CONSTITUENT PROCESSES CONTRIBUTING TO STRESS INDUCED ß-CAROTENE ACCUMULATION IN *DUNALIELLA SALINA*.

THESIS

Submitted in fulfilment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY
at Rhodes University

by

LESLEY GAIL PHILLIPS

December 1994

To Mom and Dad

This thesis is dedicated to you both in appreciation for your absolute faith in me and for the unfailing support you have provided in everything I have ever undertaken.

Lesley

CONTENTS

ABSTRACT

The alga *Dunaliella salina* possesses the unique ability to accumulate up to 14% of it's dry weight as ß-carotene in response to stress conditions. This hyper-accumulation of ß-carotene has led to the commercial exploitation of this alga for the biotechnological production of this important carotenoid. In order to maximise ß-carotene production, a dual-stage process which separates the distinctive growth phase from the ß-carotene accumulating stress phase has recently been patented. Preliminary laboratory studies showed that although stress factors such as high salinity and nutrient limitation enhance ß-carotene accumulation in *D. salina* ($\pm$ 10 pg.cell^{-1}), high light intensity was the single most important factor contributing to the induction of ß-carotene accumulation in this alga ($\pm$ 20 pg.cell^{-1}). Moreover, it was demonstrated that ß-carotene accumulation can be further stimulated by exposing the alga to a combination of high light intensity, salt and nutrient stresses ($\pm$ 30-60 pg.cell^{-1}).

The response of *D. salina* to stress was shown to occur in two phases. The first phase occurred within 24 hours and was characterized most importantly by higher rates of ß-carotene accumulation for all the stresses investigated. In cells exposed to multiple stress factors in mass culture, the ß-carotene accumulation rate was as much as 9.5 pg.cell.$^{-1}$.day^{-1} in the first phase compared to only 3 pg.cell^{-1}.day^{-1} in the second phase.

Since the rate of ß-carotene accumulation was higher within the first 24 hours after exposure to stress, the first phase was considered crucial for stress-induced ß-carotene accumulation. Characterization of this phase revealed that the stress response was multifaceted. Transition of cells from the growth stage to stress conditions was characterized by the following:

1) Change in cell volume.

Hypersalinity caused cell shrinkage while cells exposed to nutrient limitation and/or high light intensity caused cells to swell. Restoration of cell volume was shown to occur within 8 hours for all stresses investigated.

2) Altered photosynthesis.

Inhibition of both carbon fixation and oxygen evolution was demonstrated in cells immediately after exposure to multiple stress factors.

3) Production of abscisic acid.

Intracellular ABA levels increased within 6-8 hours after exposure to all stresses investigated. The rise in intracellular ABA levels coincided with an increase or return to starting cell volume. High intracellular ABA levels were however transient and within 24 hours ABA began to partition into the culture medium.

4) Change in pigment composition.

Changes in xanthophyll cycle pigment content was demonstrated soon after exposure to stress conditions. In hypersalinity shocked cells, initial epoxidation of zeaxanthin to violaxanthin and subsequent de-epoxidation to zeaxanthin occurred, whereas exposure to high stress resulted in an immediate and continued decrease in the epoxidation state indicating accumulation of zeaxanthin. A rapid rate of chlorophyll depletion was also demonstrated.

In addition to the above responses a rapid decrease in growth rate during this phase was also noted.

An interrelationship between cell volume change, ABA production, maintenance of xanthophyll cycle operation and ß-carotene accumulation therefore appeared to exist.

ABA production was shown to be stoichiometrically related to changes in xanthophyll content with $r^2 = 0.84$ and slope of the curve = 0.91 being achieved for high light stressed cells. This study therefore presents strong circumstantial evidence in support of a carotenoid origin for ABA in *Dunaliella*. In addition, enhanced ß-carotene content was achieved by the application of exogenous ABA and related compounds suggesting a role for ABA as a regulator of the overall stress response. Furthermore, zeaxanthin accumulation was shown to be positively correlated ($r^2 \geq 0.81$) to ß-carotene accumulation for all the stresses investigated.

The second phase was characterized by a return to homoeostasis of the physiological processes mentioned above, indicating acclimation of the cell to prevailing conditions. This stage was characterised by continued ß-carotene accumulation and a decreased epoxidation state of the xanthophyll cycle which together appeared to sustain photosynthesis, allowing this organism to tolerate stress conditions.

ACKNOWLEDGEMENTS

I wish to express my appreciation to the following people for their support throughout the course of the practical study and compilation of this thesis.

Keith Cowan - words cannot express the gratitude I feel for your support, direction and constant encouragement throughout this work. You really proved to be a supervisor in a million.

Peter Rose - thank you for giving me the opportunity of pursuing a dream, and for your contagious enthusiasm which was a constant source of strength.

Winston Leukes, Debbie Adamson and Penn Lloyd - thanks must be extended to you all for your time and effort in editing this thesis.

My husband, Trevor - without your love and support this thesis would definitely not have been possible. Thank you !

Finally, I would like to thank SASTECH Ltd., and in particular Andrew Jarvis and Peter Stegmann for the financial and academic support during this project.

FIGURES

TABLES

ABBREVIATIONS

ABA - abscisic acid

ABAMe - abscisic acid methyl ester

ATCC - American Type Culture Collection

Bq - becquerel

CCAP - Culture collection for Algae and Protozoa

cbr - carotene biosynthesis-related gene

DMAPP - dimethylallyl pyrophosphate

Elips - early light-induced proteins

FPP - farnesyl pyrophosphate

GPP - geranyl pyrophosphate

GGPP - geranylgeranyl pyrophosphate

GC - gas chromatography

GC-MS - gas chromatography mass spectrometry

HPLC - high performance liquid chromatography

IPP - isopentyl pyrophosphate

LHC - light harvesting complex

MVA - 3,5-dihydroxy-3-methylpentanoic acid (mevalonic acid)

PAC - polyaluminum chloride

PFD - photon flux density

PPPP - prephytoene pyrophosphate

PS I - photosystem I

PS II - photosystem II

rpm - revolutions per minute

spp. - species

TLC - thin layer chromatography

10E - exponential

CHAPTER 1

Introduction.

Algal biotechnology dates from the 1940's with the production of single-cell protein from *Chlorella* species (Burlew, 1953; Tamiya, 1957). Since then many other potential products from large-scale algal culture have come to the fore. These include the production of alternative food sources (Soeder and Binsack, 1978), health-promoting algal preparations (Kawaguchi, 1980) and fine chemicals (Aaronson, *et al.*, 1980) as well as the use of algae in aquaculture (Persoon and Claus, 1980) and waste-water treatment (Oswald, *et al.*, 1965). Two high-value speciality products are currently being produced in significant amounts from microalgae. The first, "linablue", is a phycobiliprotein concentrate produced from *Spirulina* species, and is used as a colourant in the food industry. The second, ß-carotene, is produced from *Dunaliella* spp. and is used as a food colourant, a source of provitamin A and as a therapeutic anti-oxidant (Borowitzka, 1986; Benemann, 1989; Ben-Amotz and Avron, 1989).

ß-Carotene occurs naturally in all vegetables and fruits (Ziegler, 1991). It is also produced synthetically, (Isler, *et al.*, 1956, cited in Curtain, *et al.*, 1987), however, the perception of the finiteness of non-renewable feedstocks and low levels of undesirable residues of reaction products remaining in synthetic ß-carotene, together with an increasing demand for natural products, has led to alternative sources of ß-carotene attracting attention (Curtain, *et al.*, 1987). Natural ß-carotene commands a market price of more than double that of synthetically produced ß-carotene (Ben-Amotz and Avron, 1990). Furthermore, clinical and experimental studies have shown that natural ß-carotene is more effective as an anticancer agent because of increased biological activity (Ben-Amotz, *et al.*, 1989a).

Research has shown that ß-carotene reduces cancer rates in humans (Peto, *et al.*, 1981; Ziegler, 1991) and in both mice and rats (Nagasawa, *et al.*, 1989a, 1989b). Furthermore, high serum ß-carotene levels have been associated with the induction of tumour necrosis factor in hamsters (Shklar and Schwartz, 1988). ß-Carotene rich algae have also been evaluated as a source of retinol in chick diets (Ben-Amotz, *et al.*, 1986).

The biological actions of carotenoids are comprehensively covered in two review articles by Bendich and Olson (1989) and by Krinsky (1993). Of all the known carotenoids, ß-carotene possesses the highest provitamin A activity, exhibiting a biopotency double that of other provitamin A carotenoids (Mordi, 1993). Furthermore, Levin and co-workers, (1992) identified separate retinoic acid receptors for the all-*trans* and 9-*cis* isomers of retinoic acid. The implication of this finding is that natural ß-carotene containing both 9-*cis* and all-*trans* isomers may be a superior source of provitamin A.

Natural ß-carotene was initially obtained from carrots (Barnett, *et al.*, 1958) and the fungus *Blakeslea trispora* (Ciegler, *et al.*, 1962). It was later realised that microalgae of the genus *Dunaliella* offer greater potential for ß-carotene production (Aasen, *et al.*, 1969), but commercial production from this source has only been developed in the last decade (Moulton, *et al.*, 1987; Schlipalius, 1991). Most of the preliminary studies were undertaken by groups in Israel (Ben-Amotz and Avron, 1980; Ginzburg and Ginzburg, 1985) and Australia (Borowitzka and Brown, 1974; Borowitzka, *et al.*, 1977). Currently at least five companies are reported to be cultivating *Dunaliella* spp. commercially for ß-carotene production (Borowitzka, *pers comm.*).

1.1. *Dunaliella.*

The Chlorophyte, *Dunaliella*, is classified under the order Volvocales. Members of this genus are motile, ovoid and biflagellate. The alga contains one large chloroplast and typical extrachloroplastic organelles, but unlike other green algae, *Dunaliella* lacks a rigid cell wall. Instead, the cell is covered by a plasma-membrane, which allows for rapid volume changes in response to extracellular osmolarity changes (Avron, 1992; Ben-Amotz and Avron, 1992).

In hypersaline lakes, the *Dunaliella* strain which predominates is orange rather than green in colour due to the production of ß-carotene. Two species, *Dunaliella bardawil* and *Dunaliella salina* Teod., have been shown to accumulate large amounts of ß-carotene when cultivated under appropriate conditions.

Intracellular ß-carotene hyper-accumulation can be induced by high light intensity (Ben-Amotz, 1986; Ben-Amotz, *et al.*, 1989b), nutrient limitation (Ben-Amotz, 1987), or other environmental stresses such as high salt concentrations (Mil'ko, 1963a, 1963b; Ben-Amotz and Avron, 1980; Borowitzka, *et al.*, 1984, 1990), and high and low temperature (Mil'ko, 1963a; Semenenko and Abdullaev, 1980; Ben-Amotz and Avron, 1983). ß-carotene accumulation up to 14% dry wt. has been reported (Borowitzka, *et al.*, 1986; Ben-Amotz and Avron, 1989). The accumulated ß-carotene is contained within oily globules in the interthylakoid spaces of the chloroplast and comprises a mixture of the 9-*cis* and all-*trans* stereoisomers. Both the total amount of ß-carotene accumulated and the all-*trans* to 9-*cis* ratio is dependent on the integral amount of light absorbed by the cell during one division cycle (Ben-Amotz, *et al.*, 1982, 1988).

