PHYSIOLOGICAL SIGNAL TRANSDUCTION FROM THE PHOTOSYNTHETIC APPARATUS IN THE GREEN ALGA *Dunaliella salina*

THESIS

Submitted to Rhodes University in fulfilment

of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

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December 1994

For Gael and Jessica

ACKNOWLEDGEMENTS

All affirmation to Professor P.D. Rose for excellent supervision, and interminable coercion to improve. Also for providing the funding and facilities, which without this research would not have been possible.

To Dr. A.K. Cowan for useful thoughts, criticisms and assistance.

To Professor D.A. Hendry for proof reading.

Professor H. Degani (Department of Physical Chemistry, Weitzmann Institute, Israel) for an introduction to NMR and help with interpreting NMR spectra.

Mr. B Kemp (Electronic Services Unit, Department of Physics, Rhodes University) for technical expertise in the development of the 'single-sample' chlorophyll fluorometer and fast data acquisition programme.

Professor H.K. Lichtenthaler (Department of Plant Physiology & Plant Biochemistry, Botanical Institute, University of Karlsruhe, Germany) for discussion on chlorophyll and carotenoid calculations.

Professor H. Parolis and Dr. L. Parolis (Department of Pharmacy, School of Pharmaceutical Science, Rhodes University) for discussions and unending help with NMR.

Professor H.Y. Yamamoto (Department of Plant Molecular Physiology, University of Hawaii, Hawaii, USA) for discussion and references on the xanthophyll cycle and trans-thylakoid pH.

Drs. Trevor and Lesley Phillips for hours of discussion during our years of study, and for interaction with the research group sponsored by SASOL.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	Antheraxanthin
ABA	Abscisic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
Chl	Chlorophyll
Chl*a*	Chlorophyll *a*
Chl*b*	Chlorophyll *b*
Elip	Early light induced protein
EPS	Epoxidation state
F_M	Maximum fluorescence
F_O	Basal fluorescence
F_V	Variable fluorescence (F_M-F_O)
G_o	Zero growth state
G_1	First log growth state
HPLC	High performance liquid chromatography
KDa	Kilodalton
LHC	Light harvesting complex
NMR	Nuclear magnetic resonance
PAR	Photosynthetic active radiation
PFD	Photosynthetic flux density
P_i	Inorganic phosphate
PS	Photosystem
Q_A	Quinone acceptor protein
q_E	Energy-dependent quenching
q_N	Non-photochemical quenching
q_P	Photochemical quenching
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S	Stationary growth state
SV_E	Stern Volmer energy-dependent non-photochemical quenching
V	Violaxanthin
Z	Zeaxanthin

ABSTRACT

The transduction of stress signals in plants is known to involve complex physiological responses. In *D. salina* a range of stresses results in hyper-accumulation of ß-carotene and an understanding of stress responses in this organism has important biotechnological implications. In this thesis an attempt was made to elucidate the physiological components involved and establish a role for pH in response to high light stress. In order to achieve this the effect of high light stress on photosynthesis and cell productivity was measured. Results showed that photosynthetic carbon assimilation, oxygen evolution and cellular productivity was initially inhibited by exposure to high light intensities, but this inhibition was transient and was overcome by a rapid increase in all three parameters. The response of the carbon pool intermediates was also investigated. It was shown that on exposure to high light ß-carotene declined but then showed a rapid increase after about 4 hours of exposure. It was also demonstrated that the initial loss of ß-carotene was due to loss of this pigment from the photosynthetic pigment bed and that the hyper-accumulation of ß-carotene was due to accumulation of ß-carotene in lipoidal globules located in the chloroplast stroma. It was further demonstrated that there was mass movement of carbon in the xanthophyll cycle shortly after exposure to high light. This was characterized by the de-epoxidation of violaxanthin to antheraxanthin with a further de-epoxidation to zeaxanthin, thereby decreasing the epoxidation state of the cycle. Furthermore, it was shown that there was relocation of carbon from violaxanthin to the plant growth regulator abscisic acid. It was also shown for the first time in *D. salina* that the production of ß-carotene and operation of the epoxidation state of the xanthophyll cycle has a periodicity which is established after

exposure to successive cycles of a light regime. Chlorophyll fluorescence was used together with well established ammonia stress responses to acquire a general overview of energy dissipation from the photosynthetic pigment bed. In conjunction with an understanding of xanthophyll cycle operation during exposure to high light stress it has been possible to establish a relationship between chlorophyll florescence, xanthophyll cycle operation and intracellular pH. It was also shown using chlorophyll fluorescence that after 4 hour exposure to high light a maximum fluorescence peak could no longer be induced indicating a transition at about this point from a state of reversibility to commitment of the full stress response. Nuclear magnetic resonance was used to follow intracellular pH fluxes during exposure to high light. A novel technique was developed for studying photosynthetically active organisms in the dark using nuclear magnetic resonance. These results showed that on exposure to high light stress there is rapid acidification of the chloroplast stroma and to a lesser degree of the acidic vacuole. The pH of these compartments is re-established after about 4 hours which is co-incident with the onset of β-carotene hyper-accumulation and the loss of the induction of the chlorophyll fluorescence peak indicating an intimate relationship for β-carotene, chlorophyll fluorescence, xanthophyll cycle operation and pH. The results from this study allow for the proposal of a general physiological stress transduction response mechanism for *D. salina* which is common for a range of different stresses and where intracellular pH plays a central role.

Chapter 1

INTRODUCTION

The unicellular alga *Dunaliella* has attracted investigators of various scientific backgrounds since the discovery of the alga during the 19th century. *Dunaliella* is probably the most halotolerant eukaryotic organism known and occurs in a wide range of marine habitats, including oceans, brine lakes, salt mashes, and salt ditches near the sea. It is found predominantly in water bodies with salt concentrations exceeding 10%, and proliferates in concentrated saline lakes where the salt concentration often reaches saturation level. The early ecological and taxonomic descriptions of *Dunaliella* were followed by studies aimed at the elucidation of the mechanisms that enables the alga to cope with harsh environments. Recently, studies of *Dunaliella* have focused around several major topics: osmoregulation, ion transport, ß-carotene accumulation, and the biotechnology of ß-carotene production (Avron & Ben-Amotz, 1992, and refs. therein). The ability of the halotolerant alga to thrive in media with high salt concentrations allows outdoor cultivation in relatively pure cultures with a low presence of predators and grazers. This makes *Dunaliella* species highly suitable candidates for biotechnological development. It has been proposed (Avron & Ben Amotz, 1992) that in the case of *D. salina*, the high levels of ß-carotene found in the chloroplast may protect this alga from intense solar irradiation in the areas where such cultivation is practical, i.e., arid or desert areas with access to brackish water or seawater.

CULTIVATION

Both intensive and extensive systems for the cultivation of *D. salina* have been developed. Intensive culture of *Dunaliella* is undertaken in open oblong raceways operated with paddle wheels with a culture depth of 10-25 cm and a surface area ranging from 1000 to 4000 m^2. Maximal productivity of ß-carotene of 400 $mg.m^{-2}.day^{-1}$ in these systems has been reported (Ben-Amotz, 1993). The extensive cultivation system (Borowitzka *et al.*, 1984) is based on unmixed, very large ponds of 5000-50000 m^2 in locations that provide a high yearly average solar radiation and where the ß-carotene levels in *Dunaliella* are controlled by using salt concentrations approaching saturation. The extensively grown *Dunaliella* are usually motile, round, large volume cells containing more than 50 pg ß-carotene per cell and a very low content of chlorophyll. Maximum productivity of ß-carotene in these systems is around 10 $mg\ m^{-2}.\ day^{-1}$.

Rose (1992) and Phillips (1994) have successfully demonstrated the viability of a dual-stage system for the commercial production of ß-carotene from *D. salina*. The process involves an initial stage where conditions for cells growth and development are optimized. The cells are then transferred to a second stage where conditions favour the accumulation of ß-carotene in stressed cells. This system is now under industrial pilot-scale evaluation.

Effective control of the stress process in the production of ß-carotene in *D. salina* is the key to the economic success of this organism in biotechnology (Rose 1992). Clearly, an understanding of the physiological processes involved in the transduction

of the stress signal, in connection with the physiological responses of stress, could allow more stringent control over the production of ß-carotene, and perhaps improved economic viability of the biotechnology of *D. salina*.

STRESS SIGNAL TRANSDUCTION

Osmoregulation in plants and in *Dunaliella* has been extensively reviewed (Hellebust, 1976; Ben-Amotz & Avron, 1978; Borowitzka, 1981; Avron, 1989). In contrast to other Chlorophyta, cells of *Dunaliella* do not have a cell wall (Oliveira *et al.*, 1980; Hoshaw & Maluf, 1981; Chardard, 1987). The cell is enclosed by a thin elastic plasma membrane and therefore responds rapidly to changes in osmotic pressure by changes in cell volume. *Dunaliella* can withstand 3- to 4-fold increases and decreases in osmotic pressure, shrinking or swelling in response. The exceptional ability of *D. salina* to accumulate massive amounts of ß-carotene, primarily in response to high light stress (Ben-Amotz & Avron, 1983; Smith *et al.*, 1990; Lers *et al.*, 1990; Cowan & Rose, 1991), makes it an ideal organism for studying photosynthetic stress physiology. Also, salt stress of cells of *Dunaliella* results in the accumulation of ß-carotene (Ben-Amotz & Avron, 1983). These physiological responses would appear to function as mechanisms designed to protect the organism against high intensity irradiation (Ben-Amotz & Avron, 1989) and either hypo- or hypersalinity (Kirst, 1990), and could therefore be mediated by a signal transduction mechanism involving pH (Cowan *et al.*, 1992).

Since all life is based on aqueous chemistry and because water spontaneously ionizes, protons cannot be excluded from the intracellular milieu. Yet an equivalent role of

pH as a messenger has been less widely accepted (Felle, 1989). Besides being both the substrate and product of metabolic pathways, protons have an apparent potential to communicate information about the cellular energy balance to enzymes and structures that may share no other common effector (Felle, 1989). Since small internal pH changes cause large effects on the activity of enzymes (membrane-bound transport proteins included), the potential impact on intracellular processes, in principle, need not be mediated by specialized receptors such as those required by cytoplasmic free Ca^{2+} and cyclic AMP, but could possibly be regulated by ATPases (Pick, 1992).

In animal systems, an acidic cytoplasm appears to be associated with dormant or quiescent states, whereas an increase in cytoplasmic pH signals cellular reactivation (Roos & Salvik, 1981; Nucitelli & Deamer, 1982; Busa & Nuccitelli, 1984; Frelin *et al.*, 1988). A number of reports indicate that modifications of cytoplasmic pH are important for the control of the cell cycle, division, and growth (Gillies & Deamer, 1979; Gillies *et al.*, 1981; Gross *et al.*, 1983). An increase in pH is necessary to bring cells from G_0 to G_1 and into S phase for echinoderm eggs, protozoa, slime moulds, and mammalian cells (Madshus, 1988). More recently, Ober and Pardee (1987) reported that tumorigenic Chinese hamster embryo fibroblast cell lines maintain an internal pH significantly higher than the one of normal cells. Evidence has also been provided that cytoplasmic pH can determine the choice between alternative pathways of cell differentiation in *Dictyostelium discoideum* (Gross *et al.*, 1983). These results suggest that cytoplasmic pH may behave as a signal in the control and regulation of cell division.

Changes in cytoplasmic pH and cytoplasmic Ca^{2+} are often associated in animal cells (Nuccitelli & Deamer, 1982; Busa & Nuccitelli, 1984; Frelin *et al.*, 1988). Correlative evidence shows that in some systems the cytoplasm is acidified when Ca^{2+} levels rise, while direct evidence comes from the cytoplasmic acidification induced by injection of Ca^{2+} in snail neurons (Frelin *et al.*, 1988, and refs. therein). Changes in Ca^{2+} concentration in response to cytoplasmic pH modifications have been reported for a variety of cell types such as rat lymphocytes, where the hormonal activation of the Na^+/H^+ antiport induces an increase in cytoplasmic pH (Grinstein & Goetz, 1985) paralleled by an increase in cytoplasmic Ca^{2+}. This suggests an important role for cytoplasmic pH behaving as a messenger controlling Ca^{2+} levels in the cytoplasm. In plants reports on simultaneous variations of cytoplasmic Ca^{2+} and pH are available (Felle, 1989 and refs. therein). In *Zea mays* coleoptiles, an increase of free Ca^{2+} has been shown to be associated with the decrease of cytoplasmic pH induced by indoleacetic acid (Felle, 1988). Illumination of *Nitellopsis* has been shown to elicit an increase of cytoplasmic Ca^{2+} which is reversed by darkness (Schaller & Sussman, 1988). Such treatment is known to induce a reversible alkalization of the cytoplasm. These results suggest that in plant cells, as in animal systems, pH and Ca^{2+} messages may be associated. Certainly, the importance of Ca^{2+}, for instance, as an activator and regulator of numerous intracellular processes has been clearly demonstrated (Allan & Trewavas, 1987).

The phosphoinositides have been suggested to act as messengers in plant cells (Drobak, 1993, and refs. therein) and in *D. salina* (Thompson, 1994) but this particular theory is still extremely controversial (Einspahr, 1993; Trewavas, 1993,

in reply to Einspahr). Recent advances have confirmed that phospholipase C-catalyzed hydrolysis of inositol phospholipids constitutes a major avenue for transducing extracellular signals across the plasma membrane in animal cells. Such transduction leads to changes in cytoplasmic Ca^{2+} levels and changes in protein phosphorylation patterns. Despite the expected participation of a plasma membrane-associated receptor in plant phosphatidylinositol-4,5-bisphosphate (PIP_2) mediated signal transduction, no such receptor has been identified (Einsphar & Thompson, 1990). Extensive research has focused on PIP_2 metabolism as a transducing mechanism for the control of leaf movement by light in the legume *Samanea saman*. The effects of light on phosphoinositide turnover in the *S. saman* motor organ responsible for leaf movement were assessed in [^{3}H]-inositol labelled tissue (Morse *et al.*, 1987). It was shown that after 15 seconds, light causes an increase in PIP_2 levels, and that after 30 seconds these levels had dropped to almost 40% of the control. In *D. salina* hypoosmotic shock induced rapid degradation of PIP_2 with a concomitant increase in phosphatidic acid (Einsphar *et al.*, 1988) However, the physiological significance of these results is not clear since neither changes in cytoplasmic Ca^{2+}, altered protein kinase activity, nor cytoplasmic pH were assessed as potential means of linking PIP_2 turnover to the pronounced metabolic responses of these cells to osmotic stress. It has also been shown that stimulation of some photosynthetic cell types by environmental or plant growth regulator challenge is accompanied by a degradation of poly-PIP_2. The products of this breakdown, inositol 1,4,5-trisphosphate and diacylglycerol, appear to be capable of releasing organelle-bound Ca^{2+} (Thompson, 1994). However, a direct cause and effect relationship between stimulated PIP_2 breakdown and changes in intracellular Ca^{2+}, protein

phosphorylation, or cell function has not yet been unequivocally established (Einsphar & Thompson, 1990; Ha & Thompson, 1991, 1992; Thompson, 1994).

Except in the controversial domain of the action of plant growth regulators on intracellular pH, there are few reports concerning cytoplasmic pH variations in relation to growth and development in plants. In Japanese artichoke (Tort & Gendraud, 1984), slow-growing tubers and fast-growing sprouts from non-dormant tubers display a difference in intracellular pH measured with the DMO (5,5-dimethyl-oxazolidine dione) technique. In Jerusalem artichoke, dormant and non-dormant tubers grown *in vitro* also display a difference in their intracellular pH values. The cell sap pH and the cytoplasmic pH estimated with DMO are both higher by 0.4 pH units in dormant tubers than in non-dormant tubers (Gendraud & Lafleuriel, 1983).

Although the concept of signal transduction and amplification has been accepted for animal cells for quite some time, plant physiologists have until recently found it difficult to transfer these principles to their systems. The hypothesis that pH could act as the secondary messengers of plant growth regulators is the subject of much controversy. In animal systems, the Na^+/H^+ antiport may be regulated by hormones (eg. insulin) and several growth factors, and in *D.salina* (Pick, 1992). Evidence has also been provided that bicarbonate exchange systems are also under hormonal control as revealed by their sensitivity to phorbol esters (Frelin *et al.*, 1988). The development of plants has been shown to be under the control of plant growth regulators, and concerns the influence of intracellular pH on the distribution of weakly acidic plant growth regulators between the extracellular medium and cells,

as well as between different cell compartments. This has been demonstrated to some extent for ABA and auxins (Kurkdjian *et al.*, 1979; Kaiser & Hartung, 1981; ; Rubery, 1987, Hartung & Baier, 1990; Heatherington & Quatrano, 1991).

The question of the action of plant growth regulators on intracellular pH in plant cells has raised much debate due to contradictory experimental results obtained (Kurkdjian & Guern, 1989). Discrepancies amongst these results are difficult to interpret, particularly because of the different experimental conditions, methods used (invasive and non-invasive) and differences in biological material as well as physiological state of the material. Talbott *et al.* (1988) have rejected the hypothesis that protons could act as messengers in auxin-induced cell elongation on the basis of the absence of measurable cytoplasmic pH shifts in auxin-treated pea internodes. However, the possibility should be considered that local pH variations in the vicinity of the inner surface of the plasmalemma could be functionally significant in terms of growth control. The activity of the H^+ pump could create local intracellular H^+ domains where the pH is increased in such a way as to induce the events involved in growth (Kurkdjian & Guern, 1989). Such local pH variations cannot be demonstrated using conventional techniques of intracellular pH measurement, but their occurrence is suggested by the existence of local pH domains at the external surface to the plasmalemma (Price & Badger, 1985; Sentenac & Grignon, 1987).

The possibility that non-plant growth regulator signals use intracellular pH variations as messengers has also been investigated. In the alga *Eremosphaera viridis*, the acidification of the cytoplasm induces an 'action potential-like response' (Kohler *et*

al., 1986; Steigner *et al.*, 1988) corresponding to an increase in the conductivity of K^+ channels. This suggests that in plant cells, as in excitable animal cells, intracellular pH modifies the properties of ionic channels (Moody, 1984) and plays an important role in the signal transduction from photosystems in the chloroplast to K^+ channels in the plasmalemma. In *Phaseolus vulgaris* cells in suspension culture, a glucan treatment that elicits the synthesis of a phytoalexin induces rapid and transient decreases of vacuolar and cytoplasmic pH (Ojalvo *et al.*, 1987). The mechanisms of these pH variations are not known, and the hypothesis that they could act as messengers of elicitor action remains to be tested. This system offers an interesting parallel with the chemotactic behaviour of human neutrophils reacting to a foreign intruder by cytoplasmic acidification (Yuli & Oplatka, 1987). The early expression of chemotaxis can be induced or suppressed by simply manipulating the H^+ concentration in the cytoplasmic. This suggests that the interaction of chemoattractant with its specific receptor is translated into cytoplasmic acidification, which then triggers the chemotactic transduction cascade.

STRESS SIGNALLING IN *Dunaliella*

Glycerol is produced in *Dunaliella* as an osmoticum either by photosynthetic CO_2 fixation or by starch degradation. The contribution of these metabolic pathways to glycerol synthesis depends on the availability of light, the starch reserve pool, and the size of the salt stress (Ben-Amotz & Shaish, 1992). The important role of photosynthesis as a carbon source for glycerol production has been demonstrated by [^{14}C]-labelling experiments which show that over 80% of the photosynthetically fixed CO_2 following hyperosmotic shock is recovered in glycerol (Ben-Amotz & Avron,

1978) In the dark *Dunaliella* produces glycerol exclusively by degradation of starch, and the capacity of the cells to recover from hyperosmotic shock depends on their starch reserve (Gimmler *et al.*, 1981; Oren-Shamir *et al.*, 1989). Hyperosmotic shock in the light greatly enhances the rate of glycerol production and in parallel enhances starch degradation, indicating that starch degradation also has a significant contribution to glycerol production in the light (Gimmler *et al.*, 1981).

Attempts to identify the signal transduction mechanisms whereby *Dunaliella* species overcome salt stress have focused on ATPase activity (Oren-Shamir *et al.*, 1990), phosphate metabolism (Ginzburg *et al.*, 1988; Kuchitsu *et al.*, 1989; Bental *et al.*, 1990), and phospholipase-C mediated hydrolysis of PIP_2 to diacylglycerol (Maeda & Thompson, 1986; Einsphar *et al.*, 1988; Ha & Thompson, 1991, 1992; Thompson, 1994). All except one of these studies centred around either osmotic up- or down-shocks, whilst Ginzburg *et al.* (1988) concerned themselves with the effects of oxygenation on light and dark adapted cells and related phosphate metabolism. These authors identified two phosphodiesters (glycerophosphorylglycerol and glycerophosphorylcholine) for the first time in *Dunaliella*. Using ^{31}P NMR they also identified one P_i pool within the cell and used it to monitor intracellular pH in response to oxygenation of light and dark adapted cells.

Kuchitsu *et al.* (1989) using ^{31}P NMR elucidated two intracellular compartments from the P_i signals, in *D. tertiolecta*. The pH of the compartments were estimated at pH 7.1 and 6.0, and assigned to cytoplasm and vacuole respectively. During adaptation to salt stress (0.17 to 1.0 M) both cytoplasmic and vacuolar pH showed transient

increases to pH 8.0 and 6.5 respectively. Subsequently, cytoplasmic P_i, sugar phosphates and terminal phosphate group levels increased. These authors concluded that these changes in cytoplasmic pH and P_i may be regulatory factors for osmoregulation that control synthesis and inhibit breakdown of glycerol. Bental *et al.* (1990) developed a new hypothesis for the mechanism of metabolic response during osmoregulation in *D. salina*. They proposed that the osmotic response is initiated by differential volume changes of the cytoplasm and the chloroplast which alter the cytoplasmic P_i concentration. This triggers a flow through the P_i/triose-phosphate shuttle, activating chloroplast enzymes in the direction of either starch or glycerol synthesis.

Oren-Shamir *et al.* (1990) used the fluorescent cyanine dye diS-C_3-(5) which is sensitive to the membrane potential, to follow changes in plasma membrane potential in *D. salina* during hypertonic shock. These authors showed that following hypertonic shock, the plasma membrane was rapidly hyper-polarized. This hyper-polarization was transient, and the alga reverts to its resting membrane potential after 30 minutes. This transient hyper-polarization was linked to ATPase activation, thus these authors concluded that activation of the plasma membrane ATPase may be the trigger for osmoregulation in *D. salina*.

Thompsons' group (Maeda & Thompson, 1986; Einsphar *et al.*, 1988; Ha & Thompson, 1991, 1992, Thompson, 1994) have focused on the role of phospholipid metabolism and diacylglycerol, in phospholipase-C mediated response to osmotic stress in *D. salina*. They have shown that phospholipase-C mediated hydrolysis of

PIP_2 is triggered by hypoosmotic stress in *D. salina*, and have developed a hypothesis whereby the products of this hydrolysis act as messengers in this signalling pathway. They have also shown that the formation of diacylglycerol is biphasic and culminates in the *de novo* synthesis of diacylglycerol as well as phosphorylation of a 29 kDa protein which is as yet still unidentified.

