that are different from those synthesized in young leaves (Garcia *et al.*, 1983). Synthesis of these proteins is stimulated by leaf detachment and incubation in the dark (Martin *et al.*, 1986). This enhanced protein synthesis activity has allowed for identification of several polypeptides specifically synthesized by senescent chloroplasts (Guéra *et al.*, 1989; Martin and Sabater, 1989; Vera *et al.*, 1990). In

the present investigation, despite a decrease in the intensity of some polypeptide bands, polypeptide patterns in chloroplasts isolated from leaves incubated in either light or darkness were very similar (Chapter 4, Section 4.3.2.1). A decrease in the intensity of bands might suggest that protein synthesis in chloroplasts either decreased or the rate of protein breakdown could have been higher than that of synthesis. Although chloroplasts are the main site of Rubisco synthesis, no polypeptide of 54 kDa equivalent to the large subunit of Rubisco was detected among the proteins in chloroplasts from senescent leaves of *H. vulgare*. Similar findings have been reported in related studies (Garcia *et al.*, 1983; Martin and Sabater, 1989).

The main thylakoid polypeptide bands were less affected by dark-incubation than the chloroplast and stromal polypeptides, suggesting that thylakoid polypeptides may play a role in maintaining membrane integrity. Membrane integrity might be important in facilitating orderly degradation of the photosynthetic apparatus during foliar senescence. The apparent stability of thylakoid polypeptides may be due to accumulation of carotenoids needed in the assembly of photosynthetic complexes. Lutein, the major carotenoid of the antenna of PSII, accumulates in the dark (Chapter 3). Although tentative, lutein might play a role in the assembly of photosystems during foliar senescence. A similar role for lutein has been suggested in a related study (Markgraf and Oelmüller, 1991).

Intensity of stromal polypeptide bands was highly reduced in the dark. Interestingly, the intensity of a 45.2 kDa band which co-migrated with Rubisco standard declined gradually in light whereas it decreased rapidly in the dark. This observation is consistent with the findings that synthesis and/or levels of Rubisco decline during senescence (Garcia *et al.*, 1983; Kasemir *et al.*, 1988; Martin and Sabater, 1989). An apparent new band with a molecular mass 20.7 kDa was observed only in the dark. The functional role of this polypeptide is presently unknown.

5.2 INTEGRATING THE CONSTITUENT PROCESSES OF LEAF SENESCENCE

Although complex, the process of leaf senescence is a carefully timed sequence of events. The senescence process is controlled by environmental factors, plant growth regulators, secondary messengers, structural proteins and enzymes. These factors are probably not exclusive but interactive in bringing about a response. Figure 5.1 presents a hypothetical model proposed to account for the interaction of constituent processes during leaf senescence with emphasis on molecular events.

Light is a decisive factor directing growth and morphogenesis including retardation of senescence in higher plants (Biswal and Biswal, 1984). The process by which light influences plant development includes reception of light by phytochrome, followed by transduction of the light signal leading to alterations in gene expression. Phytochrome regulates the expression of nuclear genes including those required for its own synthesis, and it does so in either a positive or negative fashion (Tobin and Silverthorne, 1985; 1987). Nonetheless, phytochrome-mediated photoperiodicity may ultimately trigger the onset of autumnal and/or dark-induced leaf senescence. It has recently been proposed that phytochrome (Pfr) may act primarily by stimulating the synthesis of acetylcholine which functions to alter phospholipid metabolism allowing for increases in membrane permeability to H^+, K^+, Na^+ and Ca^{2+} (for review see Tretyn and Kendrick, 1991).

It has been demonstrated that cytokinin interacts with phytochrome to enhance expression of light-harvesting chlorophyll *a/b* protein (Flores and Tobin, 1986). Because cytokinins retard senescence, it is likely that the senescence-delaying effect of light could be achieved by promoting the synthesis of cytokinins, and possibly auxins and GAs. These plant growth regulators appear to retard senescence by controlling the level of free radicals, an idea supported by the observation that cytokinins retard senescence by stimulating synthesis of polyamines which are scavengers of free radicals (Pell and Dann, 1991). Because the conversion of ACC to ethylene is a superoxide-mediated reaction (Drolet *et al.*, 1986), scavenging of free radicals by polyamines inhibits ethylene production (Pell and Dann, 1991). Furthermore, polyamines could be more effective in