1.2. Biosynthesis of ß-carotene.

Carotenoids constitute the most widespread group of pigments in nature. They are present in all photosynthetic organisms where they form an essential part of the photosynthetic apparatus. In addition, they are found in certain fungi (Dandekar, *et al.*, 1980) and some non-photosynthetic bacteria (Harrison, 1986).

Carotenoids are tetraterpenoids consisting of eight isoprene units joined together so that the linking of the units is reversed at the centre of the molecule (Goodwin, 1979). The early stages of biosynthesis, up to geranyl geranyl pyrophosphate (GGPP) (Figure 1.1.), are considered to be identical for all terpenoids. The specific precursor is mevalonic acid (MVA, 3,5-dihydroxy-3-methylpentanoic acid), which is formed by the condensation of acetyl-CoA and acetoacetyl-CoA, in an irreversible reaction catalyzed by HMG-CoA reductase (E.C. 1.1.1.34.) (Andrews and Ohlrogge, 1990), and requires NADPH (Goodwin, 1980a; Jones and Porter, 1985). MVA is converted to isopentenyl pyrophosphate (IPP), the universal isoprene unit, via a three-step sequence involving soluble enzymes, MVA kinase, MVA 5-

reactions are catalysed by a prenyl transferase referred to as GGPP synthase. Phytoene, the first committed compound *en route* to carotenoids, is formed by condensation of two molecules of GGPP via the intermediate prephytoene pyrophosphate (PPPP) (see Figure 1.1.) and is usually produced as the 15-*cis* isomer in a reaction catalysed by phytoene synthase (Britton, 1988). In higher plants and algae, phytoene undergoes a series of desaturation reactions to form phytofluene, ζ-carotene, neurosporene, and finally, lycopene (Bramely, 1985). Desaturation of phytoene is catalysed by the enzyme phytoene desaturase and the desaturation of ζ-carotene by ζ-carotene desaturase. Phytoene desaturase is thought to utilize each side of the symmetrical phytoene molecule as substrate and to yield ζ-carotene with phytofluene as an intermediate. ζ-carotene desaturase is then thought to carry out a similar reaction with ζ-carotene to form lycopene via neurosporene (Bramley and Mackenzie, 1988).

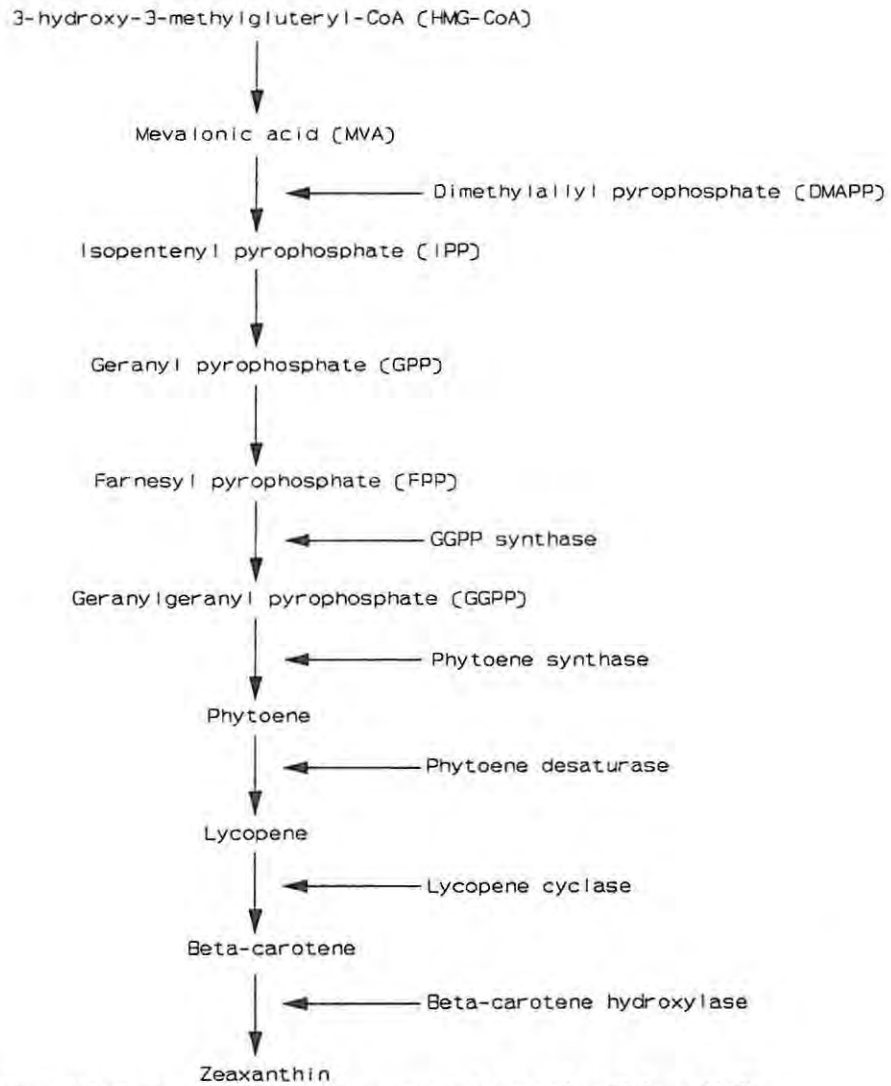


Figure 1.1. The biosynthetic pathway of isoprenoid compounds (adapted from Britton, 1988).

Whilst it is generally accepted that lycopene is the substrate for cyclization (Sandmann, 1991), the existence of ß-zeacarotene in maize and tomato mutants indicates that ß-carotene could be produced in higher plants via cyclization of neurosporene (Goodwin, 1979).

Where lycopene is the precursor, it is converted to γ-carotene and then to ß-carotene by two successive cyclization reactions. When neurosporene is the precursor, it is converted to ß-zeacarotene by cyclization and only after desaturation of ß-zeacarotene to γ-carotene, does the second cyclization reaction occur (Bramley, 1985), (see Figure 1.2.). To date, there is no evidence of direct conversion of ß-zeacarotene to ß-carotene (West, 1990).

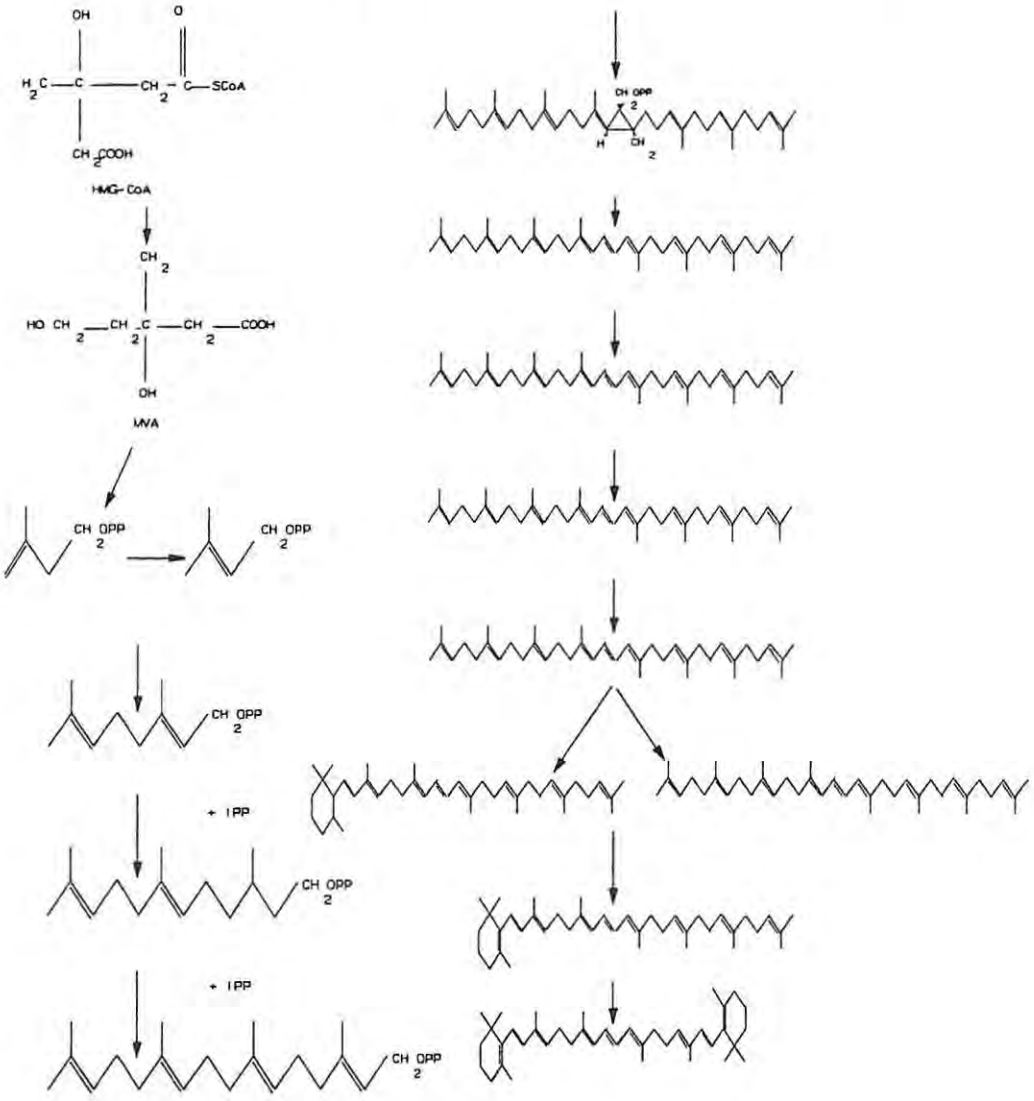


Figure 1.2. The ß-carotene biosynthetic pathway (adapted from Britton, 1988)

The oxygenated products of carotenes are called xanthophylls. Hydroxyl groups are introduced in position C-3 and C-3' of the ionone rings and epoxy groups are subsequently formed at the 5,6 and 5',6' positions. All oxygen groups originate from molecular oxygen (Goodwin, 1980b). The initial reaction, catalysed by ß-carotene hydroxylase, results in the formation of zeaxanthin via the intermediate ß-cryptoxanthin (see Figure 1.1.).