Synthesis of protein during salt stress has also been the topic of investigation by Sadka *et al.* (1991). These authors investigated the protein profiles of cells grown at different salt concentrations. A 150 kDa (p 150) protein increased in amount with salt concentration, and when cells were subjected to drastic hyperosmotic shock, the concentration of p 150 started to rise, long after completion of the osmotic response, but coincident with re-initiation of cell proliferation. This protein was identified as a detergent soluble glycoprotein which plays no role in salt stress signalling, but rather as a protein required for cell proliferation in high salinity environments.

THE ROLE OF pH IN SIGNAL TRANSDUCTION

In plants, despite the scarcity of information about the control exerted by pH on physiological functions, the main features of the distribution of protons in plant cells are now reasonably established (Raven, 1985). Large transmembrane pH gradients are established across membranes such as the tonoplast (pH 7.5 cytoplasmic side, pH 5.0 vacuolar side), and of the thylakoid membrane in the light (pH 4.0 lumenal, pH 8.0 stromal) (Kurkdjian & Guern, 1989). The pH of the cytoplasm is maintained at relatively constant values close to neutrality despite the existence of pH-perturbing processes which have been extensively analyzed in several reviews (Raven & Smith,

1974; Raven & Smith, 1976; Smith & Raven, 1979, Raven, 1990). The idea that intracellular pH acts as a regulatory signal implies that some critical steps in cell activity are highly sensitive to pH variations. Kurkdjian & Guern (1989) have suggested that small changes in pH should be sufficient to induce drastic shifts in the activity of some critical proteins, and the various systems responsible for intracellular pH regulation most likely constitute a network and can compensate each other (Kurkdjian & Guern, 1989). It is also possible that pH may play a role in two-component pathways similar to the two-component pathways recently discovered in *Arabidopsis thaliana* by Chang *et al.* (1993) involving the ethylene response.

Various methods are available for measuring intracellular pH in plant cells. Most were first developed to study animal systems (Roos & Slavik, 1981; Nuccitelli & Deamer, 1982) and were adapted for plants. These methods include (i) chemical probes - based on the preferential diffusion through membranes of undissociated molecules of a radiolabelled or fluorescent probe. The corresponding accumulation ratio of the chemical probe is a function of the extracellular and intracellular pH, and the pKa of the probe. Examples included DMO, 9-aminoacridine, and radiolabelled methylamine and benzylamine. (ii) Proton selective micro electrodes -these micro electrodes are built with a H^+-exchange carrier and allow the measurement of an electron motive force related to H^+ ion activity in the compartment where the electrode is inserted. Recent progress has been made in the use of these electrodes, in that double barrelled electrodes allow simultaneous measurement of voltage and pH (Felle, 1987; Reid & Smith, 1988). (iii) Sap pH measurements - where the cellular sap is extracted and the pH is measured directly by conventional means. (iv)

^{31}P Nuclear magnetic resonance (NMR) - this technique relies on the fact that the chemical shift (resonance frequency relative to a standard) of ^{31}P compounds is pH dependent. With the exception of ^{31}P-NMR, the above techniques have the disadvantage that they are invasive to cellular physiology. An advantage of ^{31}P-NMR is that besides intracellular pH measurements, it also produces interesting information about changes in the energetic status and phosphate compartmentation of the cells.

In recent years numerous researchers have focused on the importance of temporal resource fluctuations in algal nutrition and growth. Goldman & Peavey (1979), Goldman *et al.* (1979) and Gilbert and Goldman (1981) have provided physiological evidence for this nutrient problem, whilst Turpin (1983) has concentrated on the effect of nutrient stress and photosynthetic carbon assimilation. The nature of the interaction between NH^+_4 assimilation and photosynthetic carbon fixation is of major importance in understanding algal growth in fluctuating environments. Much work has focused on the long-term responses of photosynthetic carbon assimilation to addition of NH^+_4 to the growth medium (Thomas *et al.*, 1976; Collos & Slawyk, 1979; Healey, 1979; Quarmby *et al.*, 1982; Turpin, 1983). Conflicting evidence has been produced from these studies. Quarmby *et al.* (1982) has shown that fluctuating NH^+_4 regimes are capable of resulting in marked periodic fluctuations in carbon fixation in nitrogen limited algae. On the other hand, Goldman *et al.* (1981ab) suggest that additions of NH^+_4 to nitrogen limited algae resulted in no short-term photosynthetic interactions. Pick *et al.* (1990) have reported that influx of ammonia into cells of *D. salina* induces rapid alkalization of the cytoplasm, followed by a

recovery of the cytoplasmic pH, which occurs in parallel with massive hydrolysis of polyphosphates. It is not clear how photosynthetic cells regulate intracellular pH and avoid uncoupling of photosynthesis during amine uptake and metabolism. However, it has been suggested that in *D. salina* acidic vacuoles serve as a high-capacity buffering system for amines, and as a safeguard against cytoplasmic alkalization and uncoupling of photosynthesis (Pick *et al.*, 1991).

PHOTOSYNTHETIC STRESS RESPONSE AND pH

pH-dependent regulation of PS II occurs in plants when photosynthesis is limited by the biochemical reactions of carbon metabolism and excess light energy is absorbed (Kreiger *et al.*, 1993). Under such conditions the ΔpH across the thylakoid membrane increases to values higher than that required for maximal ATP synthesis. A high ΔpH exerts control to PS II and ΔpH-dependent down-regulation may adjust PS II activity to the actual requirement of electrons by carbon metabolism (Weis & Berry, 1987; Horton & Hague, 1988; Krause & Weis, 1991). This energy-dependent decrease in PS II activity is accompanied by a decline in chlorophyll fluorescence quenching (Krause & Weis, 1991), and excess light energy absorbed by PS II is dissipated non-photochemically and as fluorescence. By this control process, the reaction centre is protected against photodestruction. It has been proposed that quenching in the antenna is related to the formation of zeaxanthin from violaxanthin (Demmig-Adams, 1990) or by aggregation of the LHC II complexes (Horton *et al.*, 1991). Gilmore & Yamamoto (1993) have demonstrated that non-photochemical quenching is xanthophyll dependent, and that this quenching requires a threshold lumen pH that varies with xanthophyll composition. After the threshold, non-

photochemical quenching is linear with lumen acidity and xanthophyll composition. These authors have also shown that SV_E (Stern Volmer energy-dependent non-photochemical quenching) correlates with the production of zeaxanthin plus antheraxanthin and lumen acidity over the entire range of de-epoxidation and lumen pH. The pH of the lumen is dependent on light driven electron transport for translocation of H^+ into the lumen.

Gilmore & Yamamoto (1992) have also demonstrated dark induced zeaxanthin formation from violaxanthin. Thus the direct control of xanthophyll cycle activity by lumenal pH intimates pH as the transducer mechanism by which stress affecting the photosynthetic machinery is manifested to protective metabolism, as well as perhaps ultimately regulating general cell metabolism.

It is widely accepted that β-carotene protects the cell against injury by high intensity radiation under limited growth conditions by acting as a screen to absorb excess radiation (Borowitzka & Borowitzka, 1988; Ben-Amotz, 1989). In *D. salina*, total carotenoid concentration has been shown to increase linearly with increasing light intensity (Loeblich, 1982), however the Chl*a* and Chl*b* concentrations decrease with increasing light intensity up to a saturation point (Ben-Amotz & Avron, 1983; Borowitzka, *et al.*, 1984). Many workers have suggested that β-carotene has a photoprotective function in *Dunaliella* and in other algae that grow in high light environments, by absorbing light energy in the blue region of the spectrum (Shaish *et al.*, 1991, and refs. therein). It has been shown that Chl*a* can be protected from photobleaching in high light by β-carotene, and that *cis*-β-carotene effectively

quenches chemically generated singlet oxygen and is transformed to all-*trans*-ß-carotene (Foote *et al.*, 1970ab).

It has also been suggested that ß-carotene protects against Chl*a* catalyzed singlet oxygen and possibly other excited Chl damaging agents. However, due to the large distance between the ß-carotene globules and the thylakoid located Chl, and due to the short lifetime of these damaging compounds, it has been argued that the massively accumulated ß-carotene in the globules cannot be effective in this mechanism (Ben-Amotz & Shaish, 1992). It is however, possible that thylakoid bound ß-carotene may function in such a mechanism. Recently, Strzalka & Gruszecki (1994) revealed that ß-carotene affects structural and dynamic properties of model dipalmitoylphosphatidylcholine membranes more than the polar carotenoid lutein. These authors showed that this was effected by (1) a decrease in the order of the crystalline state of the membrane, (2) an increase in penetration of a polar molecules into the membrane and, (3) an increase in the electron paramagnetic resonance correlation times. In a continuation of this work, Jezowska *et al.* (1994) demonstrated using NMR that ß-carotene increases motional freedom of lipid head-groups in these model membranes and increases motional freedom of alkyl chains forming the hydrophobic core of the membrane greater than that of a choline moiety. The results from both these studies provided evidence that ß-carotene plays a physiological function in the fluidization of chloroplast membranes during stress to the photosynthetic apparatus.

Lers *et al.* (1990) investigated the photoinduction of ß-carotene in *Dunaliella bardawil* exposed to high light stress. These authors showed that in cells exposed to high light, ß-carotene accumulates in two stages: the first stage, lasting 24 hours, starts shortly after exposure; the second stage starts concomitantly with the onset of stationary phase and persists until the cells collapse. They also showed, using inhibitors of protein synthesis, that gene activation plays a role in the accumulation of ß-carotene. Levy *et al.* (1992; 1993) have cloned a nuclear gene named *Cbr*, that is co-induced with accelerated ß-carotene synthesis. Subsequently, these authors showed that the *Cbr* gene product is structurally similar to early light-induced proteins (Elips) of higher plants and the it binds to zeaxanthin to form a photo-protective complex within the light-harvesting antennae.

Although much work has been done on processes co-incident with the synthesis of ß-carotene (light stress) and glycerol (salt stress) in *Dunaliella*, mechanisms mediating stress signal transduction remain to be explained.

RESEARCH HYPOTHESIS

Because photosynthesis is the primary reaction of all green plants, any perturbation of this reaction will have major consequences for the general cell metabolism. Thus protection of the photosynthetic machinery is primary to the survival of the cell and, as such, changes in the environment resulting in conditions potentially detrimental to the cell must be communicated rapidly to allow initiation of protective mechanisms.

It is hypothesized that perception of high light stress and salt stress by the photosynthetic machinery may be transduced via an alteration of pH (the signal) to the systems responsible for initiating protection mechanisms, namely de-epoxidation of violaxanthin to zeaxanthin in the short term (carbon pool), and massive accumulation of globular β-carotene in the chloroplast stroma in the long term (protection).

RESEARCH OBJECTIVES

The following research programme was formulated in order to examine the validity of the research hypothesis:

1. To measure the response to stress of the carbon pool intermediates.
2. To elucidate the rapid and short term events leading to induction of β-carotene accumulation in the chloroplast stroma.
3. To determine whether intracellular pH plays a role in the stress signal transduction mechanism.

Chapter 2

STRESS EFFECTS ON PHOTOSYNTHESIS

INTRODUCTION

Dunaliella has numerous advantages as a model system for research in the fields of photosynthesis and stress physiology (Cowan *et al.*, 1992). Continuous culture in the laboratory is simple and the growth rate is relatively high (Goldman *et al.*, 1979). Using such advantages, synchronous culture under controlled environmental conditions such as light, temperature, salinity, and nutrient concentrations are possible (Baas-Becking, 1931; McLachlan, 1960; Ukeles, 1961; Eppley, 1972; Eppley & Sloan, 1966; Bruggemann *et al.*, 1978; Aizawa, 1984). Photosynthetic responses to harsh changes in environmental conditions are more readily studied as *Dunaliella* is capable of rapid adaption to such changes.

Stress is the result of any constraining force or influence which limits normal growth and development (Seeley, 1990). This is reflected in physiological responses, of which the effects are characteristic of the particular stress imposed. The significance of stress factors on the accumulation of β-carotene and glycerol in *D. salina* has already been noted. An understanding of stress at the physiological level provides a clear advantage for the biotechnological manipulation of this organism for the production of metabolites.

Plants have developed regulatory mechanisms to be able to adapt their photosynthetic apparatus to variations in light intensity, from dim light to full sunlight. This is

especially true for planktonic algae, which are capable of moving vertically through the photic zone of their environment. In plants, carotenoids are essentially components of the light-harvesting complexes and reaction centres of the photosynthetic apparatus. They are involved in light harvesting, energy transduction and protection against damage by photo-oxidation (Koyama, 1991). Their synthesis is co-ordinately regulated with chloroplast development (Kleinig, 1989), but the mechanism of regulation is unknown. In *Dunaliella*, carotenoid accumulation is brought about high light intensities, nutrient stress or high salinity conditions. Each of these factors has an effect on carotenoid biosynthesis, but when applied together, their effects are additive (Loeblich, 1982). Clearly these environmental conditions will also affect the efficiency of the photosynthetic apparatus.

Here the effect of high light intensity on photosynthesis in *D. salina* is evaluated with respect to fluctuations in chlorophylls, β-carotene, O_2 evolution and carbon assimilation.

MATERIALS AND METHODS

Growth medium:

Dunaliella salina (Teodoresco) (strain CCAP 19/30) obtained from Culture Collection of Algae and Protozoa (Argyll, Scotland, UK) were grown in artificial sea water medium supplemented with nutrients (Table 2.1.). The salinity of the medium was adjusted to 1.5M NaCl and the pH corrected to 8.2 with either HCl or NaOH.

Table 2.1. Growth medium for *Dunaliella salina*.

Compound	Concentration	Mol. Weight	H_2O Conversion
NaCl	1.5M	58.44	
$NaHCO_3$	50.0mM	84.01	
KNO_3	5.0mM	101.11	
$MgSO_4$	5.0mM	120.37	$7H_2O$ - 246.48 x 2
$CaCl_2$	0.3mM	110.99	$2H_2O$ - 147.02 x 1.32
KH_2PO_4	0.2mM	136.09	
$FeCl_2$	1.5μM	159.21	$6H_2O$ - 267.21 x 1.68
EDTA	6.0μM	372.24	
H_3BO_4	185.0μM	61.83	
$MnCl_2$	7.0μM	125.84	$4H_2O$ - 197.91 x 1.57
$ZnCl_2$	0.8μM	136.28	
$COCl_2$	0.02μM	129.84	$6H_2O$ - 231.95 x 1.83
$CaCl_2$	0.2μM	134.45	$2H_2O$ - 170.45 x 1.27

Inoculation of the culture was realized by addition of the basic stock culture in its logarithmic growth phase at 10% of total growth medium. The cells were cultivated in a 2 L glass erlenmeyer flask at 25°C. The culture was illuminated by a mixture of cool white fluorescent and incandescent light. Irradiance was measured with a Skye Model photometer (Skye Incorp, Wales) with a SK 215 probe head for photon flux density (PFD) and a SK 210 probe head for photosynthetically active radiation (PAR). PFD was 180 μmol.m^{-2}.s^{-1} and PAR 300 μmol.m^{-2}.s^{-1}, both measured at the surface of the culture. All experiments were conducted in triplicate, except for those involving the use of dual radioisotopes as the cost for duplication here was excessive.

Cell counts:

Cells were immobilized with iodine and then counted using a Neubauer Haemocytometer. Cells were counted and expressed as number of cells per mL.

Chlorophyll and total carotenoid extraction:

A volume of culture (usually 5 or 10 mL) was centrifuged for 10 minutes at 2500 g. The supernatant was discarded and the pellet resuspended in 100% acetone and pigments extracted in the dark for 30 minutes. The extract was diluted to 80% acetone with distilled water and centrifuged for 10 minutes at 2500 g. Absorbance was read in a UV-160A Shimadzu spectrophotometer (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) according to Lichtenthaler (1987) and Lichtenthaler *pers. comm.* (1991).

Photosynthesis:

Cells were grown as described above. Cell density was maintained at about 30 X 10^4 cells per mL. Cells were centrifuged (2500 g) and resuspended in fresh medium the day before experimentation. Cells were transferred from a low light environment ($120\mu mol.m^{-2}s^{-1}$) to high light ($1000\mu mol.m^{-2}s^{-1}$) provided by a 400W high pressure Sodium lamp (Lascon Lighting, Port Elizabeth, SA).

Photosynthetic carbon uptake was determined using $NaH[^{14}C]O_3$ (Amersham) as inorganic carbon supplied to the growth medium. Radiolabelled $NaH[^{14}C]O_3$ ($2\mu Ci$) was added to 40 mL cell culture of *D. salina*. Samples (2mL) of cell culture were extracted, to which 1 mL of 1N HCl (pH < 0.5) and 0.5 mL of 20% formaldehyde

were added. The sample was then allow to stand for 5 hours at 15°C to permit excess $[^{14}C]O_2$ in the medium to diffuse out. Scintillation cocktail (Scintillator 299, Packard Instrument Co., CT, USA) (7½mL) was added, and the samples kept in the dark to reduce chemiluminescence, after which radioactivity was counted in a Beckman 1086 Scintillation counter with a full (0-1000) window for $[^{14}C]$. Chlorophyll ($1\mu g.mL^{-1}$) was added as internal standard to correct for quenching. Uptake of $[^{14}C]$ is expressed as:

$$mg.C\ mg.Chl_a = \frac{R\ x\ dpm\ x\ CF}{mg.Chl_a}$$

where: R = ratio of $[^{12}C]:[^{14}C]$ ($1.42 \times 10^6:1$);

dpm = disintegration per minute;

CF = correction factor (1.06).

Oxygen evolution was measured as $\mu mol.O_2\ mg^{-1}$ chlorophyll h^{-1}, using a Hansatech micro oxygen electrode in a Hansatech DW2 sample chamber(Hansatech Instruments Ltd., Kings Lynn, Norfolk, England). The DW2 sample chamber was temperature controlled via a Lauda cooler at 25°C. Light was transmitted via a fibre optic cable into the DW2 chamber from an intensity regulated halogen lamp. Oxygen evolution was measured at 1 minute intervals for 2 hours at low light, and thereafter at 5 minute intervals.

RESULTS

Figure 2.1 shows the increase in cell growth for both control and high light treated cells. Characteristic of *D. salina* cells exposed to high light stress is a lag period of about 4 hours during which there is very little increase in cell number.

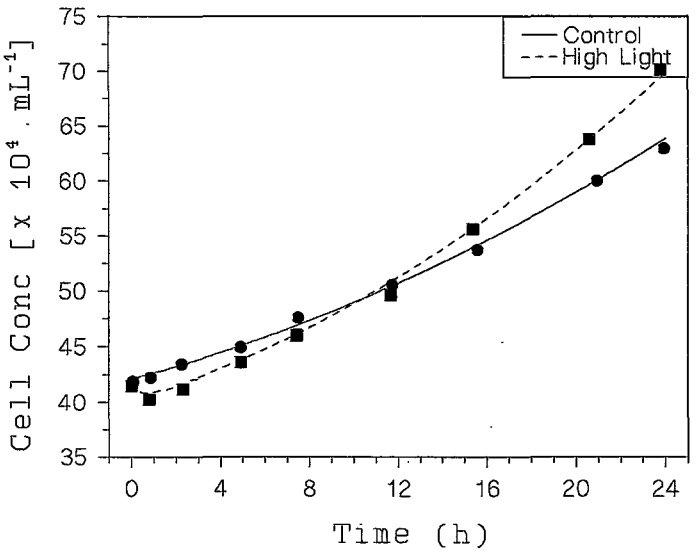

Figure 2.1. Cell growth for control and high light stressed cells.

The decrease in Chl*a* in response to high light stress is depicted in Figure 2.2. In response to high light stress cells of *D. salina* accumulate large mounts of *β*-carotene (Figure 2.3), after an initial slight decline during the first 2 hours after the imposition of high light stress. Figures 2.4 and 2.5 show the Chl*a*:*β*-carotene and Chl*a*:Chl*b* ratios respectively for control and high light stressed cells. In the first case there is a sharp decrease in the Chl*a*:*β*-carotene ratio and this is due to a rapid loss of Chl*a* after imposition of high light stress rather than an increase in *β*-carotene (see Figures 2.2 and 2.3). There is a rapid decrease in the Chl:*β*-carotene ratio in high light treated cells, however there is also a minor overall decrease in the ratio of control cells.

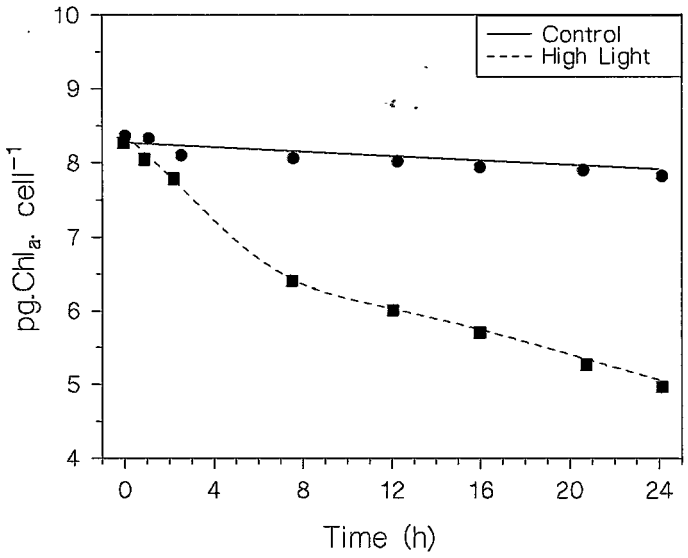

Figure 2.2. Changes in Chl*a* in control and high light stressed cells.

The Chl*a*:Chl*b* ratio in high light treated cells shows an overall decline, whereas in control cells an overall increase in the ratio is the dominant trend. Photosynthetic carbon assimilation in control and high light treated cells is shown in Figure 2.6. Cells kept under control conditions show a constant uptake of carbon. Those cells exposed to high light stress show a rapid uptake of carbon from the growth media. This rapid rate of carbon uptake reaches a plateau after about 12 hours, after which the rate of carbon uptake remains relatively constant. Figure 2.6 shows that cells exposed to high light intensity increase the rate of CO_2 assimilation compared to those cells maintained at low light. The decline in assimilation rate after 18 hours in those cells exposed to high light intensity is probably artifactual and could be attributed to a decrease in the availability of the [^{14}C] label in the medium. Evidence for this decline being artifactual is provided in Figure 2.7, where those cells exposed to high light intensity do not show a decrease in O_2 evolution rate, but levels off as the culture reaches the stationary phase of the growth curve.