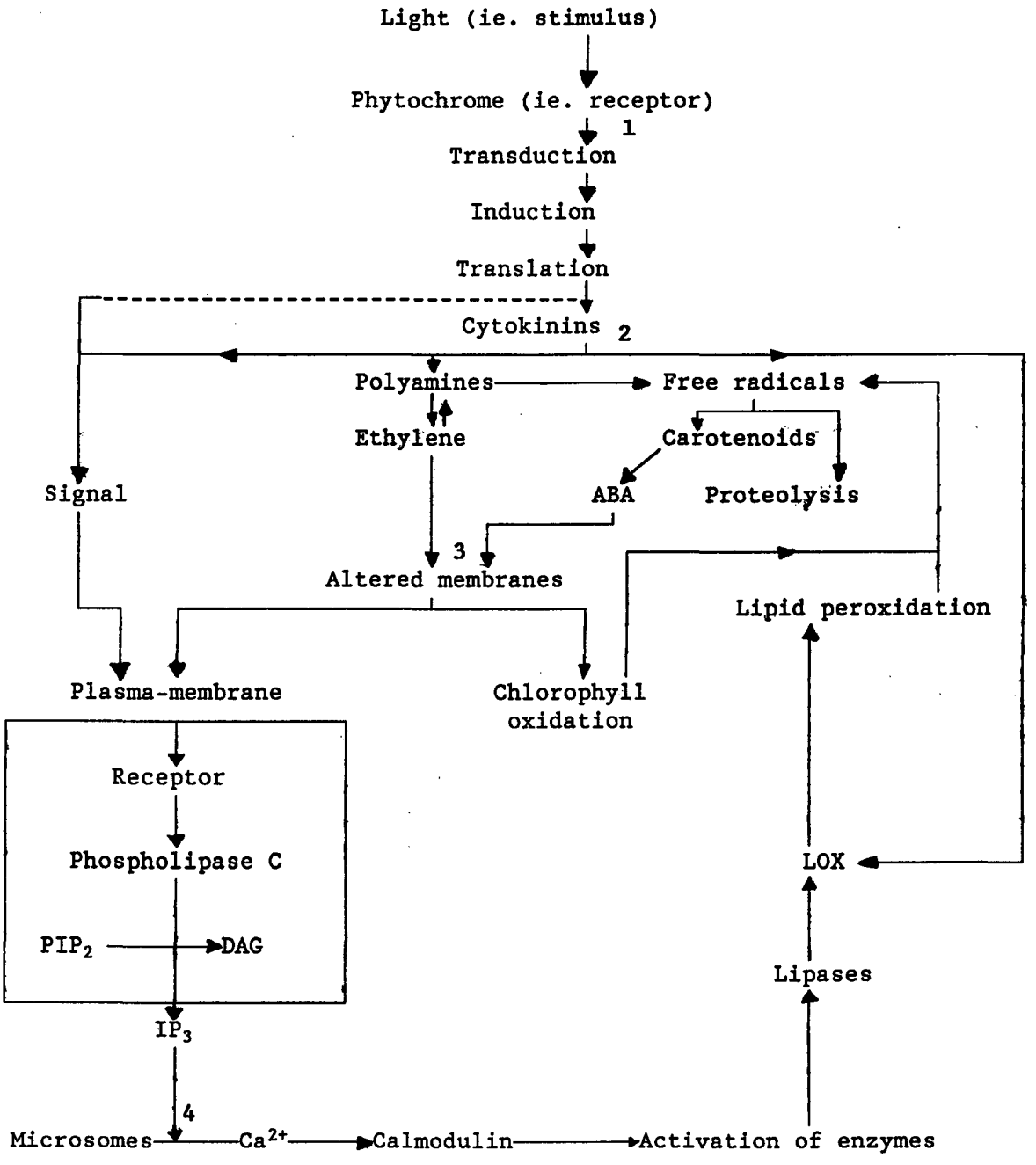

Figure 5.1 A proposed model of the pathway reflecting molecular changes leading to foliar senescence.

(1) Changes in the light signal alter gene expression which might promote or inhibit cytokinin production leading to the retardation or promotion of senescence, (2) availability or absence of cytokinins may therefore influence the level of free radicals, (3) increased free radicals may promote ethylene and ABA synthesis which might alter membrane structure and/or permeability, and (4) lead to increased Ca^{2+} flux which might alter gene expression resulting in the production of enzymes some of which may be required for senescence.

scavenging free radicals than carotenoids, thus decreasing carotenoid turnover and lowering the pool of carotenoid precursors required for ABA synthesis.