Much work has been done on the elucidation of the ß-carotene biosynthetic pathway in *D. salina* (Ben-Amotz, *et al.*, 1988). Research has focused on the effects of the herbicide norflurazon, which blocks the conversion of phytoene to phytofluene (Ben-Amotz, *et al.*, 1987; Shaish, *et al.*, 1990). These authors have shown that *D. salina* accumulates up to 8% dry weight as phytoene in the presence of norflurazon. As with ß-carotene, phytoene is accumulated in globules in the interthylakoid spaces. In addition, the isomeric ratio of phytoene is proportional to the integral irradiance to which cells are exposed during one division cycle. This observation has led to the suggestion that isomerization occurs early in the biosynthetic pathway, possibly at, or before, formation of phytoene (Ben-Amotz, *et al.*, 1988).

Because 15-*cis*-phytoene is the suggested precursor for all-*trans* carotenoids, including all-*trans*-ß-carotene, isomerization of the 15,15'-double bond must occur during desaturation or following phytoene synthesis. It is not clear at which stage the 15-*cis* intermediate is isomerized to all-*trans* phytoene, but in *Dunaliella* the conversions of 15-*cis* precursors to all-*trans* forms takes place after the phytoene stage (Shaish, *et al.*, 1990). The isomerization stage also appears to be organism specific. In *Flavobacterium* isomerization occurs at the formation of phytoene (Brown, *et al.*, 1975), in *Capsium* at the formation of phytofluene (Camara, *et al.*, 1982) and *Euglena* (Cunnigham and Schiff, 1985) and *Scenedesmus* (Britton and Powls, 1977) at the formation of ζ-carotene. Both the all-*trans* and the 9-*cis* isomer of phytoene are present in *D. bardawil* and the ratio is dependant upon the integral light intensity to which the algae are exposed during one division cycle (Ben-Amotz, *et al.*, 1988). Regulation of *cis* carotenoid formation, involvement of enzymes in isomerization and the stage of isomerization in ß-carotene biosynthesis remain obscure although recent evidence

suggests that phytoene is the branch point for formation of 9-*cis*-ß-carotene (Ebenezer and Pattenden, 1993).

1.3. The Regulation of Carotenoid Biosynthesis.

The regulation of carotenoid biosynthesis is a complex process which is often associated with important developmental processes such as fruit ripening, chloroplast and chromoplast development in higher plants, sexual mating in fungi and nutritional imbalance in algae (Bramley and Mackenzie, 1988). Natural carotenoid biosynthesis may be altered by changes in environmental conditions such as temperature, pH, oxygen, carbon and nitrogen source, and light (Bramley,1985).

Light is probably the most widely studied of all regulatory factors in carotenoid biosynthesis. Carotenogenesis is photoregulated in a wide range of organisms, particulary in fungi (Harding and Shropshire, 1980). Several different types of photoreceptor regulate carotenogenesis. In higher plants, chloroplast development leads to increased levels of carotenoids and phytochrome mediates carotenoid biosynthesis. In fungi, carotenoid biosynthesis is controlled by porphyrin. Photoinduced carotenogenesis can be divided into three phases: (1) reaction to the light source; (2) a period of protein synthesis; and (3) an accumulation of the carotenoid pigments (Jones and Porter, 1985). Recent evidence has shown that a chloroplast-encoded protein is responsible for repair to damaged photosynthetic equipment (Kim, *et al.*, 1993). Whatever the mechanism of photoinduction, the general observation is that synthesis of carotenoids is stimulated by, but is not dependant on, red light (Jones and Porter, 1985).

Several compounds have been reported to induce carotenogenesis (Bramley and Mackenzie, 1988). These include certain bio-inducers, such as trisporic acid, and related compounds such as abscisic acid and vitamin A which have been shown to stimulate carotenoid formation in a number of fungi (Dandekar, *et al.*, 1980). These compounds may interact with gene products or may act at both translational and post-translational levels (Bramley, 1985). A large number of organic compounds are also known to stimulate or inhibit carotenoid biosynthesis. They can be broadly divided into two categories: (1) those which cause

accumulation of saturated carotenoids, typically phytoene and phytofluene, and (2) those which inhibit cyclization, causing a build up of lycopene (Bramely, 1985).

Carotenoid biosynthesis is essentially genetically regulated. Studies using inhibitors of protein synthesis in bacteria have shown that the mechanism of carotene induction is dependent on transcription and translation (Howes and Batra, 1970). It is generally believed that the specific stages of carotenoid biosynthesis take place in a multi-enzyme complex which is membrane bound. The suborganellar location of carotenogenic enzymes is not clear as it is unknown whether they occur in the chloroplast envelope only, or in both the thylakoids and the envelope membranes (Kreuz, *et al.*, 1982).

Until recently, genes for the carotenoid biosynthetic enzymes had not been isolated. Within the past three years a number of genes coding for carotenoid biosynthetic enzymes have been isolated from bacteria, fungi, cyanobacteria, green algae, and higher plants (Ausich, 1994; Bartley and Scolnik, 1994), see Table 1.1.

Although little is known in general about the cyclase which catalyses the conversion of lycopene or neurosporene to ß-carotene, the cyclization of lycopene and the conversion of prolycopene into ß-carotene in *Narcissus* chromoplasts, appears to consist of two reactions; NADPH dependent isomerization to the all-*trans* form and then the cyclization reaction (Beyer, *et al.*, 1991).

The isolation of these genes will fuel the development of processes for the production of carotenoids from a number of different host organisms and a full range of carotenoid compounds from biological sources will be accessible.

Lers and co-workers, (1990) demonstrated a lag phase after light stress, before ß-carotene accumulation is accelerated, and that actinomycin D, chloramphenicol and cycloheximide inhibit this induction in *D. salina*. This suggests a role for gene activation in the process. A nuclear gene, *cbr* (carotene biosynthesis-related), has been shown to be transcriptionally activated in parallel to ß-carotene accumulation when *D. bardawil* is exposed to high light conditions. *Cbr* gene product is homologous to *Elips* (early light-induced proteins) identified

in pea and barley (Pötter and Kloppstech, 1993). Protein products of these genes also show similarity to the apoproteins of the light-harvesting complexes of photosystem I and II. It has therefore been proposed that *Cbr/Elip* genes may regulate pigment-binding thylakoid proteins and that these complexes are required during development, or recovery of the photosynthetic machinery from stress (Levy, *et al.*, 1992).

Table 1.1. Genes isolated from different organisms coding for carotenoid biosynthetic enzymes. (taken from Ausich, 1994)

Organism	GGDP synthase	Phytoene synthase	Phytoene desaturase	Zeta-carotene desaturase
Rhodobacter	crtE	crtB	crtI	
Erwinia:				
E.herbicola	crtE	crtB	crtI	
E.uredovora	crtE	crtB	crtI	
Neurospora	al-3	al-2	al-1	
Synechococcus		pys	pds	
Synechcystis			pds	
Anabena				zds
Dunaliella			pds Ref.2	
Tomato		pTOM5 pys Ref.1		
Soyabean		pys	pds Ref.3	
Pepper	GGDPS	pys	pds	
Maize		y1		
	Lycopene cyclase	**ß-carotene hydroxylase**	**Zeaxanthin glycosylase**	
Erwinia:				
E.herbicola	crtY	crtZ	crtX	
E.uredovora	crtY	crtZ	crtX	

References: 1. Bartley, *et al.*, 1991a; 2. Bartley, *et al.*, 1991b; Chamovitz and Hirschberg, 1991.

1.4. Carotenoids and Stress.

Close structural association between photosynthetic-membrane complexes and pigments means that photosynthesis and carotenoid content are not mutually exclusive. Exposure to stress results in photoinhibition, which can be defined as a phenomenon occurring when the

rate of transfer of excitation energy from light-harvesting antennae to photochemical reaction centres is in excess of the rate of transfer from the reaction centres to the electron transfer chain (Osmond, 1981). Whereas it has long been recognized that such a decreased photon efficiency may be the result of damaging effects to the photochemical apparatus, it has only recently been recognized that a decreased photon efficiency can also be the result of regulated processes leading to the dissipation of excess excitation energy within the photochemical apparatus (Demmig-Adams, 1990). Photoinhibition therefore leads to alterations in carotenoid content and composition. These include changes in isomeric composition (Ben-Amotz, *et al.*, 1989b) or carotenoid content, such as those occurring during the operation of the xanthophyll cycle or highly specific photobleaching of carotenoids (Young and Britton, 1990a).

Carotenoids and chlorophylls of photosystem I (PS I) and II (PS II) are located in the thylakoid membranes. Each photosystem consists of (1) a reaction centre and (2) an antenna complex which together comprise the light-harvesting complex (LHC). Reaction centres contain mainly ß-carotene, whereas light-harvesting complexes contain a number of xanthophylls, mainly lutein, neoxanthin and violaxanthin, and often small amounts of antheraxanthin and zeaxanthin (Clarke, *et al.*, 1982). Violaxanthin and zeaxanthin have also been reported in the chloroplast envelope (Siefermann-Harms, *et al.*, 1978).

Carotenoids associated with the antenna complexes absorb light in the 400-500 nm range, and energy is transferred from the carotenoid excited state to chlorophyll by singlet-singlet energy transfer (Krinsky, 1971). Light-harvesting ability of carotenoids allows for efficient utilization of light in the blue region. More importantly, in stress related conditions, carotenoids protect individual pigment-protein complexes and ultimately the chloroplast, against photo-oxidation (Young, *et al.*, 1989; Young, 1991). Gust and co-workers (1985) suggested that structural motion, or re-arrangement, of pigment-protein complexes could mediate triplet energy transfer from triplet excited chlorophyll molecules, hence curbing the production of destructive singlet oxygen species (Krinsky, 1978). Originally it was thought that only carotenoids with nine or more conjugated double bonds (for example ß-carotene or lutein) were able to quench triplet chlorophyll or singlet oxygen species efficiently, but Demmig and co-workers (1987) have implicated zeaxanthin accumulation as the major protective mechanism in response to photoinhibitory conditions.

1.4.1. Xanthophyll cycle operation and photoprotection.

Following Sapozhnikov and co-workers' report (1957) that the level of violaxanthin in leaves could be changed reversibly by light and dark treatments, there have been numerous studies to elucidate the mechanism of these changes, now commonly known as the xanthophyll cycle (reviewed by Hager, 1980).

This cycle consists of a de-epoxidation reaction in which violaxanthin is converted to zeaxanthin via antheraxanthin, and an epoxidation reaction which regenerates violaxanthin, again through antheraxanthin (see Figure 1.3.). The pathway is not in equilibrium because de-epoxidation and epoxidation mechanisms do not operate at the same rate (Yamamoto, 1979).