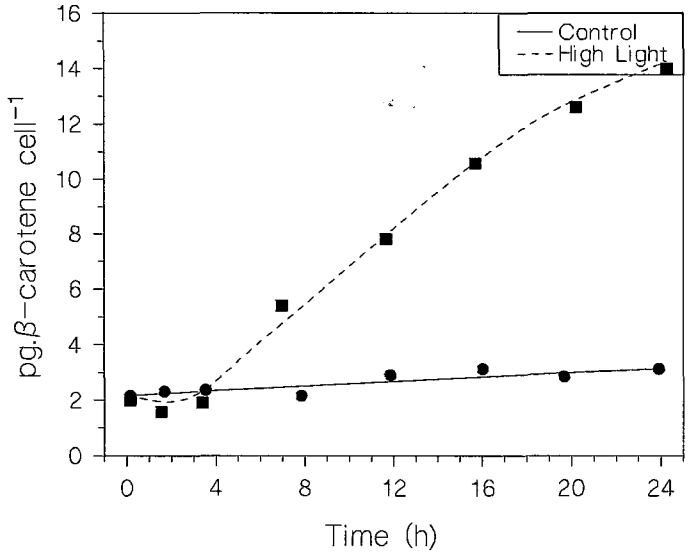

Figure 2.3. Changes in β-carotene levels in control and high light stressed cells.

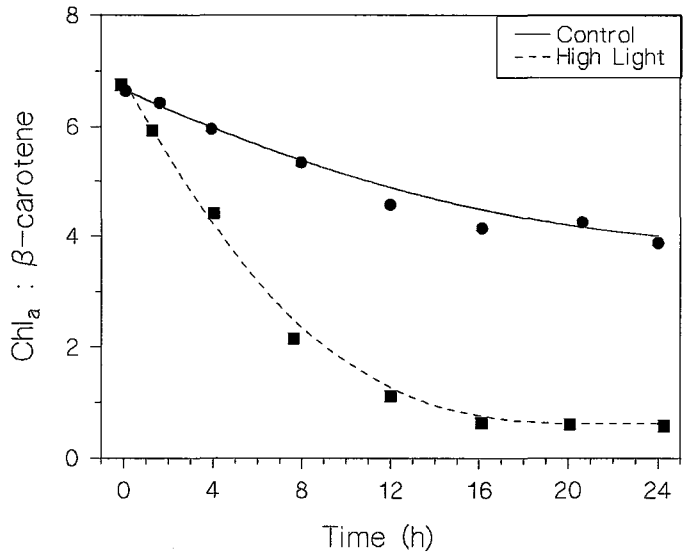

Figure 2.4. Chl*a* : β-carotene ratio for control and high light stressed cells.

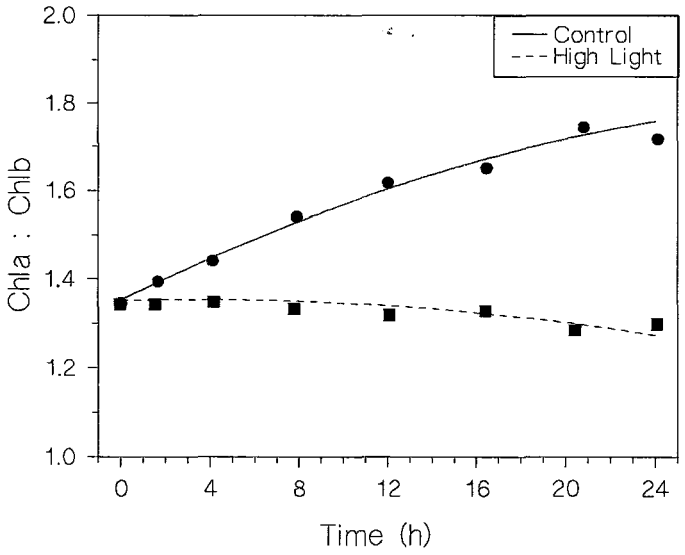

Figure 2.5. Chl*a* : Chl*b* ratio for control and high light stressed cells.

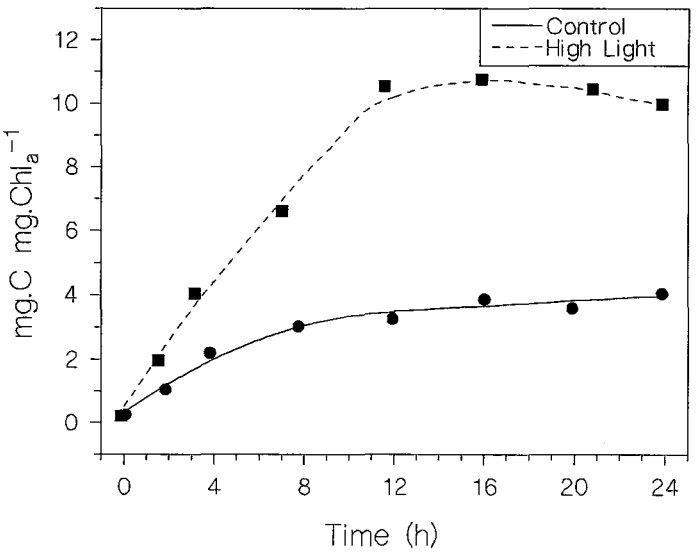

Figure 2.6. Photosynthetic carbon uptake in control and high light stressed cells.

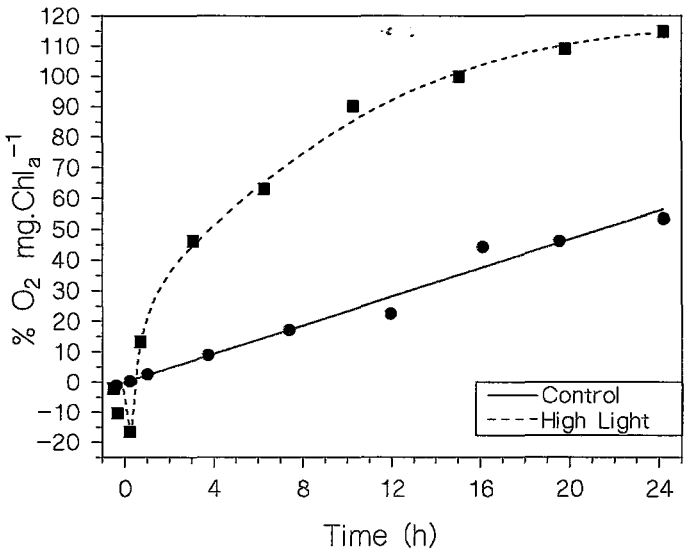

Figure 2.7. Photosynthetic oxygen evolution in control and high light stressed cells. Expressed as % O_2 evolution.

DISCUSSION

Photosynthetic organisms adapt to prolonged changes in light regime by adjusting photosystem stoichiometry and antenna size (reviewed by Melis, 1990). *D. salina* cells exposed to high light stress go through an initial phase of short term stress, followed by a period of acclimation to the stress conditions. This initial phase of stress is manifested in a decrease in cell number. Vorst *et al.* (1994) have correlated the effect of growth arrest and onset of accumulation of *ß*-carotene in *D. salina*. During this decrease in cell number there is also a sharp decrease in Chl*a* content, as well as a decline in *ß*-carotene levels and O_2 evolution rates (Rose, 1992). Results presented here confirm this trend.

It is generally agreed that the target of light-dependent reduction in photosynthetic efficiency is directed at the Q_A reaction centre protein of PS II (Kyle *et al.*, 1984;

Dementer *et al.*, 1987; Kim *et al.*, 1993). The synthesis of carotenoids which function as accessory light-harvesting pigments is co-regulated with the biosynthesis and assembly of the thylakoid photosynthetic and antenna complexes (Kleinig, 1989; Young & Britton, 1990). The decline in the Chl*a*:Chl*b* ratio, primarily in response to loss of Chl*a* by photobleaching (Aro *et al.*, 1993), is indicative of reassembly and acclimation of the photosynthetic machinery to high light stress. This pattern of loss of Chl*a* is confirmed here by an overall decline in the Chl*a*:Chl*b* ratio. Furthermore, the increase in *ß*-carotene in the chloroplast is thus intimately linked to stress perception by the photosynthetic machinery, more specifically degradation of Q_A. Studies on the organization of PS II following strong illumination *in vitro*, have indicated a partial disassembly of the hydrophobic core of this photosystem (Aro *et al.*, 1993). The size of the PS II has also been shown to undergo changes. When exposed to high light stress PS II_α become smaller within 4 hours (by loss of peripheral LHC II complexes associated with the core of PS II and, lateral movements to non-appressed regions of the thylakoid membrane) and these smaller reaction centres are designated PS $II_ß$ (Harrison *et al.*, 1992).

Thus it appears that the reduction in cell growth, and the lag phase in the synthesis of *ß*-carotene, primarily to shield the photosynthetic machinery from excess light, may be directly regulated by reorganization within the photosynthetic machinery itself. Furthermore, the appearance of Elips (early light-induced proteins) has been correlated with the photoinactivation of PS II, degradation of Q_A, and changes in the levels of photosynthetic pigments (Adamska *et al.*, 1993). One possible role of Elips could be their involvement in light-induced changes in the pigment content and/or

composition in photosynthetic membranes (Adamska *et al.*, 1993). It has been demonstrated that a decrease in photosynthetic CO_2 assimilation occurs because of photodestruction of Q_A, chlorophyll binding proteins, and LHC II is so significant that re-synthesis of these proteins cannot cope with the degradation rate. Also synthesis of Q_A may also be impaired to the same extent by photooxidation of chlorophyll, as chlorophyll is an obligatory component of Q_A (Sandmann *et al.*, 1993).

Previous reports (Portis *et al.*, 1987; Salvucci *et al.*, 1987; Campbell & Ogren, 1990) suggest involvement of the trans-thylakoid pH gradient in light activation of Rubisco activase. Associated with the requirement of a pH gradient is the requirement for electron transport through PS I (Campbell & Ogren, 1990). Maintenance of a pH gradient of continued electron flow from PS II to PS I is portrayed by the increase in $[^{14}C]O_2$ assimilation by cells exposed to high light intensity (Figure 2.6).

It has been previously shown on the imposition of high light stress there is a decline in *ß*-carotene in the pigment bed, and that after a lag phase of about 2-4 hours massive synthesis of *ß*-carotene in the chloroplast stroma is initiated. The decline in the *ß*-carotene associated with the pigment bed results in an increase in the Chl*a*:*ß*-carotene ratio. However, once this loss of *ß*-carotene has been compensated for by newly synthesized *ß*-carotene, the massive accumulation of *ß*-carotene in the stroma results in a sharp decline in this ratio. The Chl*a*:Chl*b* ratio in high light stressed cells shows an overall decline, whereas those cells kept under low light showed an

increase in this pigment ratio. The Chl*a*/Chl*b* ratio in *D. tertiolecta* (Falkowski & Owens, 1980; Falkowski *et al.*, 1981) and *D. salina* (Guenther & Melis, 1990; Smith *et al.*, 1990) increases as the cells adapt to higher irradiance. LHC comprises about half of Chl*a* and most of Chl*b* in plant cells respectively (Bennett, 1983), indicating that the changes in the amount of LHC results in changes of the Chl*a*/Chl*b* ratio in these cells. The Chl*a*/Chl*b* ratio of 8.1 in these cells grown under high light conditions (1200 μmol.m^{-2}.s^{-1}) is 1.5-fold higher than that of 5.4 observed in cells grown at low light (70 μmol.m^{-2}.s^{-1}). The Chl*a*/Chl*b* ratio of LHCII apo-protein is 2.4 and 4.7 in low- and high-light grown cells respectively (Sukenik *et al.*, 1989). These authors also suggested that efficiency of excitation transfer from carotenoid to Chl*a* declines in cells grown under high light intensity. Sukenik *et al.* (1987) conclude that both the reduction in Chl*b* content and the screening effect of poorly connected auxiliary pigments should diminish the photoinhibition under high light intensity.

The results presented in Figures 2.6 and 2.7 provide good evidence that cells of *D. salina* exposed to high light intensity are capable of altering the operational status of the photosynthetic machinery. It is also possible that the accumulation of β-carotene in those cells exposed to high light intensity contributes to a high photosynthetic rate, as a direct consequence of shielding blue light from the photosynthetic pigment bed (Shaish *et al.*, 1991, and refs. therein). Furthermore, Gomez-Pinchetti *et al.*, (1992) have demonstrated that cells of *D. salina* rich in β-carotene are more resistant to inhibition of O_2 evolution at high light intensities than green cells. However, these authors have also shown that when the same data is

expressed on a cell number basis the rate of oxygen evolution is significantly higher in the green cells. However, such an interpretation would be incorrect as it does not take into account the concept of the photosynthetic unit (Wilhelm, 1993).

CONCLUSION

The fluctuations in photosynthetic pigments in cells of *D. salina* are well described. The results of pigment fluctuations presented here give a clear indication that on exposure to high light intensity, cells of *D. salina* undergo a protective modification in photosystem stoichiometry. Photodegradation of Chl*a* by high light intensity is the first phase in the changing of the stoichiometry. Synthesis of *β*-carotene is perhaps the most noticeable consequence, although there is a 4 hour lag phase before the initial loss of *β*-carotene is made up (due either to photodegradation or destruction by active oxygen species). Irrespective of the mechanism by which the photosynthetic efficiency is temporary reduced, it is possible that the relatively large demand for reducing equivalents required for carotenoid biosynthesis might influence the photosynthetic efficiency. Vorst *et al.* (1994) have shown that this demand of reducing equivalents by carotenoid synthesis may in fact be blocked using diphenylamine, and go on to suggest that the accumulation of *β*-carotene in globules in the chloroplast stroma may in fact serve as an electron sink for the photosynthetic machinery when under stress. The results presented here demonstrate the remarkable ability of *D. salina* to rapidly conform to harsh changes in environmental conditions. Also, it is apparent from the [^{14}C] and O_2 evolution data, that *D. salina* not only accommodates a change to high light intensity but also increases its photosynthetic energy conversion efficiency.

Chapter 3

STRESS RESPONSE OF THE CARBON POOL INTERMEDIATES: Xanthophyll cycle activity, β-carotene and abscisic acid

INTRODUCTION

Upon absorption of more light energy than can be utilized by the photosynthetic electron transport system, several photoprotective mechanisms can prevent the potentially damaging accumulation of excitation energy in the photochemical apparatus. One of the most recently discovered of these is the xanthophyll cycle (Yamamoto, 1979) which involves the controlled and harmless thermal dissipation of excessive energy directly within the photochemical system, presumably in the pigment bed. This dissipation process more specifically involves the de-epoxidation state of the xanthophyll cycle pigments (Figure 3.1).

The xanthophyll cycle consists of light-dependent conversions of three xanthophylls in a cyclic reaction involving a de-epoxidation sequence from the di-epoxide violaxanthin via the mono-epoxide antheraxanthin to the epoxide-free form zeaxanthin, and an epoxidation sequence in the reverse direction (Demmig-Adams & Adams, 1992). These two reaction sequences are catalyzed by two different enzymes, and can occur simultaneously in light. The dependence of the reactions of the xanthophyll cycle on light is the consequence of the regulation, and not the

biochemistry of the cycle (Siefermann-Harms, 1977; Yamamoto, 1979; HÄger, 1980). This regulation is exercised by several factors associated with photosynthetic electron transport; it results in the accumulation of zeaxanthin under conditions of excess light and the reconversion of zeaxanthin to violaxanthin upon return to non-excessive light levels. Several factors regulate the fine-tuning of the formation of zeaxanthin from violaxanthin to the amount of excessive light absorbed. The de-epoxidation of violaxanthin to zeaxanthin requires an acidic thylakoid lumen pH and reduced ascorbate (Gilmore *et al.*, 1994).

The de-epoxidase reaction is light-dependent at physiological pH values but can also be driven in the dark at low pH, both *in vitro* and *in vivo* (Yamamoto, 1979; Pfundel & Strasser, 1988). A pH optimum for violaxanthin de-epoxidase of 4.8 - 5.2 has been established (HÄger, 1969), and that the rate of de-epoxidation depends on the structural organization of the cycle components in the membrane (Pfundel & Dilley, 1993). Also it has been recently shown that LHC II is in fact a zeaxanthin epoxidase (Gruszecki & Krupa, 1993).

It is well established that in response to both high light intensity and salt stress, cells of *Dunaliella salina* accumulate massive amounts of *ß*-carotene (Ben-Amotz & Shaish, 1992, and refs, therein; Lers *et al.*, 1990). Furthermore, the accumulation of *ß*-carotene appears to be related to the production of abscisic acid (ABA), at least in salt stressed cells (Cowan & Rose, 1991). There is now considerable evidence to support an indirect 'apo-carotenoid' pathway for the production of ABA biosynthesis (Parry & Horgan, 1991; and refs. therein) with the most likely precleavage

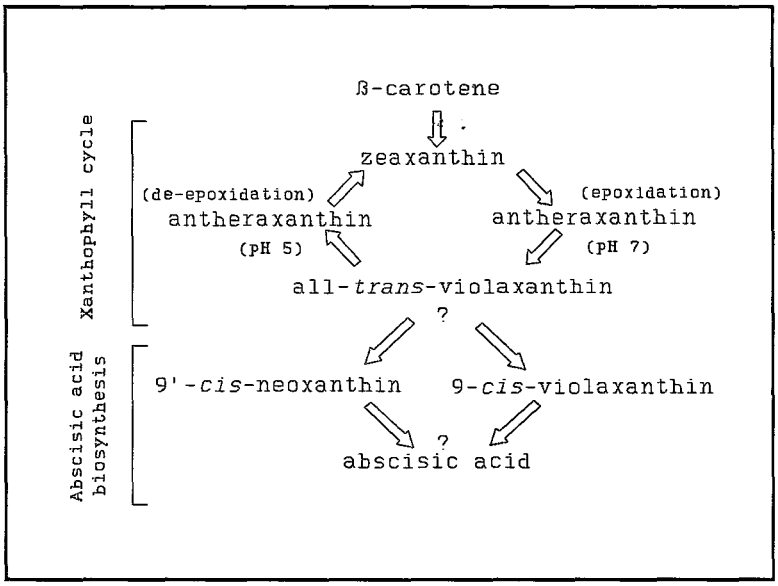

Figure 3.1. The xanthophyll cycle and biosynthesis of abscisic acid.

precursors being 9'-*cis*-neoxanthin or 9-*cis*-violaxanthin (Figure 3.1) (Parry *et al.*, 1990; Li & Walton, 1990). Lipoxygenase cleavage of violaxanthin across the 11, 12 (11', 12') double bond produces xanthoxin (Lutz & Winterhaler, 1992) which, is readily converted to ABA by plant tissues (Taylor & Burden, 1974; Parry *et al.*, 1988). The regulation of ABA biosynthesis occurs prior to the production of xanthoxin, and could involve control of violaxanthin and neoxanthin interconversion and/or cleavage reaction (Parry *et al.*, 1988; Parry, 1989).

An attempt is made to describe a more detailed interrelationship between the xanthophyll cycle, *ß*-carotene, and ABA production in cells of *D. salina* exposed to both high light intensity and salt stress.

MATERIALS AND METHODS

Cells of *D. salina* were grown as described in Chapter 2.

Radioactive labelling:

Dual Label study: After centrifugation, cells were resuspended in 400 mL of fresh growth medium with 1.47 μCi $NaH_2[^{14}C]O_2$ (Amersham International, UK). After 24 hours cells were centrifuged and washed twice in 1.5M KCl, then resuspended in 4 L of fresh growth $[^{12}C]O_2$-medium, and allowed to recover for 1 hour. $[^3H]$-mevalonic acid lactone (1.5 μCi) was added to the growth medium and 1 hour allowed for uptake. The culture was then split into two: one for high light treatment (1000 $\mu mol.m^{-2}.s^{-1}$) and the other as a low light control (20 $\mu mol.m^{-2}.s^{-1}$). At preset time intervals samples were removed for analysis of pigments, abscisic acid, and for determination of membrane/stroma β-carotene distribution.

Pulse chase study: Axenic cultures of *D. salina* (at a cell density of 4-7.5 x 10^4 cells per ml.) were exposed to either high light intensity (1000 $\mu mol.m^{-2}.s^{-1}$) or hypersalinity (transferred from 1.5 M to 3.5 M NaCl) and at intervals, samples taken for analysis of carotenoids and ABA. $NaH[^{14}C]O_2$ (sp. act. 1.92 GBq/mmol, obtained from Amersham International, UK) for 24 hours prior to exposure to either high light intensity or hypersalinity.

Extraction of pigments:

Samples for analysis were removed at preset time intervals and quickly chilled on ice, and centrifuged at 2500 g for 15 minutes. The supernatant was discarded and the

pellet resuspended in redistilled, ice-cold acetone containing 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, (BHT), 20mg.L^{-1}) added as an antioxidant, and stored at -20°C in the dark until extraction. The pellet was resuspended in acetone 20mg.L^{-1}, BHT) and sonicated for 2 minutes and reduced to dryness *in vacuo* at 30°C. The residue was redissolved in 70% aqueous methanol and applied to a pre-rinsed Sep-Pak C_{18} cartridge (Waters Chromatography Division, Millipore Corp., Milford, MA) and the carotenoids eluted with 10 mL of 100% acetone. The acetone eluates were dried under nitrogen.

Separation of stromal and membrane β-carotene:

A cell culture (10mL) was centrifuged at 4°C at 2500 g for 10 minutes. The pellet was then resuspended in 4 mL of distilled water and stirred vigorously for 15 minutes for complete cell lysis. This preparation was then centrifuged at 12000 g for 15 minutes. The pellet containing membrane bound β-carotene and the supernatant stromal β-carotene, were separated and 20 mL of ice-cold acetone (20mg.L^{-1}, BHT) was added to each (Ben-Amotz & Avron, 1989). Samples were stored at -20°C in the dark until extraction was performed as described above.

Extraction of ABA:

To a cell culture of 20 mL, [^{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% ethyl acetate (65:35, v/v). Eluates

were dried *in vacuo*, and stored at -20°C in the dark until analysis.

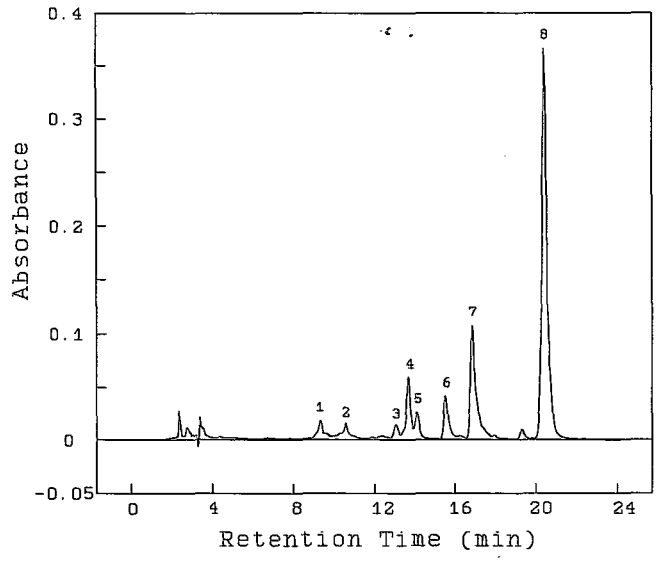

Figure 3.2. HPLC profile of photosynthetic pigments in *D. salina*: 1. neoxanthin; 2. violaxanthin; 3. antheraxanthin; 4. lutein; 5. zeaxanthin; 6. Chl*b*; 7. Chl*a*; 8. *β*-carotene.