Cytokinins may inactivate LOXs by altering an active site on the enzyme, an idea supported by the observation that purified LOXs were inactivated by cytokinins (Pell and Dann, 1991). A decrease in cytokinin levels probably results in a reduction in concentration of polyamines, leading to elevated levels of free radicals. The availability of free radicals would thus enhance the synthesis of ethylene and ABA. Although the mechanism by which ethylene brings about senescence is not known, it is thought that ethylene action depends on binding to membranes (Goren *et al.*, 1984) and altering membrane transport processes (Rhodes, 1987).

The distribution of ABA within cellular compartments is controlled by pH (Cowan *et al.*, 1982) and darkness or a reduction in photoperiod is known to elevate the apoplastic concentration of free ABA (Radin and Hendrix, 1988; Kubik and Michalczuk, 1993). ABA is thought to interact with receptor proteins at the plasma-membrane (Hartung, 1983; Hornberg and Weiler, 1984) to inhibit H^+-ATPase activity and promote efflux of Cl^-, K^+ and malate. There is also evidence to suggest that the interaction of ABA with receptor proteins in the plasma-membrane influences Ca^{2+} flux (for review see Hetherington and Quatrano, 1991).

Several lines of evidence show that Ca^{2+} plays an important role in regulating leaf senescence. Low concentrations of Ca^{2+} are essential for structural integrity of the plasma-membrane (Poovaiah, 1988). Without such protection, the plasma-membrane fails to discriminate between ions, the active pumping mechanism fails and senescence is accelerated. Low cytosolic Ca^{2+} concentration is maintained by active Ca^{2+} pumping out of the cytoplasm by Ca-transporting ATPases located on the plasma-membrane. Recent evidence suggests that Ca-transporting ATPases activity of microsomal membranes is controlled by the calcium- modulated protein, calmodulin (Paliyath and Thompson, 1987). Stimuli such as light or plant growth regulators interact with receptors at the plasma-membrane to activate phospholipase C. The latter is responsible for cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and

inositol 1,4,5-triphosphate (IP_3). DAG and IP_3 play a key role in signal transduction (Poovaiah, 1988). IP_3 stimulates the release of Ca^{2+} from intracellular stores, thereby raising the concentration of cytosolic free Ca^{2+}. Ca^{2+} binds calmodulin and the Ca^{2+}-calmodulin complex activates target enzymes. Membrane-associated lipases are also stimulated by Ca^{2+}-calmodulin interaction (Thompson *et al.*, 1987). Through de-esterification of membranes, substrates for LOX are released, resulting in increased LOX activity. LOX initiates lipid peroxidation which leads to formation of free radicals (Lynch and Thompson, 1984; Thompson *et al.*, 1987).

The oxygen radicals have been implicated in modifying proteins, thereby rendering proteins susceptible to proteolysis (Dalling, 1987). Lipid peroxidation and vulnerability of proteins to degradation leads to altered membrane structure, thus exposing chlorophyll-protein complexes which are normally embedded in thylakoid membranes (McRae and Thompson, 1983). Once chlorophyll is exposed, it reacts with oxygen generating excited triplet chlorophyll (^3Chl) which may stimulate xanthophyll cycle epoxidation leading to an increase in free ABA. Increased ABA acts at the level of gene expression to generate the synthesis of novel proteins. Since ABA is known to induce changes in polypeptide patterns during senescence, it is likely that ABA- induced proteins may alter membrane structure. Alternatively, these proteins may be representative of enzymes which act on membranes.

5.3 CONCLUSION

The present research programme has revealed several interesting aspects regarding the role of ABA in leaf senescence. Firstly, ABA appears to be derived via increased carotenoid turnover during the course of leaf senescence in *Hordeum vulgare.* Therefore, increased ABA occurs as a consequence of senescence, suggesting that this plant growth regulator may fulfil a second messenger role in the senescence process. Secondly, the senescence-induced increase in ABA was not associated with any visible change in polypeptide pattern and did not appear to correlate with increased hydrolytic enzyme activity in leaves of *H. vulgare*. The primary mechanism of action of ABA during leaf senescence may therefore involve alterations in membrane transport processes similar to its mode of action in regulating stomatal aperture.

Clearly, there is a need to carry out a detailed study on ABA metabolism in senescing tissues and to determine whether sensitivity to ABA increases during the course of this process. Such studies should assist in elucidating more fully the role of ABA in senescence at the cellular and/or molecular level.

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