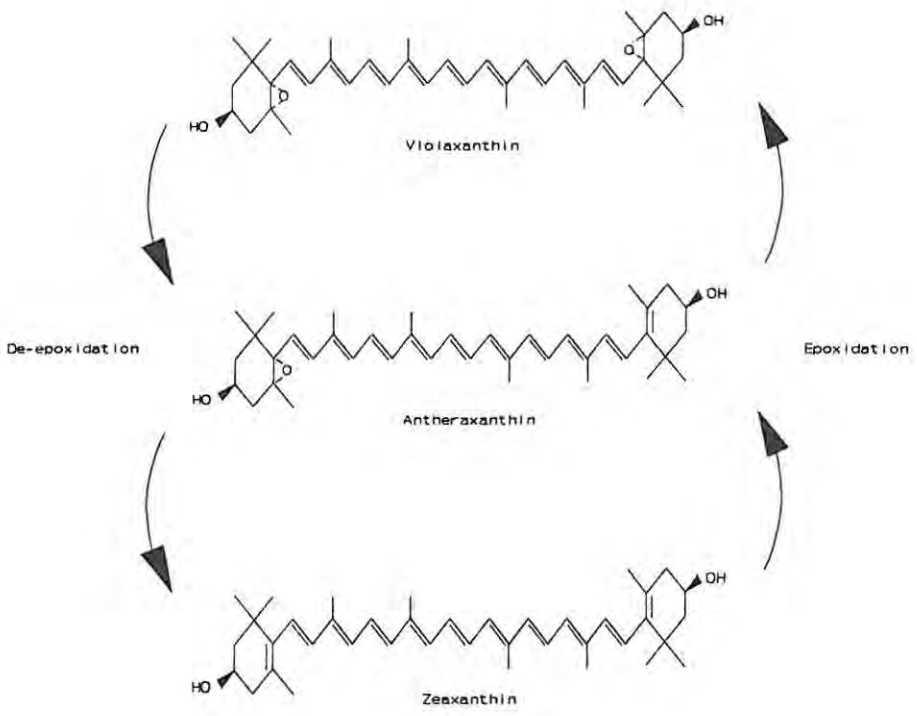


Figure 1.3. The xanthophyll cycle involving the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin and the epoxidation of zeaxanthin to violaxanthin via antheraxanthin (taken from Demmig-Adams, 1990).

The arrangement of the cycle in the thylakoid is transmembraneous with the de-epoxidation system situated on the lumen side and the epoxidation system on the stromal side.

Violaxanthin de-epoxidase has an optimum pH of 5.2 which means that the pH in the thylakoid compartment must decrease in order for conversion to take place. It has been established that light energy generates the acidic pH through the proton pump in the lumen and that it also induces a conformational change in the inner membrane surface which determines the amount of violaxanthin that is available for de-epoxidation (Yamamoto, 1979; Hager, 1980).

Numerous roles for the cycle have been proposed. Firstly, the cycle may improve the final ATP/NADPH ratio by quenching reducing agents at their site of formation (Hager, 1980). Secondly, a specific role for zeaxanthin or the de-epoxidated state of the cycle in the dissipation of excess energy within the photosynthetic apparatus has been proposed (Demmig, *et al.*, 1987, Demmig-Adams, *et al.*, 1988). Thirdly, correlations have been observed between zeaxanthin accumulation and non-photochemical quenching of chlorophyll fluorescence under photon flux densities which exceed the photosynthetic capacity of the chloroplast (Demmig-Adams, 1990; Bilger and Björkman, 1991). Such excessive fluxes are generated either by high light intensity or, under low light intensities, in conjunction with stress factors such as water deficit or nutrient starvation, which lower the efficiency of photosynthetic energy conversion (Gilmore and Yamamoto, 1991; Demmig-Adams and Adams, 1992). Zeaxanthin accumulation has therefore been proposed as a protective mechanism involved in the dissipation of excess light energy which would otherwise damage the photosynthetic machinery. A correlation between the level of zeaxanthin, other xanthophylls, and ß-carotene has been noted in higher plants (Demmig-Adams, 1990).

1.5. Abscisic Acid and Stress.

Chemical and stereochemical similarity between abscisic acid (ABA) and the carotenoid end-groups led to the suggestion that these compounds could be metabolically related (for reviews see Walton, 1980; Zeevaart and Creelman,1988; Parry and Horgan, 1990). ABA is a sesquiterpenoid, consisting of three IPP residues (Addicott, 1983), and is derived via the terpenoid pathway (Noddle and Robinson, 1969). There has, however, been much controversy surrounding the biosynthetic origin of ABA, as there are two main routes whereby MVA could be converted to ABA. A direct or C_{15} pathway involving the cyclisation of a C_{15} precursor such as FPP has been proposed, however, there is little evidence to

support this biosynthetic route. The second possibility is an indirect C_{40} pathway involving the cleavage of a carotenoid to yield ABA.

The biosynthesis to ABA is now considered to occur predominantly via the metabolism of epoxy-carotenoids (Creelman, 1989; Duckham, *et al.*, 1991; Parry and Horgan, 1991) and this pathway is shown in Figure 1.4.

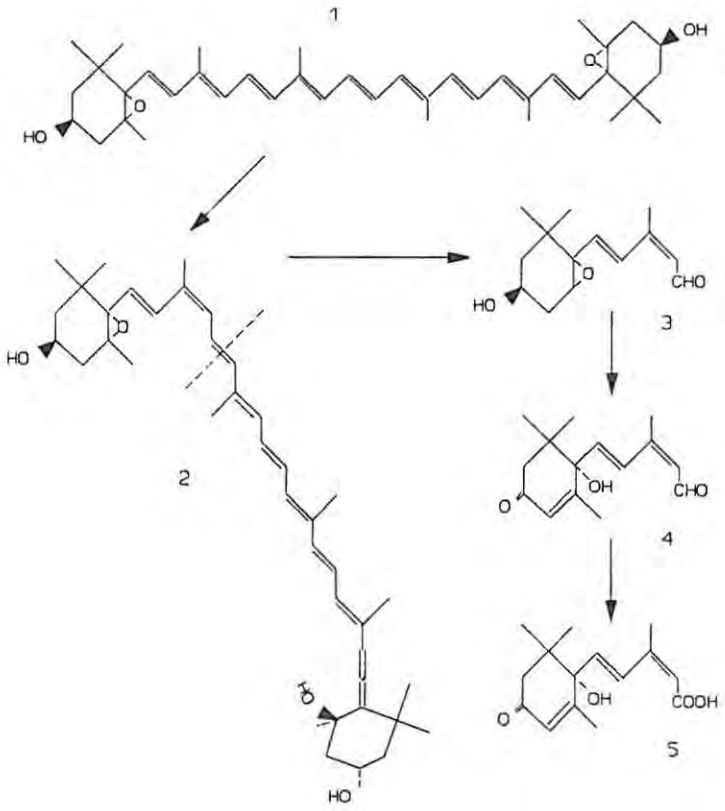


Figure 1.4. The postulated biosynthetic route to ABA in plants (1) all-*trans*-violaxanthin; (2) 9-*cis*-neoxanthin; (3) xanthoxin; (4) ABA-aldehyde and (5) ABA.

Evidence in support of this pathway includes $^{18}O_2$-labelling studies employing a wide range of tissues (Li and Walton, 1987; Gage, *et al.*, 1989), the use of inhibitors of carotenogenesis (Moore and Smith, 1985) and carotenoid-deficient mutants (Zeevaart and Creelman, 1988; Duckham, *et al.*, 1991; Rock and Zeevaart, 1991), and measurements of the levels of putative xanthophyll ABA precursors (Li and Walton, 1990). Together these studies have

revealed that, at least in higher plants, there is a universal pathway for the biosynthesis of ABA, involving the cleavage of specific xanthophylls (Parry, 1993).

Recent evidence has shown zeaxanthin to be a precursor of ABA (Duckham, *et al.*, 1991). It is well established that zeaxanthin is epoxidated to all-*trans*-violaxanthin during the operation of the xanthophyll cycle (Yamamoto, 1979). All-*trans*-violaxanthin is then converted enzymatically to either 9-*cis*-violaxanthin or 9-*cis*-neoxanthin. The enzymes responsible for this conversion are thought to exist in a dual-enzyme complex and are constitutively expressed (Parry, *et al.*, 1988). Xanthophyll interconversion, isomerisation and cleavage are all possible control sites of ABA synthesis, so an investigation of ABA biosynthesis at the carotenoid level is of obvious importance.

Both 9-*cis*-violaxanthin and 9-*cis*-neoxanthin have been considered to be ABA precursors. Levels of 9-*cis*-violaxanthin are, however, unaffected by stress in higher plants (Parry and Horgan, 1990). Cleavage of 9-*cis*-neoxanthin, thought to occur by a dioxygenase enzyme, gives rise to a neutral compound, xanthoxin (Parry, 1993). Theoretically, xanthoxin could be produced from several xanthophylls possessing a 3, (3')-hydroxy group and a 5,(5',') epoxide ring. In fact, violaxanthin, neoxanthin and antheraxanthin can all be chemically oxidised to xanthoxin (Taylor and Burden, 1973). However, the xanthoxin precursor would have to possess a *cis* configuration as *trans*-xanthoxin has been shown to be biologically inactive and is not converted to ABA (Addicott, 1983).

By using cell-free systems of various plants Sindhu and Walton (1988) demonstrated that xanthoxin was converted to ABA. It was found that xanthoxin oxidase catalyses the conversion of xanthoxin to ABA-aldehyde. ABA-aldehyde oxidase, then converts ABA-aldehyde to ABA (Rock, *et al.*, 1992).

The bulk of endogenous ABA is localized within the chloroplasts of plant cells (Heilmann, *et al.*, 1980) and it has been suggested that these organelles represent a major site of ABA biosynthesis (Milborrow, 1974). The inability to demonstrate biosynthesis of ABA from either MVA or IPP, using isolated chloroplasts (Hartung, *et al.*, 1981; Cowan and Railton,

1986), might reflect the tight link between photosynthetic carbon metabolism, plastid isoprenoid biosynthesis and ABA biosynthesis.