HPLC of pigments:

For HPLC, samples were resuspended in iso-propanol:methylene chloride and analyzed by reverse-phase HPLC on a Nucleosil 5 μm C_{18} (250 x 4,6 mm i.d.) column (Macherey-Nagal, Duren, West Germany) using a linear gradient of 0-100% ethyl acetate in acetonitrile/water (9:1; v/v), containing 0.1% (v/v) triethylamine, over 25 minutes at a flow rate of 0.8 mL/min (see Figure 3.2). Peaks were detected using a Beckman 168 diode array detector at 450nm. The coefficient of *β*-carotene was then used to derive normalized coefficients for each compound of interest, as described by de las Rivas *et al.*, (1989). The normalized coefficients used were: violaxanthin (1.53); antheraxanthin (1.41); zeaxanthin (1.34); and *β*-carotene (1.00).

To collect fractions for radioisotope analysis, a LKB Bromma 2-2 Redirac fraction collector was used. Fractions were collected at 0.3 mL.min^{-1}. Scintillator-299 scintillation cocktail (7 mL) was added and radioactivity counted in Beckman 1508 scintillation counter using 2 windows. The first window open for [^{3}H] (0-400) and the second window for [^{14}C] (400-670).

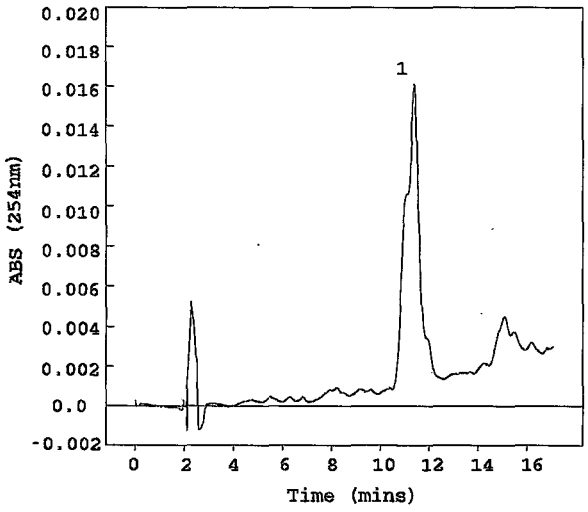

Figure 3.3. HPLC profile of abscisic acid (1) extracted from cells of *D. salina*

HPLC of ABA:

Samples were resuspended in methanol:water (50:50, v/v) and analyzed by reverse-phase on a Bondclone 10μm C18 column (150 x 3.9 mm). ABA was eluted isocratically with methanol/water/acetic acid (40:60:1; v/v) at 1 mL.min^{-1}. (see Figure 3.3).

RESULTS

Figures 3.4 and 3.5 depict the rate of cell growth and amount of chlorophyll per cell, for cells exposed to low and high light intensity in which response of xanthophyll

cycle pigments were to be measured. There is an initial decline in cell number for high light treated cells, followed by rapid cell growth. The control cells initially show a slight increase in number, after which there is a relative levelling off in cell growth. In both control and high light stressed cells there is a decline in cell number after 72 hours. This is most likely a result of a decline in the nutrient status of the growth medium. The chlorophyll concentration in the control cells has little variation over the experiment period. However, the chlorophyll concentration for the high light stressed cells rapidly declines after exposure to high light, and then maintains a consistent decline (Figure 3.5). The decline is a direct result of photodegradation of chlorophyll by high light intensity and is a well documented phenomenon in plants (Allen, 1992, and refs. therein).

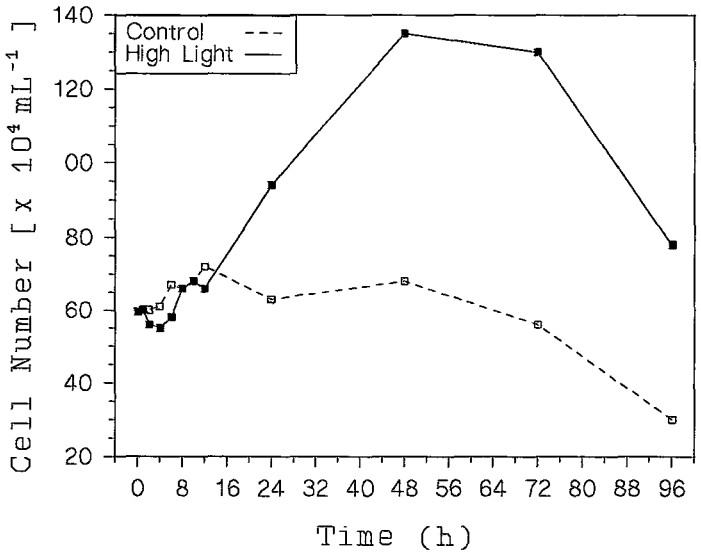

Figure 3.4. Cell growth (cells.mL^{-1}) for control (low light) and high light stressed cells of *D. salina*.

High light stress initially increases the β-carotene concentration from 3 to 4 pg.cell^{-1}, over the first 6 hours (Figure 3.6). This is followed by a rapid decline (within 2 hours) of β-carotene back to 3 pg.cell^{-1}. There is a marked increase in β-carotene

over the next 4 hours, after which the concentration remains relatively constant. After 72 hours of high light stress a third increase in β-carotene concentration is apparent and which reaches almost 10 pg.cell^{-1} at 96 hours after imposition of stress. In the control cells the β-carotene concentration shows a slight decline over the first 12 hours, after which it increases slowly reaching about 4 pg.cell^{-1} after 96 hours.

Figure 3.7 shows a large increase in zeaxanthin in light stressed cells. There is an initial rapid increase in zeaxanthin in the first hour, after which it declines for the next 2 hours. There is a second rapid increase in zeaxanthin concentration until 12 hours after the imposition of stress, and over the next 24 hours slowly declines. A third large increase in zeaxanthin concentration occurs between 48 and 72 hours with a levelling off for the remaining 24 hours. In the control cells the concentration of zeaxanthin remains constant for the first 48 hours. This is followed by a large increase in zeaxanthin concentration.

Figure 3.8 depicts changes in violaxanthin concentration for high light stressed and control cells of *D. salina*. In the control experiment there is initial (0-16 hours) variation in violaxanthin concentration, with an eventual increase over the remaining experimental time. In the high light stressed cells violaxanthin concentration declines rapidly over the first 16 hours and then remains low over the rest of the experiment.

Figure 3.9 depicts the epoxidation state (EPS) of high light stressed and non-stressed cells. Epoxidation state is calculated as:

$$\frac{\frac{1}{2}A+V}{A+V+Z}$$

where A = antheraxanthin, V = violaxanthin, and Z = zeaxanthin. In high light stressed cells the EPS rapidly declines after the initiation of stress thus favouring the formation of zeaxanthin, whereas in the control cells the EPS remains in favour of violaxanthin.

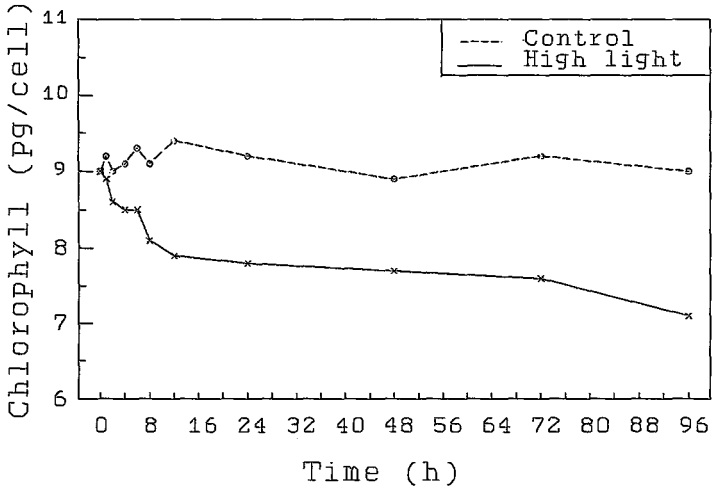

Figure 3.5. Total chlorophyll (pg.cell^{-1}) for control (low light) and high light stressed cells of *D. salina*.

Figure 3.10 illustrates the flow of both [^{14}C] and [^{3}H] specific activity in β-carotene in a time course experiment in control and high light stressed cells. In the control cells the specific activity of [^{3}H] in β-carotene rapidly peaks within the first 4 hours, after which it is not detected at significant levels. In the high light stressed cells, there is a similar early peak, however a second major peak is observed at 48 hours.

Also in these cells the specific activity of [^{3}H] in β-carotene does not go below 4KBq nmol^{-1} after the initial peak, although there is a slight tailing off at 96 hours. The specific activity of [^{14}C] in β-carotene in light stressed cells follows a very similar pattern to that of [^{3}H] in the control cells. In control cells there is the initial increase in specific activity in the first 4 hours, followed by a decline, as occurs with [^{3}H]. The second large increase at 48 hours, is followed by a decline in specific activity of [^{3}H] in β-carotene, which may be of significance.

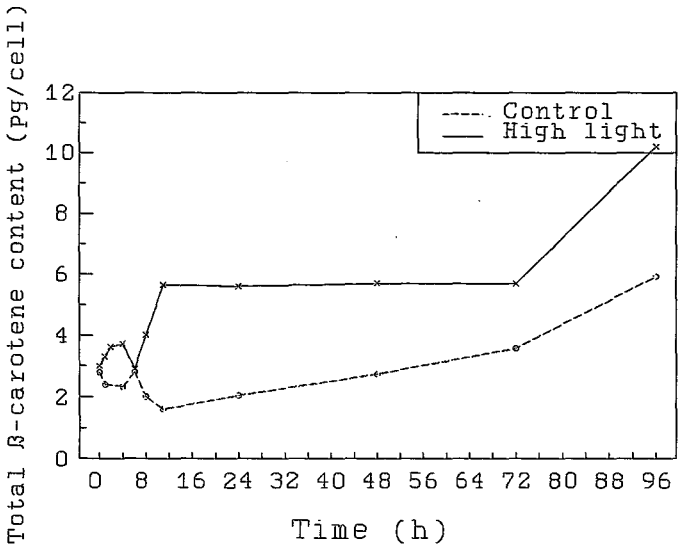

Figure 3.6. Production of total β-carotene (pg.cell^{-1}) in control (low light) and high light stressed cells.

Figure 3.11 depicts the specific activity kinetics of dual labelling in zeaxanthin, in control cells and cells exposed to high light. The specific activity of [^{3}H] in both control and stressed cells is very similar, except that activity is always higher in control cells and that there is a small peak in activity at 10 hours. The activity of [^{14}C] emulates that of [^{3}H] but with stronger emphasis on amplitude. In the control the peak in [^{14}C] specific activity at 10 hours is more marked. There is also a sharper depression in the [^{14}C] than in [^{3}H] specific activity at 2 hours.

The incorporation of [^{3}H] and [^{14}C] into violaxanthin is shown in Figure 3.12. The specific activity of radiolabel into this compound does not follow the same trend as in β-carotene and zeaxanthin.

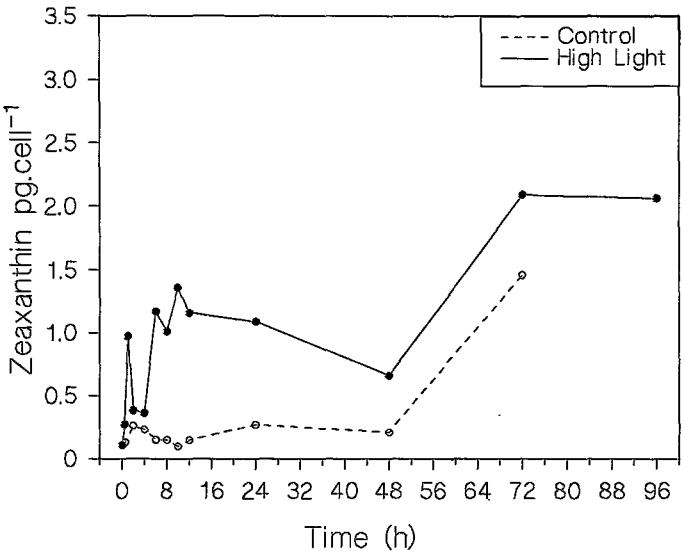

Figure 3.7. Production of zeaxanthin (pg.cell^{-1}) in control (low light) and high light stressed cells.

In the latter two compounds the control cells generally incorporated more of the radiolabels, whereas incorporation of radiolabel in violaxanthin in high light stressed cells is higher after an initial lag phase. The situation with both radiolabels is that in high light stressed cells there is a rapid increase in specific activity after 2 and 8 hours for [^{14}C] and [^{3}H] respectively.

The two major pools of β-carotene in the chloroplast are stromal β-carotene and β-carotene associated with the pigment bed in the thylakoid membrane. Figures 3.13 and 3.14 show the changes in the levels of these two pools in control and high light stressed cells. It is suggested that the decline in β-carotene in control cells is perhaps due to the increased availability of nutrient (cells were always transferred to fresh

growth medium at the start of experiments) thereby obviating nutrient stress. In control cells the ß-carotene associated with the thylakoid membrane shows an initial decrease for the first 12 hours after which there is a relatively consistent increase in ß-carotene concentration. However, the stromal pool of ß-carotene remains negligible throughout the experiment time course (Figure 3.14).

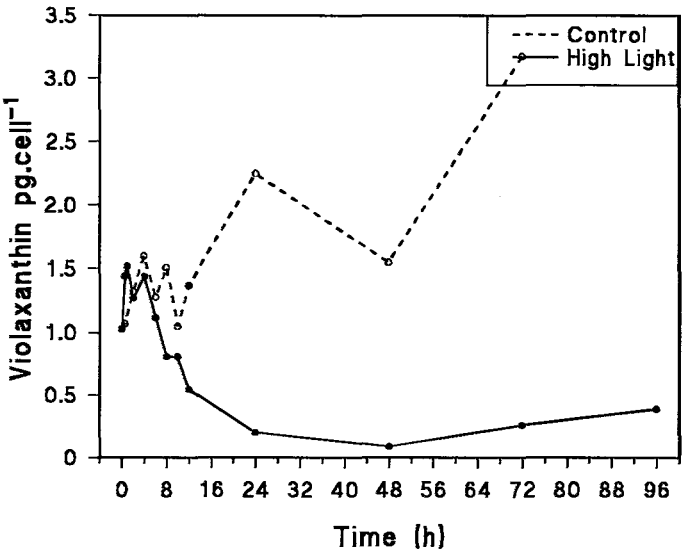

Figure 3.8. Changes in violaxanthin levels (pg.cell^{-1}) in control (low light) and high light stressed cells.

Cells illuminated under high light intensity show slight increase in ß-carotene levels associated with the thylakoid membrane and this is followed by a sharp drop in concentration after 4 hours (Figure 3.13). Following this there is a rapid increase in thylakoidal ß-carotene, with a plateau in concentration until 72 hours after imposition of stress, after which there is another increase. The stromal pool of ß-carotene in high light stressed cells shows a marked increase throughout the experimental time course from almost 0 to 5 pg.cell^{-1} (Figure 3.15).

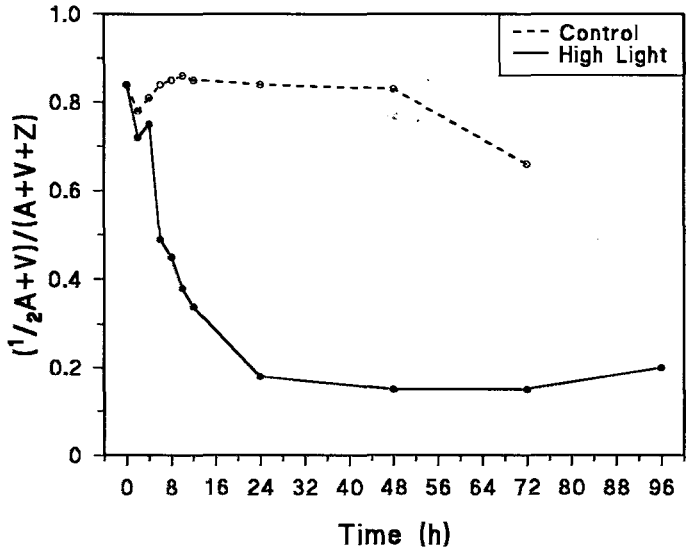

Figure 3.9. Epoxidation state of control (low light) and high light treated cells.

Partitioning of dual radiolabelling in the two β-carotene pools of the chloroplast are depicted in Figures 3.15 and 3.16. Incorporation of [^{3}H] into both the membrane and globular (stromal) β-carotene pools in high light treated cells was only significant at 4 hours following which it was negligible. However in the control cells incorporation into globular β-carotene was almost immediate and remaining relatively high. In the stromal pool [^{3}H] incorporation became significant after 24 hours but was eliminated after 72 hours. Incorporation of [^{14}C] into the membrane pool in high light treated cells was minimal, although in the control cells there was a small peak in incorporation at 48 hours. Specific activity of [^{14}C] in the globular pool in the chloroplast stroma showed a sharp peak after 4 hours after which activity was very low. Contrary to this, incorporation of [^{14}C] into the globular pool in the control cells showed a very high degree of incorporation over the first 6 hours, followed by an immediate decline and then at 24 hours a second massive increase in incorporation which remained high for the rest of the experimental period.

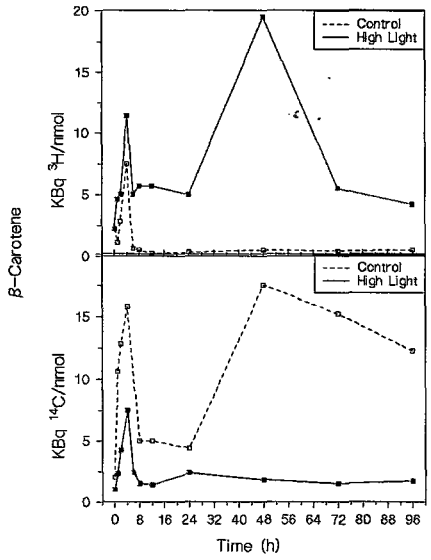

Figure 3.10. Dual labelling study illustrating the relationship between [^{14}C] (pulse) and [^{3}H] (second label) in *β*-carotene.

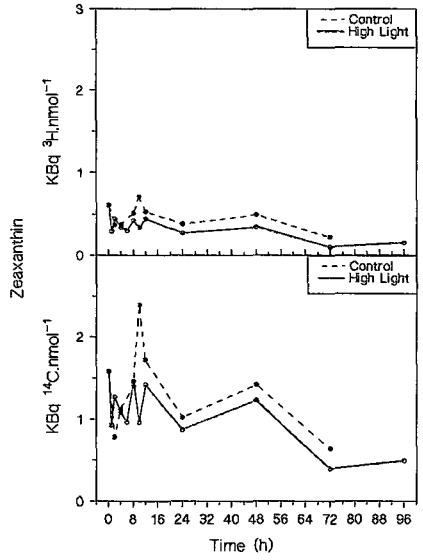

Figure 3.11. Dual labelling study illustrating the relationship of [^{14}C] (pulse) and [^{3}H] (second label) in zeaxanthin.

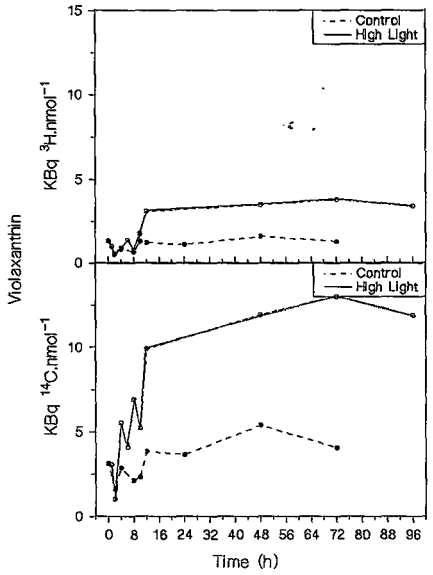

Figure 3.12. Dual labelling study illustrating the relationship between [^{14}C] (pulse) and [^{3}H] (second label) in violaxanthin.

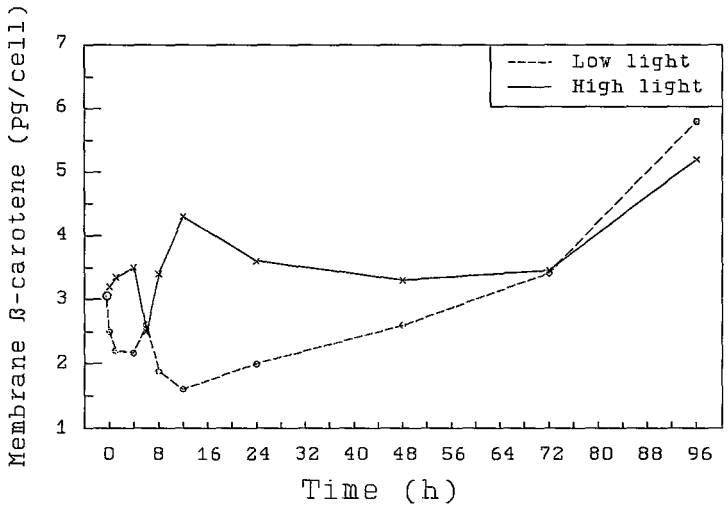

Figure 3.13. Changes in the levels of *β*-carotene associated with membranes in control (low light) and high light stressed cells.

The results presented in Figures 3.17 and 3.18 are from an independent pulse-chase study using [^{14}C]. The production of ABA in cells exposed to high light stress is very rapid and peaks within 30 minutes. This peak in production is followed by a sharp decrease although not to basal levels, but remaining at about 0.8 pg.cell^{-1}. In the control cells the concentration of ABA remains unaffected. Partitioning of ABA into the growth medium is shown in Figure 3.18. Cells exposed to high light stress partition large amounts of ABA into the growth medium compared to control cells.

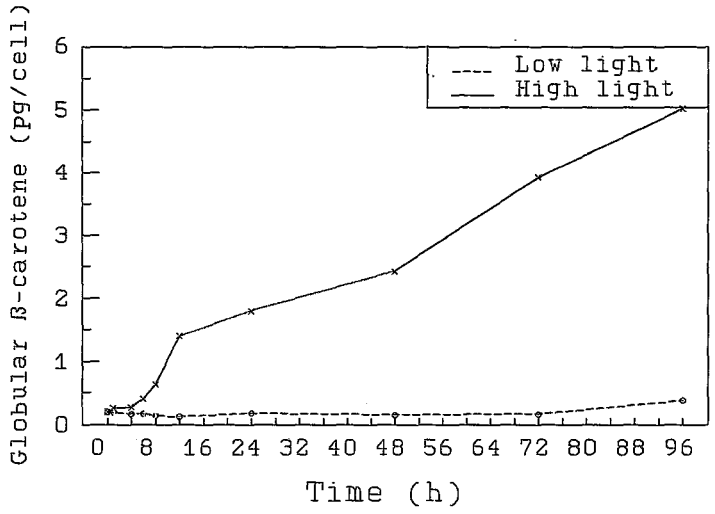

Figure 3.14. Changes in the levels of β-carotene associated with the stroma in control (low light) and high light treated cells.