Until recently, it was thought that algae did not contain ABA but rather lunularic acid, a C_{15} compound with a structure similar to ABA (Owen and Napier, 1988). ABA has unequivocally been characterized as an endogenous compound in extracts of *Dunaliella* (Tietz, *et al.*, 1989; Hirsch, *et al.*, 1989) and shown to be biologically active in algal extracts (Rose, 1992). Furthermore, levels of this growth regulator increase when algae are exposed to salt stress (Hirsch, *et al.*, 1989; Cowan and Rose, 1991). The accumulation of ABA in *D. salina* under stress suggests a similar hormonal role for ABA in this organism to that in higher plants. However, accumulation under stress conditions is only one prerequisite for a role as a growth regulator. Another prerequisite is a physiological effect of ABA which is of benefit to the alga under stress (Hirsch, *et al.*, 1989; Evans and Trewavas, 1991). An investigation into the relationship between ABA production and ß-carotene accumulation in salt-stressed *D. salina* cells by Cowan and Rose (1991), demonstrated that two stages of accelerated ß-carotene production occur, with only the first characterized by enhanced levels of ABA. In addition, ABA and its acidic catabolites were preferentially partitioned into the culture medium and partitioning was shown to increase in response to hypersalinity. The preferential partitioning of ABA into the media suggests the existence of an extracelluar ABA receptor, similar to that found on the outside of the plasma membrane of stomatal guard cells (Hartung, 1983; Hornby and Weiler, 1984). Whilst attempts to demonstrate an effect of exogenously supplied ABA on the physiology of *Dunaliella* have been unsuccessful (Hirsch, *et al.*, 1989; Tietz, *et al.*, 1989; Cowan and Rose, 1991), a possible role for ABA as a regulator of carotenogenesis in algae should not be ignored. This is particularly so given recent information to suggest that receptors for ABA are located on the outside of the plasma-membrane of higher plants (Anderson, *et al.*, 1994; Gilroy and Jones, 1994).

1.6. Integration of Metabolic Responses to Stress.

Chapin (1991) has proposed that all plants respond to stress in essentially the same way. This observation has led to the hypothesis of a "centralized system of physiological response" to stress, that involve combined changes in nutrient, water, carbon and hormonal balances in plants.

D. salina responds to salinity stress by regulating the flux of carbon between starch production in the chloroplast and glycerol in the cytoplasm (Ben-Amotz and Avron, 1973, 1990; Ben-Amotz, 1980; Bental, *et al.*, 1990). Moreover, the accumulation of ß-carotene by cells exposed to a number of stresses, indicates that carbon flux is not restricted to partition between starch and glycerol, but that flow of carbon can also be directed through the isoprenoid pathway. Based on published information on the metabolic responses of *D. salina* to stress, we constructed a biochemical and physiological framework in an attempt to integrate these responses to stress (Cowan, *et al.*, 1992).

The proposed model, which is hormone dependant, is illustrated in Figure 1.5. Based on the "stretch-activated ion channels model" (Kirst, 1990). This model suggests the following cascade of events: 1) volume changes leading to distortion of the plasma-membrane; 2) inhibition of H^+-ATPase activity; 3) change in compartmental pH; 4) increased ABA concentration in the cytoplasm; 5) opening of Ca^{2+}-channels; 6) Influx of Ca^{2+} and ABA efflux; 7) effect on metabolism through altered activity of Ca^{2+}-mediated enzymes.

The involvement of ABA in the accumulation of ß-carotene is thought to be triggered at the membrane level (Cowan, *et al.*, 1992). It has been said that the single most important factor in the recognition of stress stimuli is a change in the chemical potential of water (Kramer, 1988). In salt-stressed cells, the cascade of events is triggered by volume changes induced by alterations to the chemical potential of water. Similarly, nutrient stress may cause a substantial reduction in water uptake and loss, which in turn alters the ionic status of cells (Ben-Amotz and Avron, 1973; Beckett, et al., 1985). In contrast, the effect of high light intensity on photosynthesis appears to be direct, resulting in the accumulation of inactive PS II units and chlorophyll depletion (Powles, 1984). High light intensity induces cell swelling. This is apparently a general response of this wall-less alga, although original volume is resumed within 24 hours (Shaish, *et al.*, 1993).

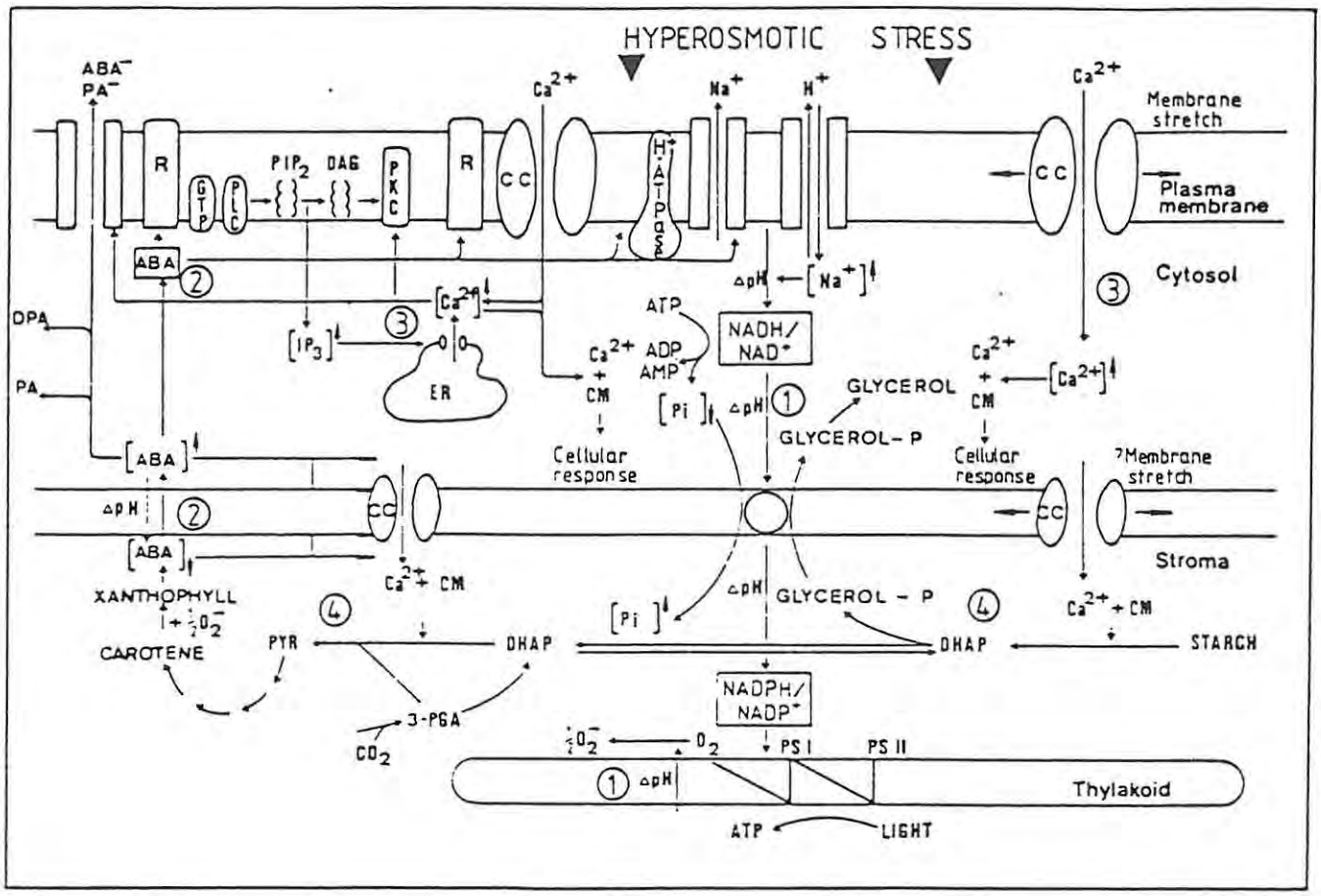


Figure 1.5. Simplified hypothetical model depicting the integration of putative second messenger systems in hyperosmotically stressed cells of *D. salina*. 1) Membrane distortion/volume changes; ΔpH transduced from cytosol to chloroplast by redox gradient through triose phosphate-Pi translocator; increase in $[Pi]_{chlp}$ in exchange for glycerol-phosphate; production of glycerol in cytoplasm. (2) ΔpH-catalysed redistribution of ABA; interaction of ABA with membrane receptor proteins or membrane lipids; inhibition of H^+- ATPase; stimulation of Na^{2+} efflux and activation of Na^+/H^+ antiporter; maintenance of ΔpH. (3) Increase in $[Ca^{2+}]_{cyt}$; influx and release of Ca^{2+} from intracellular stores through interaction with phospholipid metabolism. (4) promotion of metabolic responses through Ca^{2+}-calmodulin and protein kinase activity; increased breakdown of starch in chloroplast and synthesis of glycerol in cytoplasm; stimulation of ABA metabolism and/or increased de-epoxidation of xanthophyll cycle and accumulation of ß-carotene. Abbreviations: cc, calcium channel; CM, calmodulin; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; DPA, dihydrophaseic acid; ER, endoplasmic reticulum; GTP, guanosine triphosphate binding protein; IP_3, inositol 1,4,5-triphosphate; PA, phaseic acid PGA, phosphoglycerate; Pi, inorganic phosphate; PIP_2, phosphatidylinositol 4,5-*bis*phosphate; PKC, protein kinase C; PLC, phospholipase C: PS, photosystem; R, receptor. (From Cowan, *et al.*, 1991).

Changes in cell volume alter the internal pH of the cell and organelles and hence the partition coefficient of plastid-localized ABA (Pick, 1992). Thus alkalization might result in enhanced ABA biosynthesis *per se*, whereas acidification would bring about ABA redistribution followed by enhanced synthesis in order to restore the ΔpH of the chloroplast. The

interaction of ABA with membrane components might function to elevate intracellular Ca^{2+} levels in order to alter protein phosphorylation patterns particularly since volume changes occur coincidently with alterations in compartmental inorganic phosphate concentration (Bental, *et al.*, 1990).

Changes in pH within the chloroplast lead to the stress-induced uncoupling of photosynthesis (Gilmour, *et al.*, 1985) and hence enhanced xanthophyll cycle turnover (Demmig-Adams, 1990). The accumulation of zeaxanthin, through operation of the xanthophyll cycle, has a photoprotective function; it dissipates excess excitation energy trapped within the photosynthetic pigment bed when conditions become such that more light is being absorbed than can be utilized in photosynthesis (Demmig-Adams, 1990; Adams and Demmig-Adams, 1992). Diurnal synthesis and removal of zeaxanthin has been noted in plants exposed to changing light intensities or in plants which are exposed to unfavourable conditions (Demmig-Adams, *et al.*, 1989; Adams and Demmig-Adams, 1991). This phenomenon is now accepted as a general response exhibited by plants (Foyer, *et al.*, 1989; Demmig-Adams, 1990; Gamon, *et al.*, 1990).

The xanthophyll cycle has also been identified in green algae, although some anomalies do exist. These include organisms which possess an epoxide cycle involving lutein and lutein 5,-epoxide instead of violaxanthin, antheraxanthin and zeaxanthin (Antia and Cheng, 1983; Senger, *et al.*, 1993). Since ß-carotene is metabolically related to zeaxanthin, it is anticipated that accumulation of ß-carotene is due to reduced catabolism of zeaxanthin or increased de-epoxidation of violaxanthin to zeaxanthin via the xanthophyll cycle.

Evidence that ABA is derived from oxygenated carotenoids is now almost conclusive (Parry, 1993). In this regard, the demonstration that mutants of *Arabidopsis thaliana* are deficient in violaxanthin and also lack ABA, is highly significant (Rock and Zeevaart, 1991). These authors have demonstrated that zeaxanthin is, in all probability, a precursor of ABA. Zeaxanthin is derived from ß-carotene via ß-cryptoxanthin in a Cyt P_{450} mixed function oxidase catalysed reaction (Sandmann, 1991), and is the major component of the xanthophyll cycle in plants and green algae exposed to high light conditions. It is therefore not

unreasonable to suggest that both the production of ABA and the operation of the xanthophyll cycle might impact on the ability of an organism to accumulate ß-carotene.

Hirsch, *et al.* (1989), intimated that in order to demonstrate a functional role for ABA in algal metabolism the following requirements should be met:

1. endogenous levels of ABA must be regulated by both the rate of synthesis and the rate of catabolism or by the degradation of the hormone; and

2. variations in endogenous levels of ABA should be correlated with one or more physiological responses which allow the cell to tolerate prevailing stress.

The intracellular accumulation of ABA and release of ABA to the medium and the identification of ABA degradation products has been demonstrated in cells of *Dunaliella* exposed to salinity stress (Hirsch, *et al.*, 1989; Tietz, *et al.*, 1989; Cowan and Rose, 1991). In addition, an interrelationship between ABA production and ß-carotene accumulation in *D. salina* exposed to salinity stress has been determined (Cowan and Rose, 1991). Furthermore, the ability of *D. salina* to accumulate up to 14% of its dry weight as ß-carotene when exposed to a variety of stresses allows this organism to survive in conditions few other organisms could tolerate. (Borowitzka, *et al.*, 1986).

1.7. Biotechnology of *Dunaliella*.

D. salina has been shown to be one of the most suitable and successful microalga for mass cultivation identified to date (Ben-Amotz and Avron, 1989). Large scale cultivation of *D. salina* is based on autotrophic growth in media containing inorganic nutrients (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989). This alga can utilize either carbon dioxide or bicarbonate as its carbon source. The use of carbon dioxide sparging, which is one of the methods used in controlled mass culture, has a two-fold advantage; the maintenance of pH and the supplementation of dissolved inorganic carbon (Ben-Amotz and Avron, 1990).

Two cultivation systems are currently employed for ß-carotene production from *D. salina*, namely intensive and extensive cultivation. The intensive method involves open raceway

ponds, defined culture media and controlled conditions (Ben-Amotz and Avron, 1989; Borowitzka and Borowitzka, 1990) whereas the extensive method exploits natural populations of *D. salina* in earthen, wind-mixed ponds (Borowitzka, 1991; Schlipalius, 1991).

The basic dilemma underlying commercial ß-carotene production from *D. salina* is the conflicting requirements for biomass production and ß-carotene accumulation. In current *Dunaliella* commercial systems this problem is addressed by cultivating the alga under conditions both sub-optimal for growth and for ß-carotene accumulation, but optimised for the best overall ß-carotene yield per unit time (Borowitzka and Borowitzka, 1988). This approach termed the "single-stage" or "averaging" production process (Phillips, 1993), does not exploit the full genetic potential of the organism and leads to sub-optimum ß-carotene yields.

An alternative approach to the averaging system is the dual stage system, where the conflicting requirements for biomass production and ß-carotene accumulation observed in *D. salina* are accommodated in separate units; growth ponds in which conditions are optimized for maximum growth and stress ponds in which conditions are optimized for maximum ß-carotene production (see Figure 1.6.). Dual-stage processes using *D. salina* have been proposed for glycerol production (Avron and Ben-Amotz, 1978; Chen and Chi, 1981), the production of ß-carotene (Borowitzka, *et al.*, 1984; Rose and Cowan, 1991a) and for the simultaneous recovery of both ß-carotene and glycerol from effluent-grown algae (Rose and Cowan, 1991b).

The separation of growth and metabolite production stages is not novel in biotechnology and a number of bacterial and fungal secondary metabolite fermentation processes employ dual-stage production processes (Atkinson and Mavituna, 1991). Until recently no large scale evaluation of dual-stage processes have been undertaken.

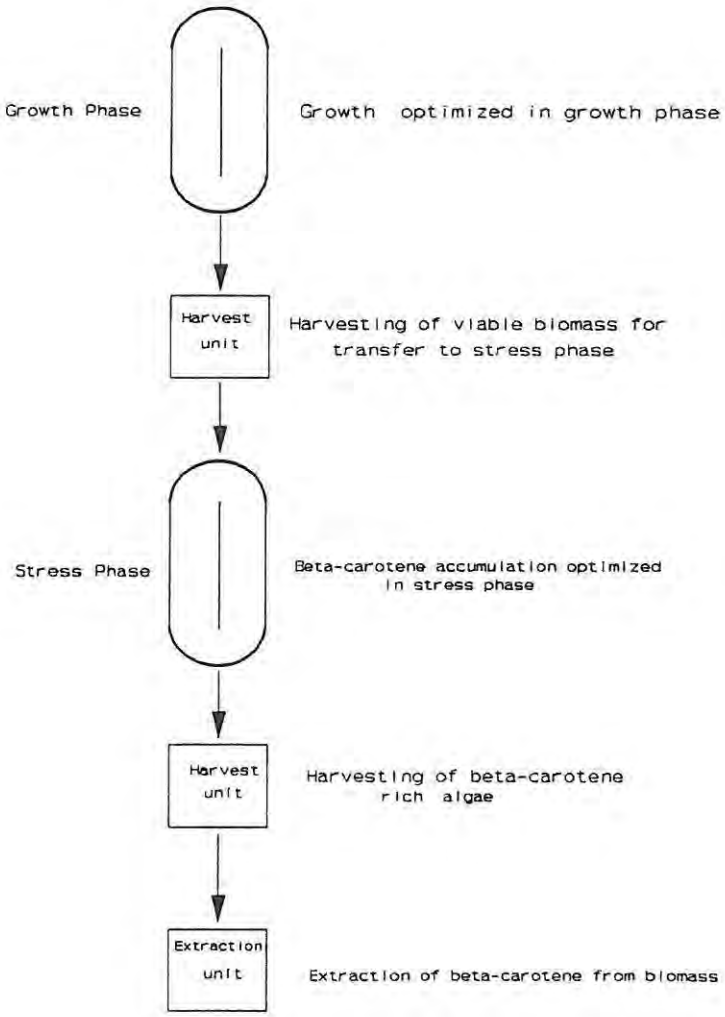


Figure 1.6. Flow chart of a hypothetical dual-stage ß-carotene production system.

Borowitzka and Borowitzka (1988) maintain that a dual-stage ß-carotene production process has inherent drawbacks. These include extra labour costs, increased retention time and the potential threat from invader species which would be encountered because of the relatively high nitrate and low salinities in the growth stage of this system.

A collaborative research program between Rhodes University and SASTECH (Pty) Ltd., the research and development section of SASOL Chemical Industries Ltd. was therefore

undertaken to evaluate the potential of the dual-stage process. It was found that due to the exceptional productivity of the dual-stage system, substantially higher ß-carotene yields are obtained which significantly reduces ß-carotene production costs (Phillips, 1993). As a direct result of this research program, a pilot plant facility situated in Upington, South Africa was designed and built to investigate the up-scale potential of this system. Due to the ideal climatic conditions experienced in Upington both biomass and ß-carotene productivity are higher than anticipated. The pilot plant thus far appears to be very successful and will hopefully lead to the construction of an industrial scale plant in the near future.

For SASOL to maintain a competitive advantage over other algal ß-carotene producers, research aimed at understanding stress induced ß-carotene accumulation in the organism is essential. A critical step in the dual-stage process involves the accelerated accumulation of ß-carotene in biomass generated during the growth phase of the system. Whilst it is well known that both environmental and biotic and abiotic stress factors induce ß-carotene accumulation, a greater understanding of the physiological response of the organism to stress may allow for physiological manipulation of the organism which will ultimately lead to the improvement of the stress stage of the dual-stage process.

1.8. Research Hypothesis.

There is a linkage between stress induced ß-carotene accumulation, xanthophyll cycle operation and ABA production in the alga *D. salina*.

Stress-induced production of ABA functions in the mediation of the stress response of this alga, and this together with alterations in xanthophyll turnover contribute to the maintenance of ß-carotene productivity in stressed cells of *D. salina*.

1.9. Research Objectives.

The objectives of this study are therefore the following:

1. To determine the influence of stress on basal physiological processes and to define these responses in both mass culture and laboratory cultured cells. Optimisation of the stress

conditions which induce ß-carotene accumulation in this alga would therefore be possible in large scale processes.

2. To determine the interrelationship between ABA production and ß-carotene accumulation in order to determine a role for ABA as a regulator of carotenogenesis in *D. salina*.

3. To evaluate xanthophyll cycle turnover as a potential regulatory mechanism contributing to ß-carotene accumulation in *D. salina* cells exposed to stress.

4. To elucidate the metabolic interrelationship between ABA, xanthophylls and ß-carotene in *D. salina*.

CHAPTER 2

Materials and Methods.

2.1. Materials.

2.1.1. Instrumentation.

The following instrumentation was used throughout the study: spectroquant model SQ 118 (Merck), Beckman LS 315 OT scintillation counter, a new improved Neubauer haemocytometer, a Coulter Electronics Multisizer II with a 70 μm orifice tube, Skye four channel light sensor Model SDL2580, DESAGA TLC spreader, Sorvall RC-5 Superspeed refrigerated centrifuge, Spectra-Physics HPLC consisting of a LINEAR UVIS 200 variable wavelength detector coupled to a SP 4290 integrator, Hewlett Packard HPLC, HP 1090 coupled to a photo-diode array detector (the system was driven by Chemstation Software), Beckman System Gold HPLC, consisting of a 126 programmable solvent module coupled to a 168 photo-diode array detector (the system was driven by Beckman Gold system driver software), Perkin-Elmer 8310 gas chromatogram, Hewlett-Packard 5890 gas chromatogram fitted with a flame ionization detector, Hewlett-Packard 5890 instrument coupled to an HP 5988A MS, Shimadzu UV-160A spectrophotometer, Hansatech oxygen microprobe.

2.1.2. General materials.

The following materials were used: in the constant environment growth chambers, cool white fluorescent tubes (Philips), incandescent bulbs (40 W) and high pressure sodium lamp (400 W) (Lascon Lighting, RSA); for preparation of the HPLC samples, 0.45μm cellulose acetate membrane filters (Millipore), sep-pak C_{18} cartridges (Waters Chromatography Division, Millipore Corp.), O.2 μm nylon centrifuge filters (Separations) and Whatman No 1. filter papers (Whatman, Laboratory Division, England); in chromatography, for TLC - silica gel

GF_{254} (Merck), for HPLC - Nucleosil 5 μm C_{18} (250 x 4.6 mm i.d.) (Macherey-Nagel) Düren, West Germany, Bondaclone 10 μm C_{18} (3.9 x 150 mm i.d.) column obtained from Phenomenex, Torrance, CA, for GC - a fused-silica capillary column (12 m x 0.32 mm i.d.) (Hewlett Packard) and a capillary column (15 mm x 0.53 mm i.d.) (SUPELCO, Bellefonte, CA, USA) and in testing the nitrate concentration of the medium, Merck Spectroquant Nitrate test cells (Cat. No. 14556).