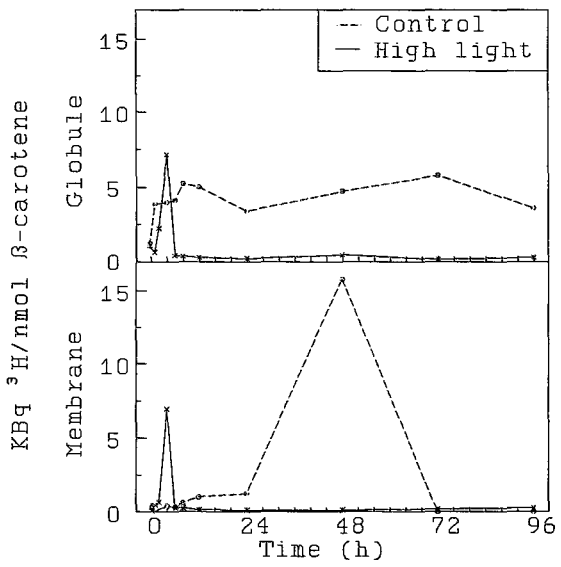

Figure 3.15. Flux of [^{3}H] through β-carotene associated with the membrane and stroma.

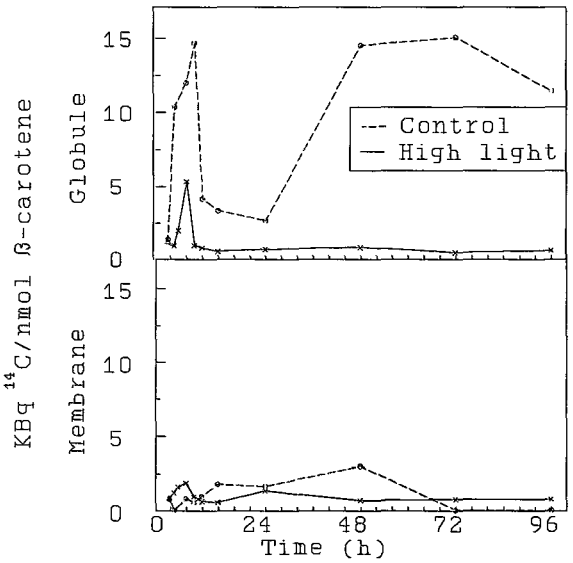

Figure 3.16. Flux of [^{14}C] through β-carotene associated with the membrane and stroma.

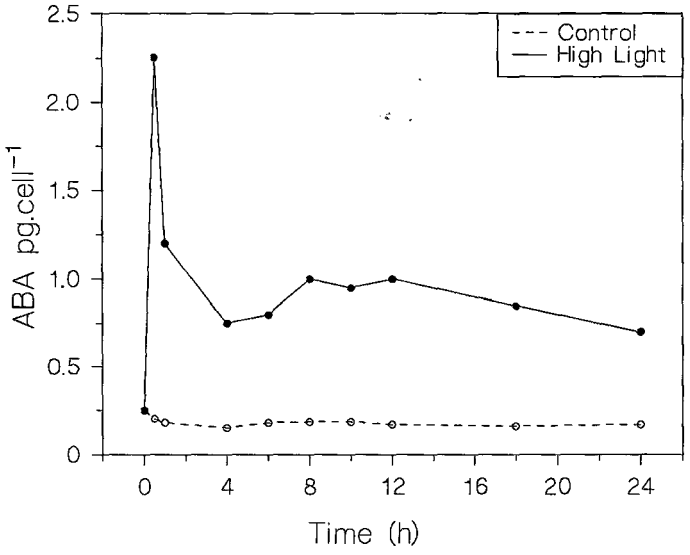

Figure 3.17. Kinetic study of changes in the levels of ABA (pg.cell^{-1}) for control (low light) and high light stressed cells.

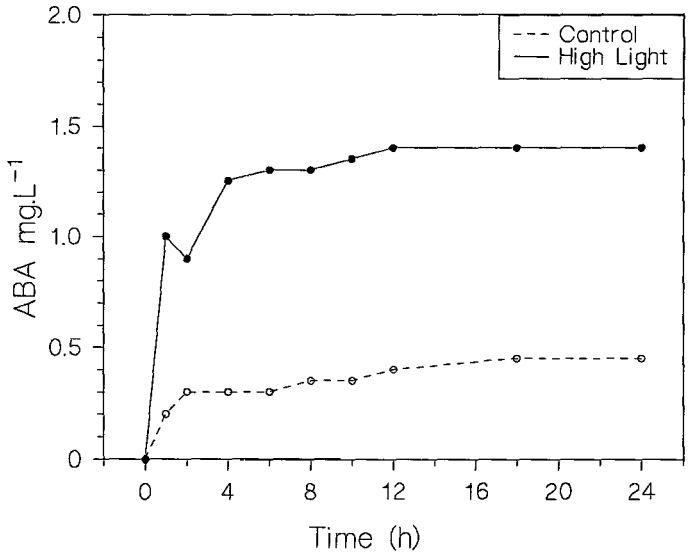

Figure 3.18. Kinetic study of partitioning of ABA into the growth medium for control (low light) and high light stressed cells.

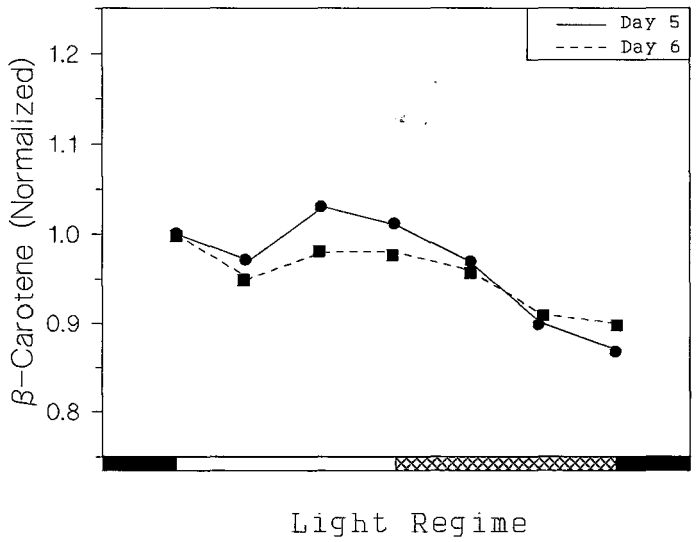

Figure 3.19. Day 5 and day 6 β-carotene accumulation pattern in cells of *D. salina*. Day 5 : dark (■), 6h high light (□), 6 h low light (▨); Day 6 no light.

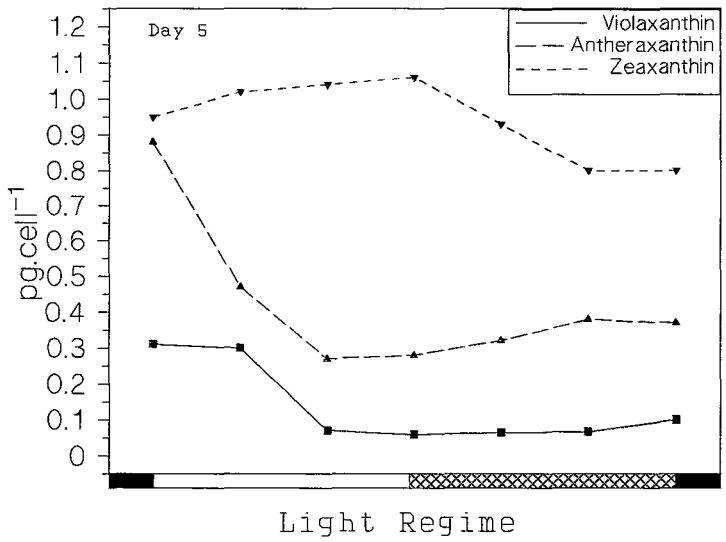

Figure 3.20. Day 5 pigment levels in cells of *D. salina* exposed to dark (■), 6hrs high light (□), and 6 hrs low light (▨).

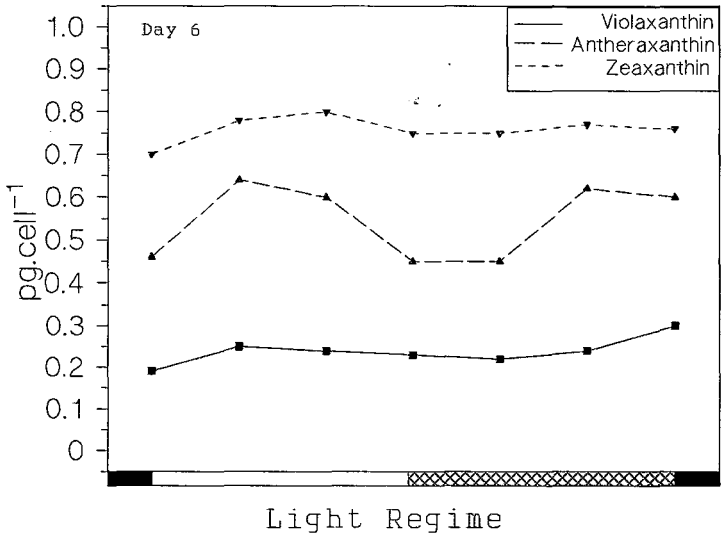

Figure 3.21. Day 6 (no light) pigment levels in cells of *D. salina* exposed to continuous dark. X-axis correlates light regimes: dark (■), 6h high light (□), and 6 h low light (▨).

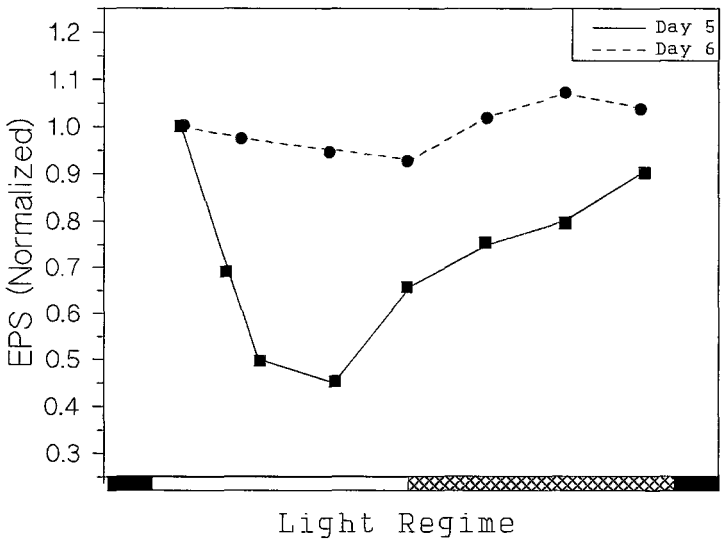

Figure 3.22. EPS state of the xanthophyll cycle in cells of *D. salina*. Day 5: dark (■), 6h high light (□), and 6 h low light (▨); Day 6 no light.

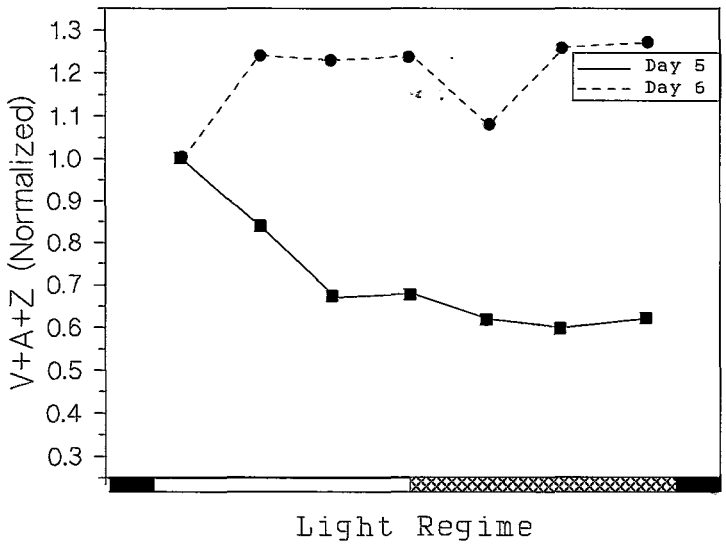

Figure 3.23. Total carbon (V+A+Z) of the xanthophyll cycle pool. Day 5: dark (■), 6h high light (□), and 6 h low light (▨); Day 6 no light.

Figure 3.19 shows *ß*-carotene levels normalized to 1.0 in cells of *D. salina* exposed for 5 days to a constant regime of light intensities using the following equation:

$$\Delta T = 1 - \frac{T_0 - T_1}{T_0}$$

On day 6 all light sources were removed, but the production of *ß*-carotene continues in the dark, albeit at a reduced level.

Figure 3.20 shows the changes in xanthophyll pigments exposed to dark-high-low-light regime. There is an accumulation of zeaxanthin at the expense of antheraxanthin and violaxanthin during exposure to high light. However, when the light intensity is reduced antheraxanthin increases, and there is also a slightly increase in

violaxanthin, as a result of epoxidation of zeaxanthin. Figure 3.21 depicts the xanthophyll pigment fluctuation when these cells are maintained in darkness. All three pigments show an increase in concentration, particularly in the period corresponding to exposure to high light. Figures 3.22 and 3.23 show the EPS state for the xanthophyll cycle pigments for the 5 and 6 days respectively. Again there is a recurrence of the trend in the absence of light. The total carbon in the xanthophyll cycle pool does not follow the same trend, in that there is a large accumulation of carbon in this pool in the time period correlating to exposure to high light, in the dark.

DISCUSSION

The results presented here reflect the interrelationship between high light stress, the xanthophyll cycle components, ß-carotene and ABA in cells of *D. salina*. It is clear that the EPS of the xanthophyll cycle is correlates with the accumulation of ß-carotene. Also the decline in ABA levels during the initial stages of ß-carotene accumulation reflects a possible close relationship between the fluctuations in photosynthetic pigments and this plant growth regulator. It is feasible that the EPS state of the xanthophyll cycle may have some regulatory control on the initiation of ß-carotene synthesis in the stroma of the chloroplast, and that changes in levels of xanthophyll pigments and their regulation of ABA synthesis (via all-*trans* violaxanthin) could be a mechanism regulating stress metabolism within the whole cell. The results presented in Figures 3.6-9 clearly demonstrate that in response to high light stress the EPS state of the xanthophyll cycle favours the production of zeaxanthin, and this effect has also been reported for higher plants (Foyer, *et al.*,

1989; Demmig-Adams, 1990; Ebenezer & Pattenden, 1993; Masamoto *et al.*, 1993). A relationship between ß-carotene and violaxanthin, and ABA has been suggested by Parry & Horgan, (1990), Parry & Horgan, (1991), Lutz & Winterhaler, (1992), and Cowan *et al.* (1993). There is now considerable evidence to support an indirect 'apo-carotenoid' pathway for ABA biosynthesis (Parry & Horgan, 1991, and refs. therein) with the most likely precleavage precursors being 9'-*cis* neoxanthin and 9'-*cis* violaxanthin. Cleavage of these xanthophylls across the 11,12 (11',12') double bond would produce xanthoxin which is readily converted to ABA by plant tissue (Taylor & Burden, 1974; Parry *et al.*, 1988). The regulation of ABA biosynthesis occurs prior to the production of xanthoxin, and could involve control of violaxanthin and neoxanthin interconversion and/or cleavage (Parry & Horgan, 1991).

Using a dual labelling technique an attempt was made to elucidate the pool fluxes of carbon in ß-carotene and the xanthophyll cycle and to determine whether the carbon used in the synthesis of ß-carotene and the xanthophyll cycle originated from a storage pool within the cell or is newly synthesized from carbon assimilated via photosynthesis. The carbon pools within the cells were preloaded with [^{14}C] as a pulse. This radioisotope was then followed by [^{3}H]-mevalonic acid as the chase isotope. The rationale behind this configuration of isotope use was that if the demand for carbon, for increased pool sizes of ß-carotene and the xanthophylls, was directed at carbon sources within the cell the pools would then show an overall increase in [^{14}C] label. However, should the demand for carbon be directed at [^{12}C] assimilated via carbon reduction and photosynthesis only the [^{3}H] label of [^{3}H]-mevalonic acid would show an overall increase in the pools of ß-carotene and the xanthophylls. It

is expected that both radioisotopes would increase during the first few hours of stress as both would be present in the prevailing pool of precursor to the carotenoids.

The results of this study provide evidence that after the initial induction of high light stress carbon responsible for the increase in the pool size of β-carotene and violaxanthin originates from newly assimilated carbon entering the cell through photosynthesis. However, the carbon making up the zeaxanthin pool increase is predominantly labelled with [^{14}C], and this suggests that violaxanthin labelled with [^{14}C] before the imposition of stress is de-epoxidated to zeaxanthin with the resultant increase in the [^{14}C] specific activity of this xanthophyll. Also the larger increase of [^{3}H] specific activity in violaxanthin may be as a consequence of [^{3}H] label rapidly moving from mevalonic acid through β-carotene and newly synthesized zeaxanthin (from cryptoxanthin - the intermediatory xanthophyll between β-carotene and zeaxanthin) into violaxanthin because of a demand for carbon by ABA, after de-epoxidation of [^{14}C]-violaxanthin into [^{14}C]-zeaxanthin.

Under low light conditions there is little necessity for carbon from these pools, consequently [^{14}C] from either a storage pool (such as starch) or from the carotenoid precursor pools is sufficient to replenish normal metabolism of the β-carotene and zeaxanthin. The lower specific activity of [^{14}C] in violaxanthin in low light treated cells is perhaps artifactual resulting from [^{3}H]-mevalonic acid not yet having equilibrated throughout the system completely because of little demand on the violaxanthin pool by its immediate products, neoxanthin and zeaxanthin.

By separating the two chloroplastic ß-carotene pools it was shown that during stress there is an initial increase then a rapid decline in ß-carotene associated with the membrane and therefore associated with the pigment bed. After this decline there is a subsequent increase followed by a levelling off in this pool size. The decline may be a result of ß-carotene loss partially due to photodegradation and/or oxidative degradation due an increase in active oxygen species. However, the significance of these processes is perhaps negligible in comparison to the larger demand for carbon via violaxanthin for synthesis of ABA. The increase in ß-carotene associated with the membrane after 72 hours in both low light and high light stressed cells is apparently due to nutrient stress described earlier. The stromal or globular ß-carotene pool shows a radical increase in high light stressed cells. After an initial lag phase of about 4-6 hours there is a rapid increase in this pool size. This increase continues throughout the stress period. It has been speculated that the function of this pool of ß-carotene is to shield the photosynthetic machinery from excess blue light (Ben-Amotz *et al.*, 1989). The ß-carotene associated with the membrane has been shown to be tightly associated with chlorophyll molecules, and can act either as light-harvesting antenna, the protection of the photosynthetic apparatus from photooxidation, as well as to protect Qa (the primary electron acceptor of PS II) from degradation in the light (Kyle *et al.*, 1984; Siefermann-Harms, 1987; Kuhn & Boger, 1990; Mimuro *et al.*, 1992; Sandmann *et al.*, 1993). This loss of membrane associated ß-carotene is significant in that it is also detectable in the total ß-carotene complement (Figure 3.6) over and above the constant increase in stromal ß-carotene.

The dual isotope labelling in membrane and stromal ß-carotene suggest that cells

stressed by high light quickly pass the [^{3}H] label perhaps through to ABA via all-*trans* violaxanthin. After this initial pulse the membrane β-carotene contains little [^{3}H] radioactivity. The similar trend in the stromal component of β-carotene clearly results from the synthesis of β-carotene in the chloroplast stroma before it is bound into the thylakoid membrane (Bramley & MacKenzie, 1988; Goodwin, 1993). Control cells maintain a somewhat high specific activity of [^{3}H]-β-carotene in the stromal component, which perhaps emanates from slower movement of the radiolabel through the various pools because of a slower demand and metabolism of the component pools. This is also manifested in the membrane bound β-carotene which shows a large peak of radioactivity at 48 hours, indicating slower or retarded movement of the label due to lower demand rates on the component pools. In high light stressed cells the [^{14}C] label remained low in both membrane and stromal β-carotene, which suggests that the demand from stored carbon was low. There was however a small increase in [^{14}C] in the stromal β-carotene pool at about 4 hours. In the control cells again little change occurred in the membrane component, however the movement of this radioisotope was prominent in the stromal component of control cells. It is perhaps possible that this is artifactual, and represents [^{14}C] already incorporated in this pool during the loading of [^{14}C] for 24 hours and which remains in this pool because of slow metabolism of the pool.

Results from an independent pulse-chase study using only [^{14}C] (Figure 3.17) shows that carbon from β-carotene/xanthophyll cycle components is rapidly metabolized to ABA within the first 30 minutes after the induction of high light stress. Results not shown from this experiment indicate that violaxanthin and lutein were synthesized

from newly formed precursors while neoxanthin was derived from an existing, labelled compound such as violaxanthin. These data support the hypothesis that neoxanthin is synthesized from violaxanthin, and this is consistent with the results of Parry & Horgan (1991). The ABA thus produced by high light stress would then influence general cell metabolism, by inducing the synthesis of specific proteins which might be important in acquired stress tolerance (Gomez *et al.*, 1988). The accumulation of ABA in the growth medium during high light stress may have an important competitive influence over competitive flora and fauna in the growth medium. It has also been reported that ABA can affect the growth and reproduction of some animals (Vissher, 1983). Thus extracellular partitioning of ABA by *D. salina* may be of particular significance in the natural environment. The extracellular partitioning of ABA is not particular to *D. salina*, and has also been reported in green algae *Chlorella vulgaris*, *Stichococcus bacillaris* (Marsalek *et al.*, 1992a) as well as in the cyanobacteria *Nostoc muscorum*, *Trichormus variabilis* and *Synechococcus leopoliensis* (Zahradnickova *et al.*, 1990; Marsalek *et al.*, 1991; Marsalek *et al.*, 1992b).

The data presented in Figures 3.19 - 3.23 demonstrate fluctuations in pigment levels occurring over a variable photoperiod. It is apparent that the xanthophyll cycle is not only intimately involved in the stress response of *Dunaliella* but functions with a periodicity which suggests the presence of a rhythm.

A circadian rhythm is defined as a regular rhythm of growth and activity that occurs on an approximately 24 hour basis. The timing of these activities does not depend directly on the day-night cycle; it can also be demonstrated in constant light or darkness and at a constant temperature and therefore must be innate. The timer responsible is known as a circadian clock (Sweeney & Borgese, 1989). The term "circadian" is derived from the Latin *circa*, (about) and *diel* (day). This term is appropriate as it stresses one of the most important properties of such rhythms, being that the period is often not exactly 24 hours under constant conditions (Sweeney, 1987). The rhythmicity of a cell parameter must also run under constant conditions, be independent of cell division, and have a period of about 24 hours to qualify as a true circadian rhythm (Prezelin & Sweeney, 1977).

An endogenous, self-sustaining or free running rhythm is a cycle that persists under constant environmental conditions for at least several periods, usually much longer, before becoming indistinguishable or "damped out." If evidence for persistence is lacking, the cycle is known as a periodicity, for example "diel periodicity" observed in a light-dark environment where the period is 24 hours. Thus, the production of β-carotene could perhaps be a periodicity and not a true circadian rhythm.