2.1.3. Specialized chemicals.

The following speciality chemicals were used during this study; (±) *cis-trans* abscisic acid (ABA) (Sigma), ABAMe marketed as (±) *cis-trans* ABA (Sigma), *trans*- ABA (Sigma), all-*trans*-ß-carotene (Sigma), ABA-Methyl ester (Sigma), formaldehyde (C.J. Chem), polyaluminum chloride flocculant (Floccotan), 2,6-Di-*tert*-butyl-*p*-cresol (Unilab, Saarchem, RSA), aqueous scintillant (Hewlett Packard 299) and N-methyl-N'-nitro-nitrosoguanidine (Sigma).

2.1.4. Radiochemicals.

$NaH^{14}CO_3$ (1.91 x 10^{-3} TBq.mmol^{-1}) and (R,S)-[G-^{3}H]- ABA (specific activity, 4.26 TBq.mmol^{-1}) marketed as DL-*cis*, *trans*- [G-^{3}H]-ABA (Amersham International, Amersham, UK).

2.1.5. Solvents for HPLC.

All solvents used in this study were HPLC grade, they included; ethyl acetate (carbonyl free), dichloromethane, diethyl ether (Baxter Healthcare Corp., McGraw Park, Illinois, USA); acetonitrile and methanol (Bio-lab Laboratories Ltd. Israel).

2.2. Algal Cultivation.

2.2.1. *Dunaliella* culture.

Dunaliella salina (Teodoresco) var *bardawil* was purchased from the Culture Collection for Algae and Protozoa, Oban, United Kingdom (CCAP 19/30). This strain was isolated near Bardawil lagoon, North Sinai in 1976. The organism has been patented (US Patent # 4,199,895) and deposited with the American Type Culture Collection (ATCC 30861) as *Dunaliella bardawil*. Borowitzka and Borowitzka (1988) maintain that *D. bardawil* is a *nomen nudum* and is actually a strain of *D. salina* Teod. All experiments reported in the present study involve the use of pure cultures, and all references to *D.salina* pertain to *D. salina* var. *bardawil*.

2.2.2. Laboratory cultures.

2.2.2.1. Culture medium.

The culture media constituents were dissolved separately in water, combined and then diluted to the desired concentration (Table 2.1.). The medium was then filtered through 0.45 μm cellulose acetate membrane filters.

Table 2.1. Constituents of Growth Medium (adapted from Ben-Amotz and Avron, 1983).

NaCl	1.5M	$MnCl_2$	7μM
$NaHCO_3$	50mM	EDTA	6μM
KNO_3	5mM	$FeCl_2$	1.50μM
$MgSO_4$	5mM	$ZnCl_2$	0.80μM
$CaCl_2$	0.30mM	$CoCl_2$	0.02μM
KH_2PO_4	0.20mM	$CuCl_2$	0.20μM
H_3Bo_4	185μM		

Where a particular medium has been referred to as stress medium details of this media are given in the appropriate chapters.

2.2.2.2. Growth conditions.

Algal cultures were incubated in a constant environment chamber at 27 °C under continuous illumination (90 μmol.m.$^{-2}$s.$^{-1}$), provided by a bank of four fluorescent tubes and supplemented with far red light from incandescent bulbs. Cultures were grown in glass vessels which were continuously agitated on orbital shakers. Control experiments were performed using these conditions.

2.2.2.3. Indoor separation of algal biomass from growth medium for transfer to stress conditions.

Algal biomass was separated from the growth medium by centrifugation at 2000 g for 10 minutes in a cold centrifuge (4 °C) (Sorvall RC-5). The algal pellet was then washed three times with 1.5 M KCl and resuspended in the specified stress medium.

2.2.2.4. Stress induction studies.

Log phase cultures at approximately 30-40 x 10^4 cells.mL^{-1}, were harvested by centrifugation (2000 g for 10 minutes) and resuspended in the specified stress medium. Stress cultures were cultured under the same conditions as outlined in section 2.2.2.2.

Where the effect of high light intensity was investigated, algae were resuspended in growth medium (unless otherwise stated) and exposed to a light intensity of 1000 μmol.m^{-2}.s^{-1}.

2.2.2.5. Cell volume changes.

Log phase cultures were harvested by centrifugation, and resuspended in growth medium (control), salt, nitrate and multiple stress medium. For cells subjected to high light and multiple stress, cells were exposed to a light intensity of 1000 μmol.m^{-2}.s^{-1}. Samples were

taken from the cultures at the designated time points and cell volume was immediately measured in a Coulter Multisizer II, equipped with a 70-μm orifice. The electrolyte used in the coulter counter corresponded to the NaCl molarity of the stress medium in which the cells were cultivated.

Application of Radiochemicals, ABA and Related Compounds.

2.2.2.6. Continuous labelling with ^{14}C.

D. salina cultures (1.2 L) were grown from early log phase in growth medium containing 10 mCi $NaH^{14}CO_3$ (0.33% enrichment) until a cell density of 40 x 10^4 cells.mL^{-1} was obtained. Cultures were then divided equally. The resulting cultures were incubated at 29 °C under 90 μmol.m^{-2}.s^{-1} (control) and 1000 μmol.m^{-2}.s^{-1} respectively. Samples were taken at the designated time intervals, extracted into acetone and stored at -20 °C. After extraction and purification (see section 2.3.8.), samples were fractionated using a fraction collector attached to a Beckman Gold HPLC in order to separate and quantify the specific radiolabelled compounds. Aqueous scintillant was added (5 mL) to each fraction and the radioactivity was counted on a Beckman LS 315 OT scintillation counter.

2.2.2.7. Pulse-chase study using $NaH^{14}CO_3$.

D. salina cultures (4 L) of approximately 50-60 x 10^4 cells.mL^{-1} were harvested by centrifugation (2000 g) and resuspended in 400 mL of growth medium containing 1.5 mCi $NaH^{14}CO_3$. This enriched culture was incubated for 24 hours under low light (90 μmol.m^{-2}.s^{-1}). Cells were then harvested again by centrifugation to remove the unutilized ^{14}C, washed twice with 1.5 M KCl and resuspended in growth medium. Cultures were allowed to acclimate for $\pm$ 1 hour, before being divided equally. The resulting cultures were incubated at 28 °C under continuous illumination; 90 μmol.m^{-2}.s^{-1} (control) and 1000 μmol.m^{-2}.s^{-1} (high light stress) respectively. Samples were taken at designated time intervals, extracted into acetone and stored at -20 °C. After extraction and purification (see section 2.3.8.), samples were fractionated using a fraction collector attached to an HPLC. Aqueous scintillant was

added (5 mL) to each fraction and the radioactivity was counted on a Beckman LS 315 OT scintillation counter.

2.2.2.8. Application of exogenous ABA and related compounds.

Log phase cultures were harvested by centrifugation (2000 g) and resuspended in growth medium. Cultures were then aliquoted (20 mL) into 50 mL erlenmyer flasks and ABA or related compounds were added to achieve a required concentration of 50 μM or 100 μM. Intracellular ß-carotene levels were measured spectrophotometrically (see section 2.3.8.1.) in acetone extracts after the culture had been exposed to the ABA for 24 hours.

2.2.3. Outdoor cultures.

2.2.3.1. Location and climatic conditions of the experimental site.

Experimental work was carried out during mid-to-late summer at SASTECH (Ltd), Sasolburg, South Africa. This site is situated 27° 50 S and 26° 48 E at an altitude of 1500m above sea level. Typical climatic conditions for this seasonal period as supplied by the South African Weather Bureau are as follows; average minimum temperature - 14.8 °C; average maximum temperature - 27.3 °C; average hours of cloudless sunshine per day - 8.2.

2.2.3.2. Culture medium.

Culture medium used outdoors was similar to that used in laboratory studies (see Table 2.1.) except that salinity was increased from 1.5 M to 2 M. This prevented contamination with the non ß-carotene accumulating species *D. viridis* and *D. minuta* which have lower salinity optima than *D. salina* (Borowitzka and Borowitzka, 1988). The medium was prepared in 200 L containers and constituents of the medium were dissolved with a drum pump.

2.2.3.3. Culture conditions.

The algae were cultured in 2 m^2 circular fibreglass ponds with a capacity of 240 L. Mixing was achieved using a central stainless steel blade (length 30 cm), driven by an electric motor facilitating a rotation speed of 62 rpm. Turbulence was created by four 20 cm baffles situated on the pond wall. These ponds were inoculated with 5 L log phase laboratory cultures. Upon inoculation, ponds were covered with shade-cloth to protect the low light adapted cultures. The shade-cloth was removed after 24 hours.

Cultures were maintained at an average areal density of 45 g dry wt.m^{-2} (log phase). This was achieved by operating the cultures as continuous cultures by daily harvesting of a specific volume of the culture and replacing it with an equal volume of fresh medium. Before daily measurements were taken, evaporative losses were made up with water. Culture density was determined using a Coulter Multisizer (see section 2.3.1.).

2.2.3.4. Outdoor harvesting for transfer of algal biomass to stress conditions.

Algal biomass produced in the growth ponds was harvested by flocculation using a polyaluminum chloride flocculant at pH 8. Flotation of the flocculated biomass was induced by sparging with carbon dioxide through a 30 cm long sintered stainless steel tube positioned at the bottom of the flocculation vessel. The algal float was recovered and the separated biomass was immediately transferred to the stress ponds. The presence of the flocculant has previously been shown to have no effect on *D. salina* growth and ß-carotene accumulation (Phillips, 1993).

2.2.3.5. Outdoor stress induction.

Cells were separated from the culture medium (see section 2.2.3.4.) and resuspended at a culture density of 0.4 g dry wt.L^{-1} (40 x 10^4 cells.mL^{-1}) in stress media. Stress cultures were grown in 0.2 m^2 circular perspex ponds operated at a depth of 12 cm. A multi-stirring unit, driven by a single motor, was used to simultaneously mix four stress ponds.

2.2.3.6. Diurnal study.

D. salina was cultured at a density of 40 x 10^4 cells.mL^{-1} outdoors in 200 L miniponds and the medium maintained at a depth of 12 cm. Non-stressed cultures were cultured in growth medium (as described in Table 2.1.) and stressed cultures were grown in media containing 3 M NaCl, 50 mM $NaHCO_3$, 0.2 mM KNO_3 (multiple stress medium). Cultures were continuously stirred as described. in section 2.2.3.3. above.