Prezelin *et al.* (1977) defined circadian rhythms in photosynthesis for the first time in the dinoflagellates *Glenodinium* sp. and *Ceratium furca* and compared them with that of *Gonyaulax polyedra*. All three phytoplankton species had photosynthetic rhythms with daily amplitudes ranging from 3 to 5 and maxima displayed about midday. The photosynthetic pigment content and absorption properties of the cells

were constant over the circadian cycle. They found sufficient similarity between the circadian rhythms of these three dinoflagellates to suggest the mechanism of regulation to be the same for each of them, however the mechanism itself is not known.

The data presented here implies that there is some signal which plays a regulatory role in carotenoid biosynthesis in this alga. Clearly a dynamic physiological mechanism which has its origin on the photosynthetic machinery plays some role. From previous biophysical *in vitro* studies, it has been postulated that zeaxanthin can deactivate the triplet (and even singlet) excited state of chlorophyll with a greater efficiency than violaxanthin (Gillbro *et al.*, 1993). These results are in agreement with the previous hypothesis (Siefermann-Harms, 1987; Demmig-Adams & Adams, 1992; Arsalane *et al.*, 1994) that certain de-epoxidated xanthophylls can provide photoprotection. Thus the increase in the total carbon of the xanthophyll cycle coupled with the maintenance of an epoxidation state favour zeaxanthin provides strong evidence for a physiologically controlled signal from the photosynthetic machinery to the xanthophyll cycle in anticipation of an excess energy load at PS II.

CONCLUSION

The results presented here suggest a defined mechanism whereby *D. salina* responds to high light stress. The overloading of PSII with energy from the pigment bed, results in mass movement of carbon in the xanthophyll cycle towards zeaxanthin. Simultaneously, photodestruction of chlorophyll and β-carotene located within the

pigment bed occurs. Abscisic acid is produced in the stroma (Parry & Horgan, 1990) and is then exported to the cytoplasm where it acts as a regulator of cellular metabolism. After an initial decline in photosynthesis carbon is rapidly assimilated, of which part is channelled into carotenoid synthesis, firstly to replace that β-carotene in the pigment bed lost due to photolysis and then other pigments. Once this sink of carbon has been saturated, mass accumulation of β-carotene in the chloroplast stroma commences. Under alternating light conditions, which may simulate natural environment conditions, this mechanism appears to establish a rhythm corresponding to the light/dark cycles and which further characterises the stress adaptation in this organism. Furthermore, the close correlation between changes in β-carotene and xanthophyll cycle pigments suggests that the stress induced alterations in xanthophyll cycle activity may contribute to elevated intracellular β-carotene content during high light stress.

Chapter 4

CHLOROPHYLL FLUORESCENCE AS A STRESS INDICATOR

INTRODUCTION

It was already assumed many years ago that the time-dependent variations of the chlorophyll fluorescence of previously dark-acclimated leaves contained information about the function of photosynthesis (Kautsky & Hirsch, 1934). Although chlorophyll fluorescence developed to be a very useful method in studies of energy transfer and primary photochemical events (Lavorel & Etienne, 1977; Butler, 1978), it was not until the last decade that real progress was made in the use of fluorescence to measure photosynthesis under physiological conditions. In studies of photoinhibition of PS II, it was realized that the photochemical efficiency expressed by the F_v/F_m ratio (the highest ratio of variable to maximum fluorescence after dark-acclimation) related very well to inhibition of PS II electron transport (Critchley, 1981; Fork *et al.*, 1981; Ogren & Oquist, 1984). Later it was shown that there is an apparent linear relationship between F_v/F_m and the quantum yield of CO_2 uptake in Scots pine (Leverenz & Oquist, 1987) and CO_2 dependent O_2 evolution in leaves (Demmig & Bjorkman, 1987). However, it was not until the introduction of equipment that allowed separation of the two major fluorescence quenching components *in vivo*, photochemical quenching (q_P) and non-photochemical quenching (q_N), that it became possible to establish a better understanding of the relationship between the fluorescence yield and photosynthetic function. This progress came with the development of new instrumentation based on a principle of modulated fluorescence:

the technique separates fluorescence caused by weak, modulated excitation light from that caused by continuous, actinic light used to drive photosynthesis (Ogren & Baker, 1985; Schreiber *et al.*, 1986; Bolhar-Nordenkampf *et al.*, 1989; van Kooten & Snel, 1990).

By quantifying the relationship between q_p and q_N in relation to photosynthesis, Weis and Berry (1987) suggested that the major component of q_N, as related to the energization of the thylakoids in light by the ΔpH gradient (Oxborough & Horton, 1988), controls the extent of thermal dissipation of excitation energy in PS II; such a thermal dissipation pathway in turn regulates the quantum yield of non-cyclic electron transport in open PS II reaction centres and the development of q_N in light was reported (Weis & Berry, 1987; Weis, *et al.* 1987; Krause *et al.*, 1988). Recently, the strict linearity between the two parameters has been questioned for non-saturating light conditions (Horton & Hague, 1988; Rees & Horton, 1990).

Further support for q_N controlling the photochemical efficiency of PS II was given by Genty *et al.* (1989), who demonstrated that there was a strictly linear relationship between the quantum yield of CO_2 uptake and the product of q_P and the steady state trapping efficiency ratio (F_v/F_m). A theoretical and experimental analysis of quenching parameters in relation to photosynthesis also showed that the yield of PS II photochemistry was modulated largely be q_N (Havaux *et al.*, 1991). It is not yet understood how q_N mechanistically regulates PS II photochemistry (Foyer *et al.*, 1990), and it is under discussion whether the thermal quenching as triggered by the

trans-thylakoid ΔpH gradient resides at the level of the reaction centre (Weis & Berry, 1987) or the antenna (Genty *et al.*, 1989).

In testing the relationship between the yield of PS II photochemistry, as defined by $q_P \times F_V/F_M$ according to Genty *et al.* (1989), and the quantum yield of CO_2 saturated O_2 evolution, Keiller and Walker (1990) observed a linear relationship during photosynthetic oscillations. Subsequently, however, Seaton and Walker (1991) reported a single curvilinear relationship between the two parameters for several unrelated plant species. In the latter study, the quantum yield of O_2 evolution was based on incident light. If the single curvilinear relationship was to be strictly tested for its universality among diverse plant species, then it is necessary rather to express quantum yields on the basis of absorbed light, so as to take into account the different chlorophyll contents per unit area of leaves in various species (Oquist & Chow, 1992).

Several recent studies seem to suggest that non-photochemical quenching of chlorophyll fluorescence originates mainly from the LHC II pigment-protein complex of PS II (Horton *et al.*, 1991; Ruban & Horton, 1992). According to Hortons group, such fluorescence quenching is related to acidification of the LHC II environment , followed by its apo-protein aggregation (Horton *et al.*, 1991; Ruban *et al.*, 1993). On the other hand, a light-induced chlorophyll fluorescence decrease in isolated LHC II was demonstrated to be independent of the aggregation state of the antenna protein (Jennings *et al.*, 1991). Fluorescence quenching of isolated LHC II was also found to be independent of the xanthophyll cycle-controlled violaxanthin to zeaxanthin

ratio, the reversibility of this quenching being much faster in the presence of zeaxanthin (Rees *et al.*, 1992).

It is generally accepted that non-photochemical quenching depends on the activity of the xanthophyll cycle and the presence of a thylakoid ΔpH (Gilmore *et al.*, 1994). The linkage between the acidification of the thylakoid lumenal space and the biophysical mechanism of chlorophyll fluorescence quenching, however, is complex and much of it remains to be explained (Krause & Weis, 1991).

It has been previously demonstrated that chlorophyll fluorescence may be enhanced using lipophilic uncouplers of photosynthesis (Finazzi *et al.*, 1993). It was further shown that this uncoupling has two components: one is the well established (Demmig-Adams, 1991, and refs. therein) reversal of energy-dependent quenching of fluorescence, which is observed upon addition of any uncoupler including NH_4Cl, and is enhanced by the de-epoxidation of the xanthophyll cycle. The other component does not require any previous light-dependent fluorescence quenching, but is associated with stimulation of PS II photochemistry and increased Emerson enhancement under low light intensity conditions, where electron transport is not limited.

Recently Pick *et al.* (1993) demonstrated that ammonia at alkaline pH induce in cells of *Dunaliella* a transient stress that is manifested by a drop in ATP and an increase in cytoplasmic pH. The time course of ammonia uptake was shown to be biphasic - a rapid influx, associated with cytoplasmic alkalization, followed by a temperature-

dependent slow uptake phase, which is correlated with recovery of cellular ATP and cytoplasmic pH. These authors suggest that acidic vacuoles in this organism serve as a high-capacity buffering system for amines, and as a safeguard against cytoplasmic alkalization and upcoupling of photosynthesis.

The neutral molecule NH_3 is a weak base and protonates rapidly to yield NH^+_4, the dissociation constant being 10^{-9} (Leive, 1969). It is generally assumed that if a molecule can exit as a neutral and ionic species, biomembranes are permeable towards the neutral form but less so for the ionic form. In the case of a membrane across which exist a pH gradient there would be rapid movement of NH_3 from the basic side to the acidic side where NH^+_4 would be formed. The movement of NH_3 across the membrane will be continuous as a gradient of NH_3 is maintained.

Thus with the addition of NH_4Cl at low light intensities the enhancement of chlorophyll fluorescence may be largely attributed to the disruption of the trans-thylakoidal ΔpH by diffusion of NH_3 into the lumenal space and forming NH^+_4 in the more acidic environment, thereby reducing the rate of electron transport from PS II to PS I as a direct result (Finazzi *et al.*, 1993).

In order to determine whether fluctuations in chlorophyll fluorescence in *D salina* could be correlated with changes in chloroplastic pH, the chlorophyll fluorescence response to NH_4Cl addition was followed in cells at low light intensity. Furthermore, cells of *D. salina* were exposed to various regimes of light intensity and darkness in an attempt to associate the commitment of β-carotene accumulation after 4 hours of

exposure to high light intensity, to changes in chlorophyll fluorescence yields.

MATERIALS AND METHODS

'Sample-population' chlorophyll fluorescence was measured with a Turner fluorometer (Model 112) equipped with a 5 mL flow-through cuvette, 415nm excitation filter and 700nm emission filter. Cells were continuously pumped through the cuvette at 100 $mL.min^{-1}$, and then returned to the incubation vessel, where cells were continuously stirred. Chlorophyll fluorescence traces were recorded on a flat-bed chart recorder. High light (1000$\mu mol.m^{-2}.s^{-1}$) was supplied from a high intensity halogen lamp, and NH_4Cl supplied to growth medium at 0.01mM.

'Total-population' fluorescence was measured using an in-house built fibre optic-diode chlorophyll fluorometer. Light was supplied via light-emitting diodes (either 425nm or 734nm) at 16Hz, or via a 2cm fibre optic cable from a variable intensity halogen lamp. Chlorophyll fluorescence was measured using a pin-diode (734nm) with software specifically written for the fluorometer, and was acquired every 10ms. Here a single population of cells commonly 1.5mL contained in a light sealed chamber, with light supplied through select port using either light-emitting diodes or the fibre optic cable. NH_4Cl was supplied through a light sealed transfer chamber.

Variable light/dark regime responses: Cells were grown at low light intensity (80-100 $\mu mol.m^{-2}.s^{-1}$) for a 12:12 hours light:dark diurnal cycle for 4 days. The morning of the 5th day cells received either 1, 2, 4, or 8 hours of high light exposure (1000 $\mu mol.m^{-2}.s^{-1}$) followed by 11, 10, 8, or 4 hours of low light, respectively, to

complete the 12 hours light period. The following morning the cells received the same light treatment as for day 5. Chlorophyll and carotenoid content, as well as cell number were determined as described in Chapter 2. Chlorophyll fluorescence was measured using the 'total-population' procedure.

RESULTS

Figure 4.1 shows the 'total-population' chlorophyll fluorescence response for cells of *D. salina* exposed to either high light intensity or NH_4Cl stress. In both cases the

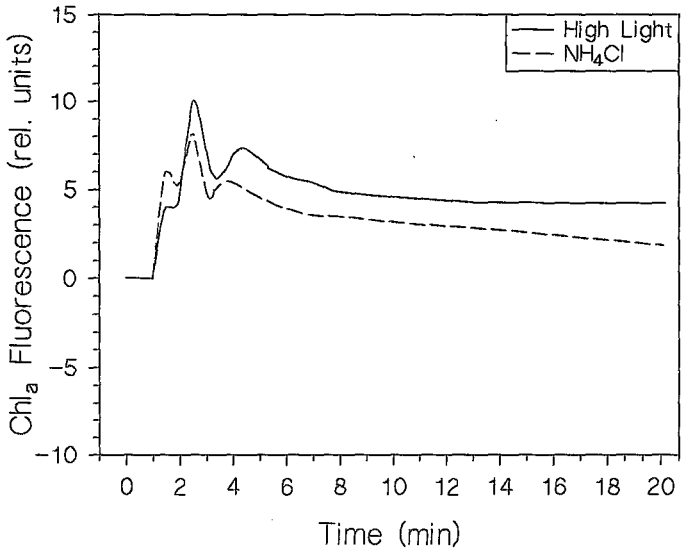

Figure 4.1. 'Total-population' chlorophyll fluorescence for cells of D. salina exposed to either high light intensity or NH4 stress.

response was similar. There is an initial rapid increase in fluorescence, followed by a small dip. This is followed by a second sharp increase to a maximum peak. The decline in fluorescence has a similar two-stage development maximal fluorescence. Overall the final fluorescence level attained after either addition of NH_4Cl or high light, was higher than the initial fluorescence level. Figure 4.2 shows the 'single-

sample' chlorophyll fluorescence response for cells of *D. salina* exposed to either high light intensity or NH_4Cl. In both cases there is an increase in fluorescence yield to a maximum followed by a smooth decline to a final fluorescence yield which is higher than the initial fluorescence level. It is suggested that the slower and smoother response for the 'single-sample' fluorometer is a result of the physical layout of the system, where those cells in the incubation vessel at the time the stress was applied would have been exposed earlier to then applied stress, than those cells in the pump lines and inside the flow-through cuvette inside the fluorometer. Also as the cells in the pump lines and flow-through cuvette mixed with those in the incubation vessel there would be a dilution of overall fluorescence yield from the population of cells. However, as specific details on the chlorophyll fluorescence induction parameters was not required and because such data is not required from a biotechnological management view point, the 'single-sample' fluorescence apparatus was used for further investigation.

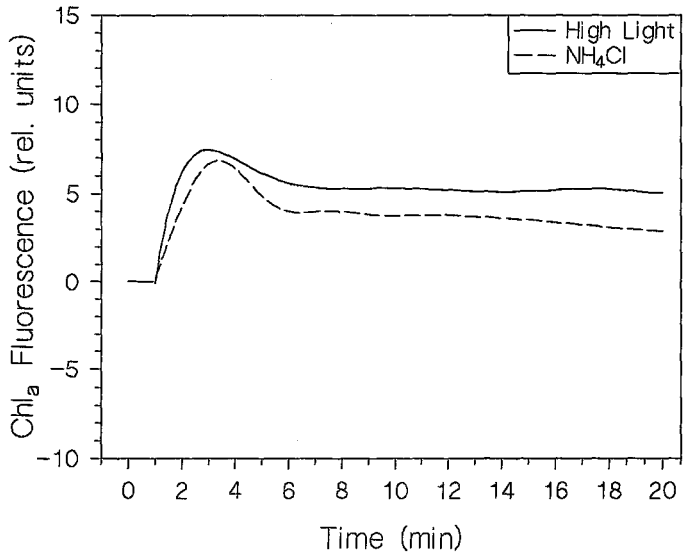

Figure 4.2. 'Single sample-population' chlorophyll fluorescence for cells of *D. salina* exposed to either high light intensity or NH_4Cl stress.

Figures 4.3-6 show diagrammatically the changes in chlorophyll fluorescence for cells of *D. salina* exposed to increasingly longer high light periods. In all the figures, after a 12 hour dark period, exposure to high light results in an immediate increase in chlorophyll fluorescence. This sharp rise is followed in all cases by a larger decrease in fluorescence to below the initial dark level fluorescence. This decrease commonly occurred within 20-30 minutes. The fluorescence signal was then maintained at this level for the remainder of the high light period.

Figure 4.3 shows the fluorescence trace for those cells exposed to only 1 hour of high light, after which the light intensity was decreased to low light. At this point chlorophyll fluorescence increases slightly, then remains constant for the duration of the low light period. After 11 hours of low light, a 12 hour period of darkness followed, which at the onset resulted in a small decline in the fluorescence signal.

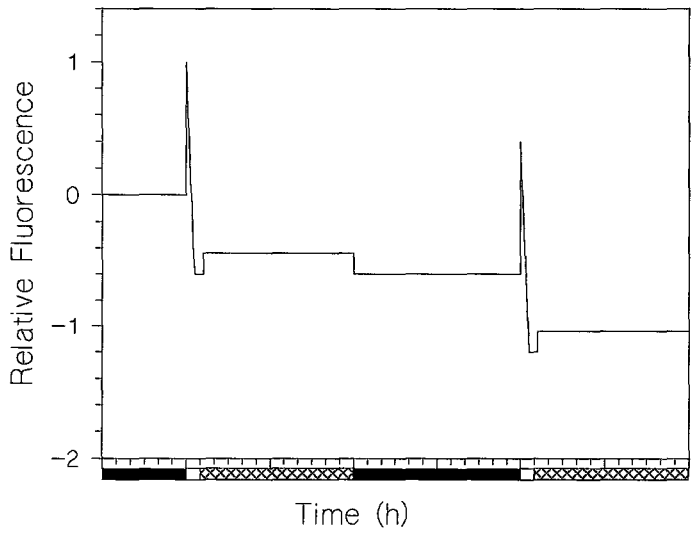

Figure 4.3. Chlorophyll fluorescence for cells of *D. salina* exposed to 12 h dark (■), 1 hr high light (□), and 11 h low light (▨).

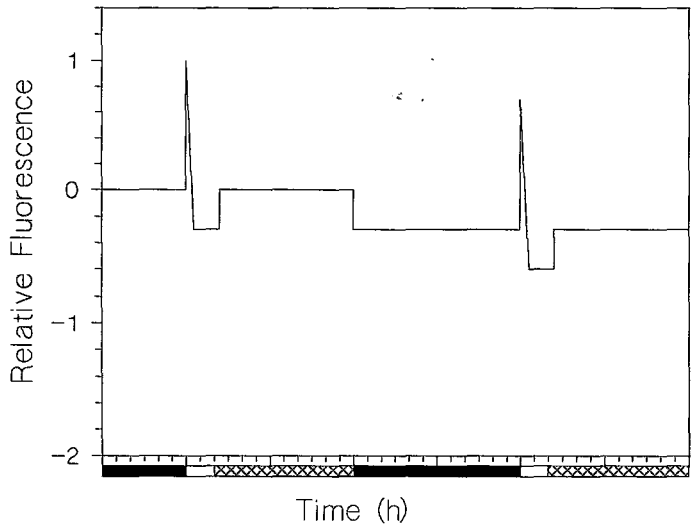

Figure 4.4. Chlorophyll fluorescence for cells of *D. salina* exposed to 12 h dark (■), 2 h high light (□), and 10 h low light (▨).

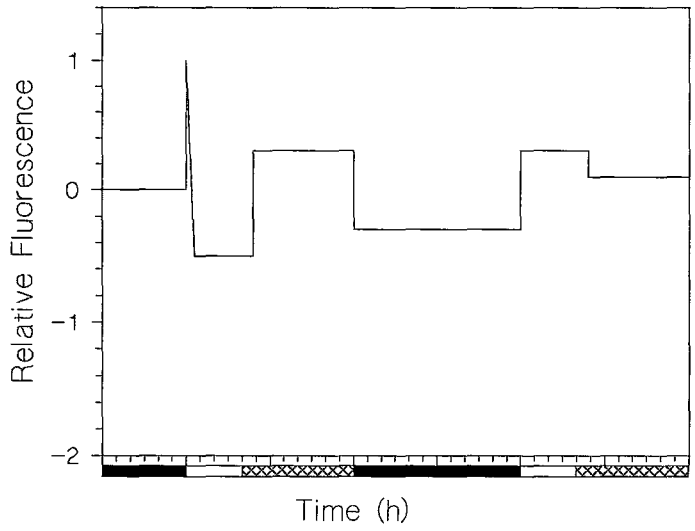

Figure 4.5. Chlorophyll fluorescence for cells of *D. salina* exposed to 12 h dark (■), 4 h high light (□), and 8 h low light (▨).

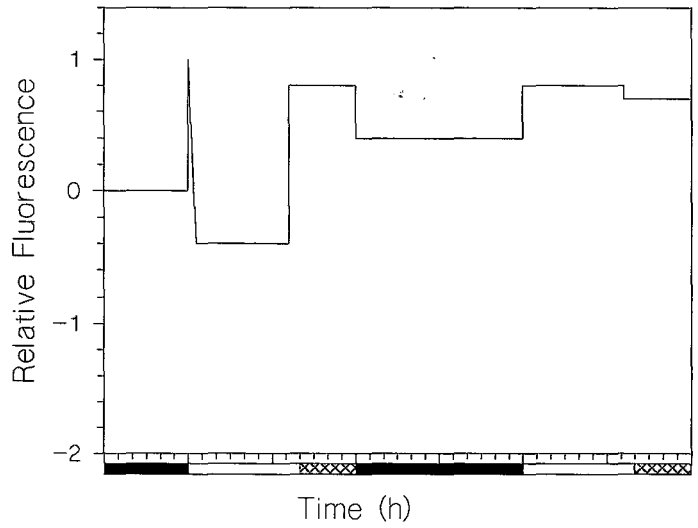

Figure 4.6. Chlorophyll fluorescence for cells of *D. salina* exposed to 12 h dark (■), 8 h high light (□), and 4 h low light (⊠).

Figure 4.4 shows the chlorophyll fluorescence trace for those cells exposed to 2 hours high light intensity. After this exposure to high light, a 10 hour low light period followed. This cycle was repeated after a 12 hour dark period. The fluorescence response from these cells was almost identical to those cells exposed to 1 hour high light intensity, except that the degree of fluorescence change between the light regimes was slightly amplified. However the overall level of fluorescence showed less decline. The level of dark fluorescence during the second dark period was higher than in cells exposed to 1 hour high light intensity. As with the cells exposed to high light intensity for only 1 hour, in the cells exposed to high light intensity for 2 hours the second high light cycle resulted in a sharp increase in chlorophyll fluorescence.

On exposure to 4 hours of high light intensity, cells of *D. salina* also showed this sharp increase in chlorophyll fluorescence, but only for the first light cycle (Figure

4.5). The second exposure to high light did not result in the sharp peak in fluorescence, characteristic of the two shorter exposure periods. The overall level of fluorescence increased to a higher level than at the start of the incubation period. Also the level of low light fluorescence for the first period of low light was higher than the initial dark fluorescence.

Figure 4.6 shows the chlorophyll fluorescence trace for those cells exposed to 8 hours of high light intensity, followed by 4 hours low light and then a 12 hour dark period. As with the previous treatments, the first period of high light exposure was characterized by a sharp increase in fluorescence followed by a rapid decline. However, on transfer to a low light intensity environment the fluorescence intensity almost reached that which was obtained during the initial period of high light intensity. As with the 4 hours high light exposure no sharp peak was produced on the second exposure to high light. The period of darkness after the first exposure to the light regimes is characterized by a fluorescence level which is higher than the initial dark level fluorescence. The second period of exposure to high light shows an increased in fluorescence without a subsequent decline. On exposure to the second period of low light, there is only a small decrease in fluorescence intensity. The overall fluorescence trend increased for cells exposed to this light/dark treatment.

DISCUSSION

Characterization of the photoprotective mechanisms by which plants dissipate excess light energy is currently a major avenue of research in photosynthesis. One mechanism that protects photosystems against excess light during stress which

restricted carbon assimilation involves the xanthophyll cycle (Gilmore *et al.*, 1994). This protective mechanism is stimulated under excess light conditions which energize the chloroplast thylakoid membrane (Demmig-Adams & Adams, 1992). Protection of the photosynthetic apparatus is achieved through harmless and radiationless dissipation of excess light energy, through release as heat. This is accompanied by a decline in Chl*a* fluorescence yield, which can be monitored as non-photochemical quenching of photosystem II fluorescence (Gilmore *et al.*, 1994). It is now generally accepted that non-photochemical quenching depends on the activity of the xanthophyll cycle and the presence of a thylakoid ΔpH. The linkage between the acidification of the thylakoid lumenal space and the biophysical mechanism of chlorophyll fluorescence quenching is however complex, and much of it remains obscure.

As yet, it is not clear whether *in vivo* photoinhibition is initiated at the donor side or at the acceptor side of PS II (Jegerschold *et al.*, 1990; Ohad *et al.*, 1990). The *in vivo* situation is complicated as closure of PS II (reduction of Q_A) is accompanied by an increase of the proton gradient, due to light-induced acidification of the lumen (Weis & Berry, 1987; Horton *et al.*, 1988). It was suggested that a low pH at the donor side of PS II can lead to limitation of electron donation to Q_A (Schreiber & Neubauer, 1987, 1990) resulting in an increase in chlorophyll fluorescence from PS II. One of the main mechanisms leading to non-photochemical F_v quenching is the trans-thylakoid membrane potential associated with a pH gradient (Krause & Weis, 1984). It is also known that lowering of lumenal pH results in the de-epoxidation of violaxanthin to zeaxanthin which is another process of non-photochemical quenching. Thus full activation of this cycle will also decrease fluorescence levels.

From the results presented here it appears that in *D. salina*, the 4-6 hours lag period before β-carotene accumulation coincides with the activity of a regulatory mechanism which controls chlorophyll fluorescence yield. It is only after exposure to high light intensity for 4 hours and longer that a sharp increase in chlorophyll fluorescence does not occur during the second exposure to such light. It is possible that alteration in cellular metabolism due to complete transduction of the stress signal may play a role in regulating the chlorophyll fluorescence yield.

These results suggest that chlorophyll fluorescence as a mechanism of energy dissipation is a primary stress response in previously unstressed cells which is suspended once other stress response mechanisms are activated. In the case of β-carotene accumulation and the xanthophyll cycle, the fluorescence data indicate an initial period of reversibility of the cells response followed after about 4 hours by a period of commitment after which these mechanisms fully absorb the stress impulse. After this period chlorophyll fluorescence appears to no longer play a role in the primary stress response mechanism.

CONCLUSION

Ammonium in millimolar concentrations has been found to strongly reduce net carbon fixation at alkaline pH (Pearson *et al.*, 1987) Undissociated ammonia molecules freely diffuse into the lumenal space during photosynthesis and act to effectively uncouple photosynthesis by breakdown of the trans-thylakoid membrane potential (Padan & Schuldiner, 1978). Furthermore, it is well documented that NH^+_4 affects the intercellular pH differentials (Weiss & Pick, 1991; Finazzi *et al.*, 1993;

Post, 1993). The results presented here (Figures 4.1 & 4.2) show that addition of ammonia to the growth medium of cells of *D. salina*, effects a degree of uncoupling of photosynthesis sufficient to cause an increase in chlorophyll fluorescence.

It is well document that when plants and green algae are exposed to an increase in light intensity there is an increase in the yield of chlorophyll fluorescence (Buchel & Wilhelm, 1993 and refs. therein). The results presented in Figure 4.1 show that an increase in the light intensity results in a stepped increase in chlorophyll fluorescence. This response, known as the Kautsky response, is also well documented although the exact kinetics are still under discussion (Buchel & Wilhelm, 1993, and refs therein). The smooth response to increased high light intensity from the sample-population fluorometer (Figure 4.2) again shows the effect of response dilution, whereby the smaller alterations in the fluorescence yield are dampened.

It is interesting that the chlorophyll fluorescence yield response for ammonia is very similar to that for high light. The linkage between the acidification of the thylakoid lumenal space and the biophysical mechanism of chlorophyll fluorescence quenching, however, although a role for trans-thylakoid ΔpH has been shown to play a major role (Buchel & Wilhelm, 1993).

The results presented in Figures 4.3-6 show that a minimal exposure of about 4 hours to high light intensity prevents the sharp increase in fluorescence on exposure to high light on the second day. This indicates that some physiological procedure has been initiated and completed whereby excess fluorescence and perhaps energy from PS II

is removed non-photochemically and that the mass accumulation of β-carotene in the chloroplast stroma and activation of the xanthophyll cycle may play an pivotal role here. What the mechanistic inter-relationship between xanthophyll cycle activity, β-carotene accumulation, intracellular pH, and chlorophyll fluorescence remains obscure, other than strong correlation in the overall changes occurring in each component.

Chapter 5

INTRACELLULAR pH

INTRODUCTION

In vivo ^{31}P-nuclear magnetic resonance (NMR) spectroscopy is a non-invasive method well suited to the measurement of intracellular concentrations of phosphate metabolites in living organisms. Special interest in the resonance of intracellular orthophosphate (P_i) arose from the pH-dependency of its chemical shift (Moon & Richards, 1973) which can be used to determine intracellular pH. A further advantage of ^{31}P-NMR spectroscopy is the ability to discriminate between different subcellular compartments. This aim is usually difficult to achieve with conventional analytical methods and requires the isolation of the organelles. In most cases only two intracellular P_i signals have been detected in ^{31}P *in vivo* NMR spectra of algae measured under aerobic conditions in the dark (Mitsumori & Ito, 1984; Sianoudis *et al.*, 1985; Kugel *et al.*, 1987; Bental *et al.*, 1988; Lundberg *et al.*, 1989). These P_i signals could originate from at least four cell compartments: chloroplast, cytoplasm, mitochondria or vacuole.

The P_i signal which indicates an acidic pH has been attributed to the vacuole (Waterton *et al.*, 1983; Sianoudis *et al.*, 1984). The assignment of the remaining extravacuolar P_i signal to a specific intracellular compartment, however is still controversial. The intensity and chemical shift of this P_i signal were found to be dependent on the energy status of the cell. The chemical shift indicated a more

alkaline pH when the experimental conditions allowed respiration or photosynthesis than when the cells were kept anaerobic in the dark (Sianoudis *et al.*, 1987). It was suggested that this signal be attributed to the chloroplast (Waterton *et al.*, 1983; Mitsumori & Ito, 1984) to explain the alkalization under illumination, an observation which is also known from isolated chloroplasts of spinach (Heldt *et al.*, 1973; Heber & Heldt, 1981).

Hentrich *et al.*, (1993) used ^{31}P-NMR spectroscopy saturation transfer experiments to measure ATP synthesis and consumption in respiring cells of *Chlamydomonas reinhardtii* and to determine the intracellular compartmentation of P_i. These authors observed that most of the flux towards ATP synthesis was catalyzed by the coupled enzymes glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase (GAPDH/PGK). The attribution of the measured flux to these enzymes is supported by the observations that (i) the magnetization transfer was strongly reduced by iodoacetate, an irreversible inhibitor of GADPH and that (ii) the unidirectional flux was much greater than the net flux through the mitochondrial F_0-F_1-ATPase as determined by oxygen consumption measurements. Also in unicellular algae such as *Chlamydomonas* and *Dunaliella*, glycolysis is divided into a chloroplastidic and cytoplasmic part with the enzymes GAPDH/PGK being located in the chloroplast stroma (Klein, 1986). This ^{31}P-NMR spectrometer signal of P_i must, therefore, originate from the chloroplast. The life time of the magnetic label transferred to P_i by these enzymes is too short for it to be transported to the cytoplasm via the phosphate translocator of the chloroplast envelope (Hentrich, *et al.*, 1993). These authors have also shown that when the intracellular compartmentation of P_i was taken

into consideration the calculated unidirectional ATP synthesis rate was equal to the consumption rate, indicating operation of GAPDH/PGK near equilibrium. Further, these authors conclude that the major P_i signal originates from the chloroplast, which is in contradiction to earlier reports which assign this signal to cytoplasm P_i.

One of major disadvantages of NMR spectroscopy for the study of metabolism in photosynthetic organisms is the methodological difficulty of acquiring sufficient light within the NMR spectrometer probe head to maintain active photosynthetic processes. Where some researchers have ignored this problem and worked with photosynthetic organism in the dark, others have used a variety of techniques to overcome this problem. Those using NMR spectroscopy to study algal physiology have used a cell slurry (Sianoudis *et al.*, 1984; Kuchitsu *et al.*, 1989), ligation of cells to polyester threads (Mimura & Kirino, 1984), centrifugation pellet (Ruyters *et al.*, 1985), perfusion of cells incorporated into agarose beads (Degani, *pers. comm.*, 1992) and cell suspension (Degani & Avron, 1982). Some researchers have also attempted to physically modify the NMR spectrometer probe to include a light source within the probe via a fibre optic cable. A major problem with this method is focusing the light throughout the NMR spectrometer sample tube so that all cells receive a similar intensity of light with similar spectral characteristics.

In the present study a ^{31}P-NMR spectroscopy "freeze-frame" technique was developed for studying intact photosynthetically active *Dunaliella salina* in the dark. Cells were incorporated into agarose beads which were then resuspended and stirred in at least 2L of fresh growth medium, thus ensuring adequate exposure to light as well as

ensuring that the cells do not become nutrient limited. The stress was imposed under natural light conditions and the response is allowed to proceed at normal temperature in the light, and at predetermined times the beaded cells were removed and rapidly chilled with the objective of holding the physiology of the beaded cells in an unchanged state. The chilled cells are then transferred to the NMR spectrometer where the lack of light would not affect the chilled physiological state, thus allowing examination of a 'photosynthetically active' cellular physiology in the dark. By using this technique it was possible to monitor stress induced metabolism as well as to follow the chemical shifts of orthophosphate in this algae.

MATERIALS AND METHODS

Cells of *Dunaliella salina* were grown under standard conditions (see Chapter 2)

Encapsulation of cells into agarose beads:

The method used was that described by Degani (*pers. comm.*) and Bental *et al.* (1990). Cells were centrifuged at 2500 g at 15°C for 15 minutes. The cells were then placed inside a 20 mL. beaker in a water bath at 28°C. A beaker containing 120 mL. of liquid paraffin oil and a magnetic stirrer, was placed inside a water bath at 28°C. A volume (commonly 20-25 mL.) of molten agarose (4% w/v - gelling point 26°C) made in full complement growth medium was cooled to 28°C. The concentrate of cells was then added to the agarose and stirred well with a glass rod previously warmed to 28°C. The liquid paraffin oil was stirred vigorously enough to allow vortexing, to which the agarose-cell mixture was added. By regulation of the stirring speed beads of agarose-cells form in the liquid paraffin. The liquid paraffin-agarose-

cells concoction was then cooled to 20°C by addition of ice to the water bath. This solidified the agarose into spherical beads containing cells. Stirring is halted allowing the beads to settle, and the liquid paraffin is removed by aspiration and replaced by full complement of growth medium. Beads were always made one day prior to use in experiments to allow for handling equilibration. Cells of *D. salina* were encapsulated into agarose beads at a final concentration of about 1 x 10^9 cells ml^{-1}. Beads were then gently stirred at low light conditions (80-100 $\mu mol.m^{-2}.s^{-1}$) at 25°C.

On the day of experimentation the beads were washed at least three times and then resuspended in P_i-free growth medium so that no P_i signal would originate from the growth medium. Cells in beads were exposed to either high light or hyperosmotic shock for predetermined times before they were quickly chilled to -4°C to freeze intracellular events and placed in the NMR spectrometer with the temperature set at -4°C. High light stress was achieved by changing the light intensity from 80-100 $\mu mol.m^{-2}.s^{-1}$ to 1000 $\mu mol.m^{-2}.s^{-1}$ by placing the cells under a 400 Watt high pressure sodium lamp (Lascon Lighting, Port Elizabeth, SA). Hyperosmotic salt stress was achieved by increasing the molarity of the growth medium from 1.5M to 3.0M by addition of NaCl. Cell viability in the agarose beads was measured as chlorophyll and carotenoid production. Also visual examination of the beads showed that cells continued to multiply whilst in the beads.

^{31}P-NMR spectrometer measurements were performed at 161.97 MHz using an AMX Brukker 400 spectrometer with proton noise decoupling. A spectrum was obtained by accumulating 6000 transients with a cycle time of 0.6 seconds and a 30° pulse.

Chemical shift values were obtained from a standard curve referenced against the γ-P_i signal of ATP set arbitrarily at -10 ppm. the acquisition and processing parameters are listed in Tables 5.1 and 5.2. A standard calibration curve was prepared by determining the chemical shift of orthophosphate at 0.5 pH unit intervals (Degani, *pers. comm.*).

Table 5.1. NMR spectroscopy acquisition parameters.

PULPROG	zgpg
SOLVENT	D2O
AQ	0.6226120 sec
FIDRES	0.803094 Hz
DW	38.0 used
RG	65536
NUCLEUS	31P
TE	269.0 K
HL1	1 dB
D1	0.7000000
P31	100.0 used
S4	15 dB
D11	0.0300000
S2	15 dB
P1	11.6 used
DE	47.5 used
SF01	161.9765204 MHz
SWH	13157.89 Hz
TD	16384
NS	6000
DS	2

Table 5.2. NMR spectroscopy processing parameters.

SI	32768
SF	161.9776061 MHz
WDW	EM
SSB	0.00
LB	20.00 Hz
GB	0.00
PC	0.20

RESULTS

A number of studies were undertaken to determine the correct freezing temperature and number of NMR spectroscopy scans necessary to obtain the best results (results not presented). From the investigations it was noted that cells incorporated into agarose beads could be frozen to -10°C and kept at this temperature for up to 4 hours, remained viable and continued to swim when equilibrated to room temperature and the agarose beads broken open. Feasibility of the 'freeze-frame' approach was also tested in the NMR spectrometer where results indicated that the metabolic state of the cell could be held in suspension for 4-5 hours before any noticeable changes started to occur. This was achieved by leaving the same cells in the NMR spectrometer and constantly monitoring any significant chemical shifts of P_i over an 8 hours period.

When cells of *D. salina* were incorporated into agarose beads the concentration of chlorophyll increased for the first 12 hours (Fig 5.1). Cells in agarose beads which were chilled for 2 hours at -4°C showed a slight increase in chlorophyll

concentration in comparison to the control beads, but after a further 10 hours showed a more significant reduction in this pigment in comparison to the control beads. Total ß-carotene levels in the beads chilled for 2 hours at -4°C showed a rapid drop in concentration, and then for the following 10 hours a steady increase but not reaching the concentration of ß-carotene in the control beads (Fig 5.2).

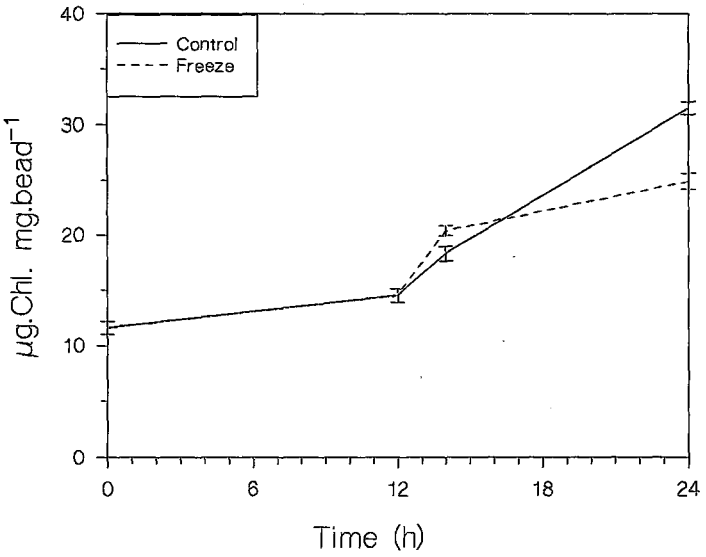

Figure 5.1. Chlorophyll *a* content of beads. Control beads kept at low light and 25°C; Freeze beads were frozen for 2 hours.

To evaluate the 'freeze-frame' method proposed here it was decided to use the NH^+_4Cl stress studies reported by Pick's group (1990; 1991) as a published indication of chemically induced shifts of P_i peaks in *Dunalliella*, using NMR spectroscopy. One minute after addition of NH^+_4Cl to the growth medium a rise in pyrophosphate (at -5 ppm overlapping with γ-NTP) and higher polyphosphates (at -20 ppm) was noted (see Figures 5.4 & 5.5). This NH^+_4 induced phosphatase activity on polyphosphates was also shown by Pick's group.

The result of NH^+_4 addition to the growth medium was the induction of an apparent chemical shift of the two major P_i peaks, with acidification of the up field peak and alkalization of the down field peak, after two minutes. These two peaks then apparently superimposed forming a single peak (Figure 5.5).

Based on replicates of the above experiments on the effect of NH^+_4Cl on cellular pH, it was evident that the 'freeze-frame' approach may be successfully applied to the study of photosynthetic physiology in the dark using NMR spectroscopy, and could be used to provide new insights into the understanding of stress responses in plants.

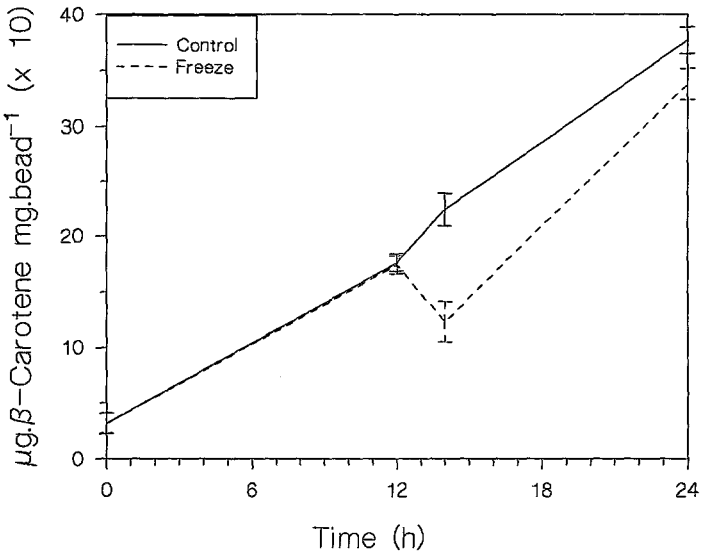

Figure 5.2. *β*-Carotene content of beads. Control beads kept at low light and 25°C; Freeze beads were frozen for 2 hours.

Figure 5.3 shows the time zero (control) ^{31}P spectrum. Along with polyphosphate signals, two P_i signals are prominent indicating two compartments within the cell

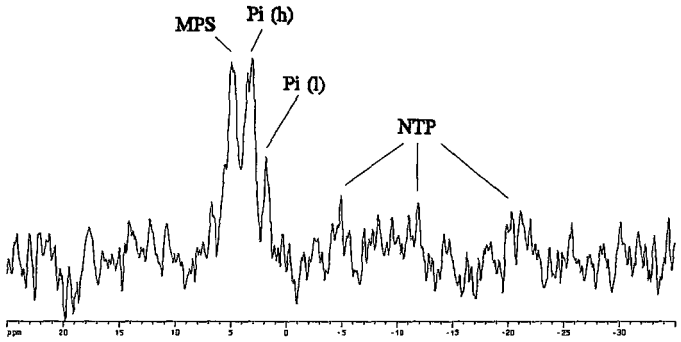

Figure 5.3. A characteristic NMR spectrum of *D. salina*. $1x10^9$ cell.mL^{-1}. MPS: monophosphate sugars; Pi (h): high field Pi; Pi (l): low field Pi; NTP: ATP/ADP.

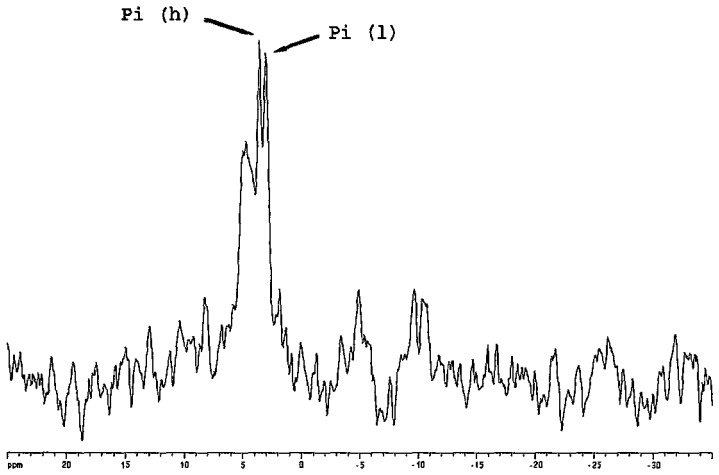

Figure 5.4. Control spectrum for the NH_4Cl stress study in *D. salina* encapsulated into agarose beads. Pi (h) and Pi (l) are high and low field orthophosphate peaks respectively.

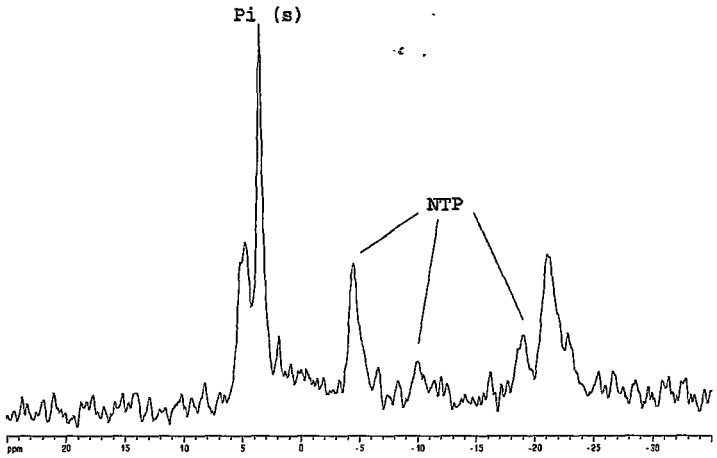

Figure 5.5. NMR spectrum of *D. salina* cells encapsulated into agarose beads, after 1 minute of $NH^{+}_{4}Cl$ stress. Pi (s): single Pi peak.

which have different pH values. A pH 6.8 for the high field P_i signal was determined from the standard curve, whilst the pH of the low signal was measured to be 6.3. Both these signals moved in response to high light irradiance (Figure 5.6).

The response of chloroplast and vacuolar pH to high light intensity is shown in Figure 5.6. Exposure of *D. salina* cells to high light intensity appeared to cause an immediate and dramatic decrease in chloroplast pH. A similar, though less pronounced effect was observed for vacuolar pH. Interestingly, recovery of vacuolar pH was reached 4 hours after exposure to continuous high light whereas full recovery of the chloroplast pH was achieved only after 8 hours of treatment.

It can be seen that there is a small but significant increase in the chloroplastic pH after treatment of the cells with 3 M NaCl-containing medium (Figure 5.7). This increase in chloroplastic pH occurred within 5 minutes of exposure to salt stress. A

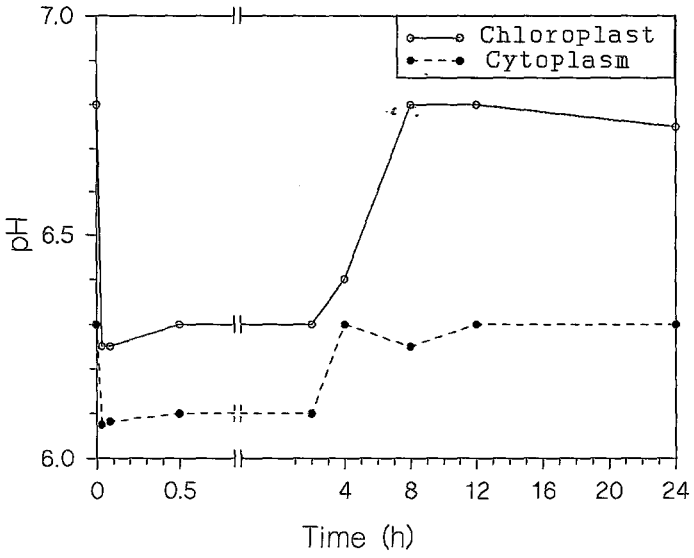

Figure 5.6: The response of compartmental pH fluxes to high light in *D. salina*.

second, albeit less pronounced increase in chloroplastic pH was evident 8 hours after the onset of salt stress. Thereafter, chloroplastic pH declined gradually throughout the remainder of the 24 hours incubation period.

By comparison, a substantial increase in pH of the vacuolar compartment was observed within 5 minutes of exposure to salt stress. A decline in pH of this compartment then followed, lasting approximately 3.5 hours. This probably reflects the effect of increased ionic strength due to cell shrinkage. Recovery of volume is complete approximately 3.5 hours after the start of treatment with NaCl at this molarity (Cowan, Weiss and Pick, unpublished data). A second rise in vacuolar pH was observed 4 hours after the onset of salt stress. Vacuolar pH then remained constant, 0.175 units above the pre-stress pH for this compartment for the remainder of the incubation period.

To reexamine the specific P_i signal and pH values reported, cells at control (low light) conditions were examined in the NMR spectrometer together with sealed capillary tubes containing either P_i at pH 6.3 or 6.8 and phosphocreatine as an external standard. This was done to eliminate any possible doubt that shifts in the pH calibration curve

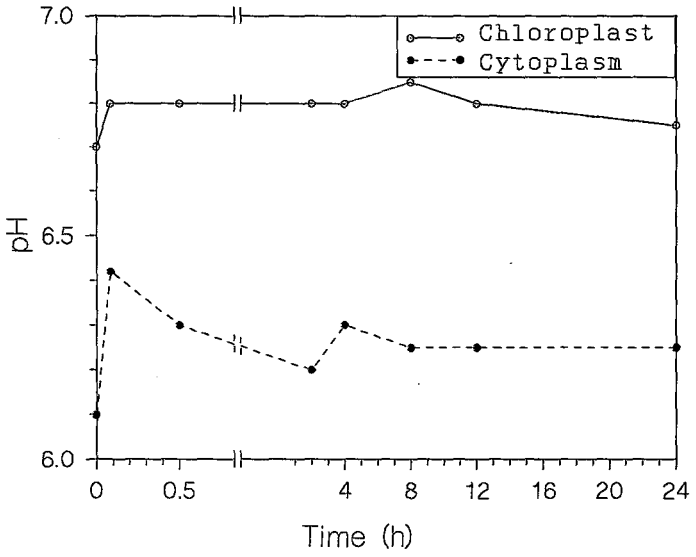

Figure 5.7. The response of compartmental pH fluxes to an increase in salinity (1.5M to 3.0M) in *D. salina*.

may result from interaction of the P_i chemical shift with other compounds in solution, as well as the osmotic potential of the cellular fluids. The NMR spectroscopy spectra resulting from these experiments showed that P_i in the capillary tubes demonstrated the same chemical shift as the P_i in the proposed chloroplastic compartment (pH 6.8) and cytoplasmic compartment (pH 6.3) (Figure 5.8).

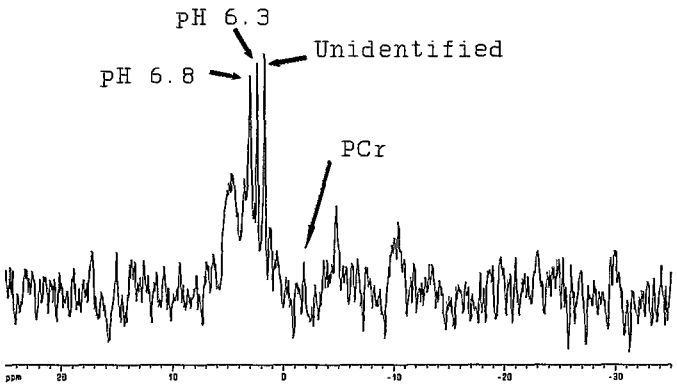

Figure 5.8. Imposition of Pi at pH 6.8 and 6.3 in sealed capillary tubes over the two major Pi signals. External standard phosphocreatine.

DISCUSSION

The major compartments in intact *D. salina* cell are cytoplasm, cytoplasmic vacuoles, the stroma of the chloroplast and the lumen of the thylakoids. In the light protons are known to move from the chloroplast stroma into the thylakoid lumen, which increases the pH of the lumen and at the same time decreases the pH of the stroma (Heldt *et al.*, 1973). Mitsumori and Ito (1984) have demonstrated that the compartments from which two P_i signals emanate in *Chlorella vulgaris* are the cytoplasm and stroma of the chloroplast. The pH for each of the compartments was shown to be 6.8 and 8.1 respectively. Further, these authors showed that addition of 10μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for 30 minutes resulted in convergence of the two P_i signals.

According to the chemiosmotic theory, the electrochemical gradient of protons across the thylakoid membrane is utilized to synthesize ATP by ATPase (Mitchell, 1966).

The pH change in the stroma of the chloroplast under illumination observed in this study represents only one side of the gradient. Similarly when exposed to a high light environment, the decreased transport of protons across the thylakoid membrane by the electron transport machinery due to photoinhibition at PSII, results in relative acidification of the chloroplast stroma for a short period of time. However, as the photosynthetic machinery adapts to the high light, the immediate inhibition of proton transport across the membrane is overcome resulting in rapid alkalization of the chloroplast stroma (Figure 5.6). The equilibrium between stroma and lumen is restored after several hours, as the pH of the stroma normalises. In *D. salina* it appears that it takes 8 hours for the system to re-establish an equilibrium, and that this time period is coincident with the production of β-carotene (see Chapters 5 & 6), either to quench singlet oxygen, scavenge free radicals (Burton & Ingold, 1984; Rich *et al.*, 1992) or to shield the PSII from excess light.

According to Klock and Kreuzberg (1991), there is no difference in the concentration of ATP in the chloroplast and in the cytoplasm. The ATP concentration determined by ^{31}P *in vivo* NMR spectroscopy can therefore be used to calculate the flux in the reaction from τ-ATP to P_i (Hentrich *et al.*, 1993), and these authors have shown that this flux is nearly identical to the unidirectional ATP synthesis in the chloroplast provided that the compartmentation of P_i is taken into consideration. Even assuming (i) a complete saturation of chloroplastic P_i pool by saturation transfer from τ-ATP and (ii) an exclusion of any reverse flow of saturated cytoplasm P_i to the chloroplast, the decline of the P_i magnetization would be limited to about 17% (Hentrich *et al.*, 1993). This is in contradiction to the requirement of rapid transport across the

chloroplast membrane, because it would take at least 5.8 times the spin-lattice relaxation time for complete exchange of P_i. These authors state that the observed magnetization transfer which they observed can not be explained by assuming an exclusive localization of the NMR spectra visible P_i in the cytoplasm. Rather most of the P_i signal intensity has to be assigned to the chloroplast. This conclusion is also supported by the recent NMR spectroscopy detection of P_i in isolated spinach chloroplasts (Bligny *et al.*, 1990) as well as by the high amount of chloroplastic P_i (85% of total cellular orthophosphate) in *Chlamydomonas* (Klock & Kreuzberg, 1991). The fact that chloroplastic P_i can be detected by ^{31}P *in vivo* NMR spectroscopy and accounts for most of the P_i signal is of special importance for understanding the response of photosynthetic organisms to stress. Based on the above discussion it is proposed that the high field P_i signal emanates from the stroma of the chloroplast, and that a thylakoid P_i signal was not observed because either the concentration of P_i within the lumen is too low, or because the fractional volume of the thylakoid in *D. salina* is too small, as suggested by Mitsumori and Ito (1984) for *Chlorella*. The fact that chloroplastic P_i can be detected by ^{31}P *in vivo* NMR spectroscopy, coupled with observations that osmotic stress induces an increase in intracellular orthophosphate concentration in *Dunaliella* (Avron & Ben-Amotz, 1992), facilitated use of the chemical shift of the P_i signal as a pH-marker for interpretation of intracellular pH changes in response to high light intensity and salt stress. Based on this, it is proposed that the alkaline P_i signal in ^{31}P *in vivo* NMR spectroscopy spectra of *Dunaliella* originates from the chloroplast whereas the more acidic P_i signal is vacuolar. Acidic vacuoles are present in *Dunaliella* and have been shown to contribute to osmoregulation in this species (Pick, 1992).

CONCLUSION

In this algae it is apparent that environmental stress in the form of high intensity irradiance and salt stress have marked effects on the intracellular pH compartmentation. High intensity irradiance appears to have a more radical effect in that it appears to effect chloroplast pH as a direct result of its effect on the photosynthetic machinery, and therefore proton exchange between the chloroplast stroma and thylakoid lumen. The increase in chloroplastic pH to its ground state at 4-8 hours is coincident with the onset of ß-carotene production in the chloroplast lumen. The reestablishment of the ground state of chloroplastic pH is also contiguous with a rapid decline in violaxanthin and accumulation of zeaxanthin in the thylakoid membrane. The relationship between pH and violaxanthin de-epoxidation to zeaxanthin has already been shown (Pfundel & Dilley, 1993).

The role pH plays in the response to salt stress is less clear. Although there is restoration of pH to the ground state after 12 hours, it is suggested that either the initial increase in pH or the restoration of pH to ground state could impact the signal for the production of ß-carotene via the xanthophyll cycle. Should compartmented pH within the chloroplast be the initial signal for ß-carotene mass accumulation, it would be reasonable to anticipate that the degree of pH flux may contain the amplitude of ß-carotene accumulation.

The present study revealed rapid compartmental acidification following high light treatment, but chloroplastic and cytoplasmic alkalization on exposure of *D. salina* to salt stress. Compartmental alkalization appears to be a characteristic response of

Dunaliella to hypersaline conditions (Goyal *et al.*, 1987; Goyal & Gimmler, 1989; Kuchitsu *et al.*, 1989). Conflicting evidence about pH changes following hyperosmotic shock in *Dunaliella* have been reported. Goyal *et al.* (1987) and Goyal and Gimmler (1989) reported a small (0.2 pH units) alkalization in *D. tertiolecta* cells within 2 hours after hyperosmotic shock, measured from ^{14}C-5,5-dimethyloxazolidine-2,4-dione (DMO) distribution. Similarly Kuchitsu *et al.* (1989) presented evidence from ^{31}P-NMR spectroscopy studies for a large cytoplasmic pH increase (from pH 7 to 8) following hyperosmotic shock. In contrast, Weiss and Pick (1990) presented indirect evidence for a rapid and transient cytoplasmic acidification, following hyperosmotic shock in *D. salina*, manifested by activation of Na^+/H^+ exchange. The different reports are not necessarily mutually exclusive since they occur in different time regimes of the osmotic response.

In comparison, very little is known about light-induced changes in compartment pH of *Dunaliella*. Nevertheless, light-induced acidification has also been demonstrated for *Chlorella* (Mitsumori & Ito, 1984) and *Eremosphaera viridis* (Thaler *et al.*, 1992). Transduction of pH changes between cellular compartments is considered to impact on metabolism by redirecting carbon flux. This aspect assumes particular significance in the stress induction of zeaxanthin and β-carotene formation in *D. salina*.

Chapter 6

CONCLUSION

The initial component of this study was to determine whether chlorophyll fluorescence could be used as an indicator of the stress response of cells *Dunaliella salina* grown in mass culture. Results confirmed the work of Rose (1992), showing that chlorophyll fluorescence provides a very rapid means of following the induction of stress (high light intensity, or NH^+_4) in this alga. Modification of the efficiency of photosynthesis by imposition of stress was confirmed by O_2 evolution studies. For the first time changes in the yield of chlorophyll fluorescence has now been correlated with de-epoxidation of carbon in the xanthophyll cycle and the initiation of β-carotene production at about 4 hours post stress, in this algae. Furthermore, a novel finding is the reversibility and commitment factors operating in the stress response, in as much that at least 4 hours of exposure to high light intensity is required before accumulation of β-carotene occurs and that the same time period is required before the chlorophyll fluorescence no longer reacts indicating energy overload at PS II. Reversibility (non-commitment) of the stress signal on a diurnal basis was demonstrated for production of β-carotene and operation of the xanthophyll cycle. Non-commitment to the mass production of β-carotene resulted in a periodicity or circadian rhythm occurring in the anabolism and catabolism of β-carotene and epoxidation state of the xanthophyll cycle in the dark. This suggests that reversibility or non-commitment is itself regulated by a physiological signal perhaps linked to pH or a phytochrome-type mechanisms. Although circadian rhythms have been previously noted for many cellular events including photosynthesis and β-carotene

(Prezelin *et al.*, 1977); Sweeny, 1987), demonstration of this rhythmic regulation of the epoxidation state of the xanthophyll cycle is now reported for the first time in this alga.

It was also shown that the carbon required for the early synthesis of ß-carotene comes from photosynthesis. The carbon requirement of the xanthophyll cycle has two sources; the carbon replenishment of the violaxanthin pool appears to be dependent of photosynthesis, whilst the initial increase in zeaxanthin diminishes the violaxanthin pool but also then depends on photosynthesis as a carbon source. Activation of de-epoxidation of the xanthophyll cycle is known to be pH dependent, and stimulated under conditions which provide inhibition to photosynthesis (Gilmore, *et al.*, 1994). Thus when cells of *D. salina* are exposed to high light intensities, acidification of the thylakoid lumen results in de-epoxidation of violaxanthin to zeaxanthin. However, the reverse reaction has recently been shown to be independent of pH (Gilmore, *et al.*, 1994). It was also shown that the initial decline in ß-carotene in cells exposed to high light intensity was due to loss of ß-carotene located in the pigment bed. The main increase in ß-carotene in cells exposed to high light intensity has a lag period of about 4-6 hours. It is suggested that the ABA pool also provides a principal sink for photosynthetic carbon during the first 30 minutes of exposure to high light intensity. This plant growth regulator is then partitioned out of the chloroplast to the more alkaline cytoplasm. Also the accumulation of ABA in the growth medium during high light stress may have important ecological consequences to other organisms in the growth medium.

Studies on photosynthesis of cells of *D. salina* exposed to high light intensity showed that these cells undergo a protective modification in photosystem stoichiometry before the mass accumulation of β-carotene. These results indicate that some physiological procedure has been initiated and completed whereby excess fluorescence and perhaps energy from PS II is removed non-photochemically and that the mass accumulation of β-carotene in the chloroplast stroma and activation of the xanthophyll cycle may play a pivotal role here.

The central factor in the above discussion is the possible regulatory role pH plays in controlling operation of the xanthophyll cycle and performance of photosynthesis in the production of reducing agents. Investigating the involvement of pH in the transduction of the stress signal revealed rapid compartmental acidification following high light treatment but alkalization on exposure to salt stress. Compartmental alkalinization appears to be a characteristic response of *Dunaliella* cells to hypersalinity. In comparison very little is known about light-induced changes in compartmental pH of *Dunaliella*, nevertheless, light-induced acidification has been demonstrated for *Chlorella* (Mitsumori & Ito, 1984) and *Eremosphaera viridis* (Thaler, *et al.*, 1992). A method was also developed whereby photosynthetically active cells are examined in the dark, using NMR spectroscopy. This new method provides an opening to a new methodology for photosynthesis research. The transduction of pH fluxes between cellular compartments impact on metabolism by redirecting carbon fluxes. This aspect assumes particular significance in the stress induction of xanthophyll cycle activity, partitioning of ABA out of the chloroplast, and perhaps also in the mass accumulation of β-carotene.

Results from the present investigation reveal the following cascade of events in cells of *Dunaliella salina* after exposure to high light intensity and are summarized in Figure 6.1: (i) Increase in chlorophyll fluorescence; (ii) Changes in chloroplastic and vacuolar pH; (iii) De-epoxidation of the xanthophyll cycle; (iv) Increase in *ß*-carotene production; (v) *De novo* synthesis of ABA and subsequent partitioning of ABA out of the chloroplast thereby initiating whole cell stress metabolism; (vi) establishment of a photoprotective mechanism against high light stress.

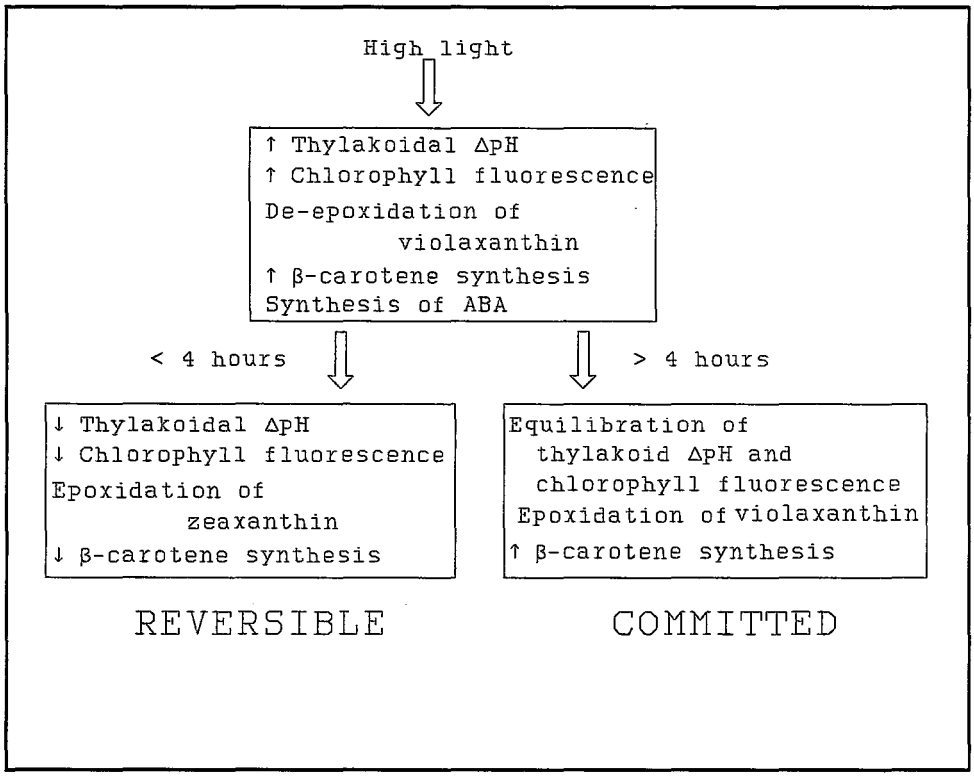

Figure 6.1. Summary of events of reversibility and commitment in the stress response in *D. salina*.

The studies reported here focus only on physiological responses to high light stress. Studies on stress signal transduction involving chlorophyll fluorescence, xanthophyll cycle responses and abscisic acid production to salinity and nutrient stress reported by Cowan & Rose (1991), Cowan *et al.* (1992, 1993ab, 1995), Logie *et al.*, (1993ab,

1994ab), Rose (1992), Phillips (1994) and Phillips *et al.* (1995) could collectively be accommodated within the above sequence of events reported in Figure 6.1. Thus it is possible to envisage the development of a general physiological stress response by *D. salina* common to a range of different stresses.

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PUBLICATIONS

The following are conference presentations of various sections of this thesis.

1. Logie, M.R.R., Cowan, A.K., Rose, P.D., 1993, Stress signal transduction and the manipulation of secondary metabolites in *Dunaliella salina* var. *bardawil*, 1st International Biotechnology Conference, Rhodes University, South Africa.

2. Cowan, A.K., Horne, L.G., Logie, M.R.R., Rose, P.D., 1993, Carotenoid metabolism and abscisic acid production in stressed cells of *Dunaliella salina*, Annual Conference of the South African Association of Botanists, University of the Western Cape, South Africa.

3. Logie, M.R.R., Horne, L.G., Cowan, A.K., Rose, P.D., 1993, Monitoring stress induced secondary metabolite accumulation in *Dunaliella salina*, Progress in Biotechnology of Phototrophic Microorganisms, Masojedek, J., Setlik, I., (eds.), 6th International Conference on Applied Algology, Ceske Budejovice, Czech Republic, 16.

4. Cowan, A.K., Horne, L.G., Logie, M.R.R., Rose, P.D., 1993, Carotenoid metabolism and abscisic acid production in stressed cells of *Dunaliella salina*, 10th Carotenoid Symposium, Trondeheim, Norway.

5. Logie, M.R.R., Rose, P.D., Cowan, A.K., 1994, Monitoring light stress induction processes leading to the accumulation of β-carotene in *Dunaliella salina*, Annual Conference of the South African Association of Botanists, University of the Witwatersrand, South Africa.

6. Logie, M.R.R., Rose, P.D., Cowan, A.K., 1994, Chlorophyll fluorescence as an indicator of changes in photosynthetic and cellular physiology in the unicellular alga *Dunaliella salina*, Annual Conference of the South African Association of Microbiologists, Rhodes University, South Africa.

The following have been accepted to the Journal of Plant Physiology for publication:

1. Cowan, A.K., Logie, M.R.R., Rose, P.D., Phillips, L.G., 1995, Stress induction of zeaxanthin formation in the β-carotene accumulating alga *Dunaliella salina* Teod., J. Plant Physiol., *In press*.

2. Phillips, L.G., Cowan, A.K., Rose, P.D., Logie, M.R.R., 1995, Operation of the xanthophyll cycle in non-stressed and stressed cells of *Dunaliella salina* Teod. in response to diurnal changes in incident irradiation: A correlation with intracellular β-carotene content, J. Plant Physiol., *In press*.