All experiments were carried out on the same day and at each assay interval, aliquots from both cultures were removed for determination of pigment composition (see section 2.4. for more details). Incident irradiation was measured using a Skye SDL 2580 four channel light sensor, photosynthetic oxygen evolution was determined using a Hansatech oxygen electrode and cell number was measured using a Coulter Multisizer (see section 2.3.).

2.2.3.7. Determination of optimal starting culture density for ß-carotene production.

Biomass produced in the growth ponds was harvested at a culture denisty of 40 x 10^4 cells.ml^{-1} and resuspended into fresh growth media at the following culture densities: 10, 20, 30, 40, 80 x 10^4 cells.ml^{-1}.

2.3. General Methods

2.3.1. Cell counts.

Cells counts were performed using either an improved Neubauer haemocytometer or a Coulter Electronics Multisizer II with a 70 μm orifice tube. Results are the mean of three readings.

2.3.2. Light intensity measurement.

Light intensity (photon flux density) expressed as μmol.m^{-2}.s^{-1} was measured with a Skye four channel light sensor Model SDL2580.

2.3.3. Generation of ethereal diazomethane.

In order to methylate compounds for detection by GC, it was necessary to generate ethereal diazomethane. Ethereal diazomethane was generated at room temperature (25 °C) without co-distillation, by the hydrolysis of N-methyl-N'-nitro-nitrosoguanidine with 5 N NaOH in a Wheaton Diazomethane Generator (Pierce Chemical Company, Rockford, Illinois, USA). N-methyl-N'-nitro-nitrosoguanidine (133 mg) and 0.5 mL distilled water were placed in the inside tube of the generator. Diethyl ether (3 mL) was placed in the outer jacket. The two parts were then assembled and held in place by a pinch-type clamp. NaOH (0.6 mL of 5 N) was injected through the teflon rubber septum, and the reaction was allowed to proceed on ice until a deep yellow colour signifying a high yield of ethereal diazomethane was obtained.

2.3.4. Preparation of ABA-diol.

A known quantity (± 5 mg) of authentic (±) *cis-trans* ABA was dissolved in 1 mL methanol/water 2:1 (v/v). Sufficient $NaBH_4$ (± 2 g) was added and the reaction was allowed to proceed on ice for approximately 2 hours. Methanol was removed under nitrogen and the remaining aqueous phase was partitioned three times with an equal volume of ethyl acetate. The sample was then dried under nitrogen. *Cis-* and *trans*-ABA-diol were separated by thin layer chromatography.

Thin layer chromatography (TLC).

TLC plates (0.25 mm) of silica gel GF_{254} (Merck) were prepared by adding 60 mL water to 30 g of the silica gel. Plates were then prepared using a DESAGA TLC spreader. The ABA-diol isomers were separated by developing the plates twice to 15 cm using the solvent system toluene/ethyl acetate/acetic acid (50:30:4 v/v/v). Based on their published R_f values as described by Tietz (1985), the isomers were tentatively identified and corresponding ABA zones were scrapped off and eluted from the silica gel with ethyl acetate. The eluates were then dried under nitrogen and stored at -20 °C.

2.3.5. Determination of relative oxygen evolution.

Log phase cultures (30-40 x 10^4 cells.mL^{-1}) were separated from growth medium and transferred to stress medium. Samples (1.5 mL) were taken at 0, 4, 8, 24, 48, 72, 96 hours after transfer to stress conditions, and immediately placed in a Hansatech oxygen microprobe illuminated at a light intensity of 90 μmol.m^{-2}.s^{-1} using optic fibres. Rate of oxygen evolution using a galvanometric oxygen probe, was monitored once a linear response was observed. The photosynthetic oxygen evolution rate (PO_2) was expressed as relative oxygen evolution rate per mg chlorophyll per hour.

2.3.6. Nitrate determination.

Nitrate concentration of the culture medium was determined with Merck Spectroquant Nitrate test cells for the determination of NO_3^- in high salinity solutions. Samples were pre-filtered through 0.45 μm cellulose acetate filters to remove cells and cell debris.

2.3.7. Determination of carbon fixation rate.

Log phase cultures of *D. salina* were separated from the culture medium by centrifugation (2000 g), washed three times with 1.5 M KCl and split into two. The resulting cultures were resuspended in growth and multiple stress media respectively. Cultures were placed into 1 L beakers at a depth of 6 cm. Representative samples (40 mL) of these cultures were taken daily and placed in narrow 100 mL beakers at a depth of 6 cm. Radiolabelled $NaH[^{14}CO]_3$ (1 μL) was added to each representative sample and 1 mL aliquots were taken at the various time intervals. The aliquots were placed in a solution containing 0.5 mL (20% v/v) formaldehyde and 1 mL HCl , and were incubated at 20 °C overnight. Aqueous scintillant was added (7 mL) and radioactivity was determined using a Beckman LS 315 OT scintillation counter. The amount of carbon fixed was determined according to Gocke and Hoppe (1977).

2.4. Extraction, Purification and Analysis of Compounds of Interest.

D.salina cells (10 mL) were separated from the medium by centrifugation at 2000 g for 10 minutes in a cold centrifuge (Sorvall RC-5). The pelleted cells were then extracted for compounds of interest.

2.4.1. Spectrophotometric analysis of ß-carotene and chlorophyll.

Chlorophyll and ß-carotene were extracted from the algal pellets with 100% acetone, diluted to 80% acetone with distilled water (v/v) and assayed according to Arnon (1949) and Jensen (1978). $E^{1\%}_{1cm}$ of 2273 at 480 nm and $E^{1\%}_{1cm}$ of 890 at 666 nm were used to calculate ß-carotene and chlorophyll concentrations respectively.

2.4.2. High performance liquid chromatography (HPLC) analysis of compounds of interest.

Following separation from the culture medium, pigments and abscisic acid were extracted from the algal pellets by repeated sonication and centrifugation in 100% ice-cold acetone containing 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHL; 20 $mg.l^{-1}$), added as an antioxidant. These acetone extracts were then divided for pigment and ABA purification.

<u>Extraction, purification and analysis of pigments.</u>

Acetone extracts were filtered through Whatman No 1. filter papers to remove any remaining cell debris. The filtrates were reduced to dryness *in vacuo*, redissolved in 70% methanol, applied to pre-rinsed sep-pak C_{18} cartridges and eluted with 10 mL of 100% acetone. Acetone eluates were filtered through 0,2 μm centrifuge filters and reduced to dryness under nitrogen. Samples were stored at -20 °C until analysis on HPLC. All manipulations were carried out in dim light to minimise photo-oxidation and isomerisation of the pigments of interest.

Separation of pigments on HPLC.

Samples were resuspended in a known amount of isopropanol/dichloromethane (9:1 v/v) and analyzed by reversed-phase HPLC on a Nucleosil column using a linear gradient of 0-100% ethyl acetate in acetonitrile/water (9/1 v/v), containing 0.1% (v/v) triethylamine, over 35 minutes at a flow rate of 0.8 $mL.min^{-1}$. Peaks were detected at 410 nm using one of three different HPLC systems. **System 1**- Spectra-Physics- consisting of a LINEAR UVIS 200 variable wavelength detector coupled to a SP 4290 integrator, **System 2**- Hewlett Packard-HP 1090 coupled to a photo-diode array detector, the system was driven by Chemstation Software, and **System 3**- Beckman System Gold, consisting of a 126 programmable solvent module coupled to a 168 photo-diode array detector, the system was driven by Beckman Gold system driver software.

Typical HPLC traces of carotenoid separation using these HPLC systems are shown in Figure 2.1.-2.3.

For analysis of ß-carotene stereoisomers, separation was carried out using **System 2** comprising a Nucleosil 5 μm C_{18} (250 x 4.6 mm i.d.) column eluted isocratically with (9:1 v/v) methanol:acetonitrile over 25 minutes at a flow rate of 1.0 $mL.min^{-1}$. A typical HPLC trace of ß-carotene isomer separation is presented in Figure 2.4.

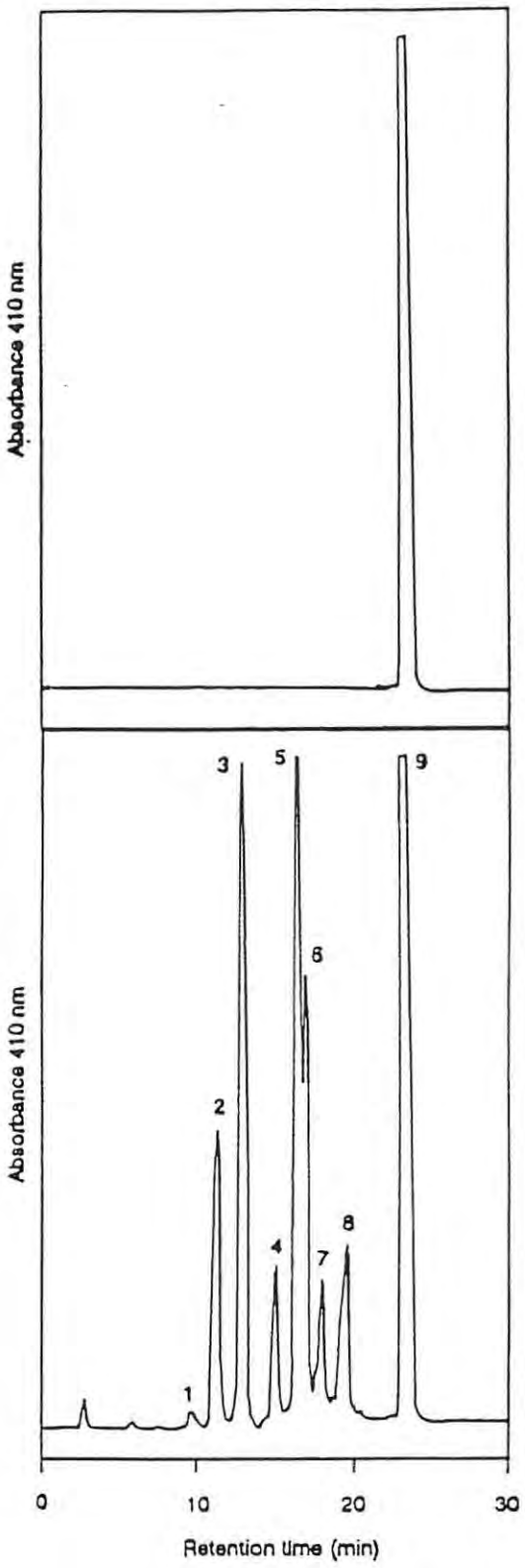


Figure 2.1. A typical HPLC trace obtained using System 1 (Spectra Physics) showing separation of pigments of interest extracted from algal samples. (top - ß-carotene standard, bottom - algal sample). Refer to Table 2.2 for identification of peak numbers.

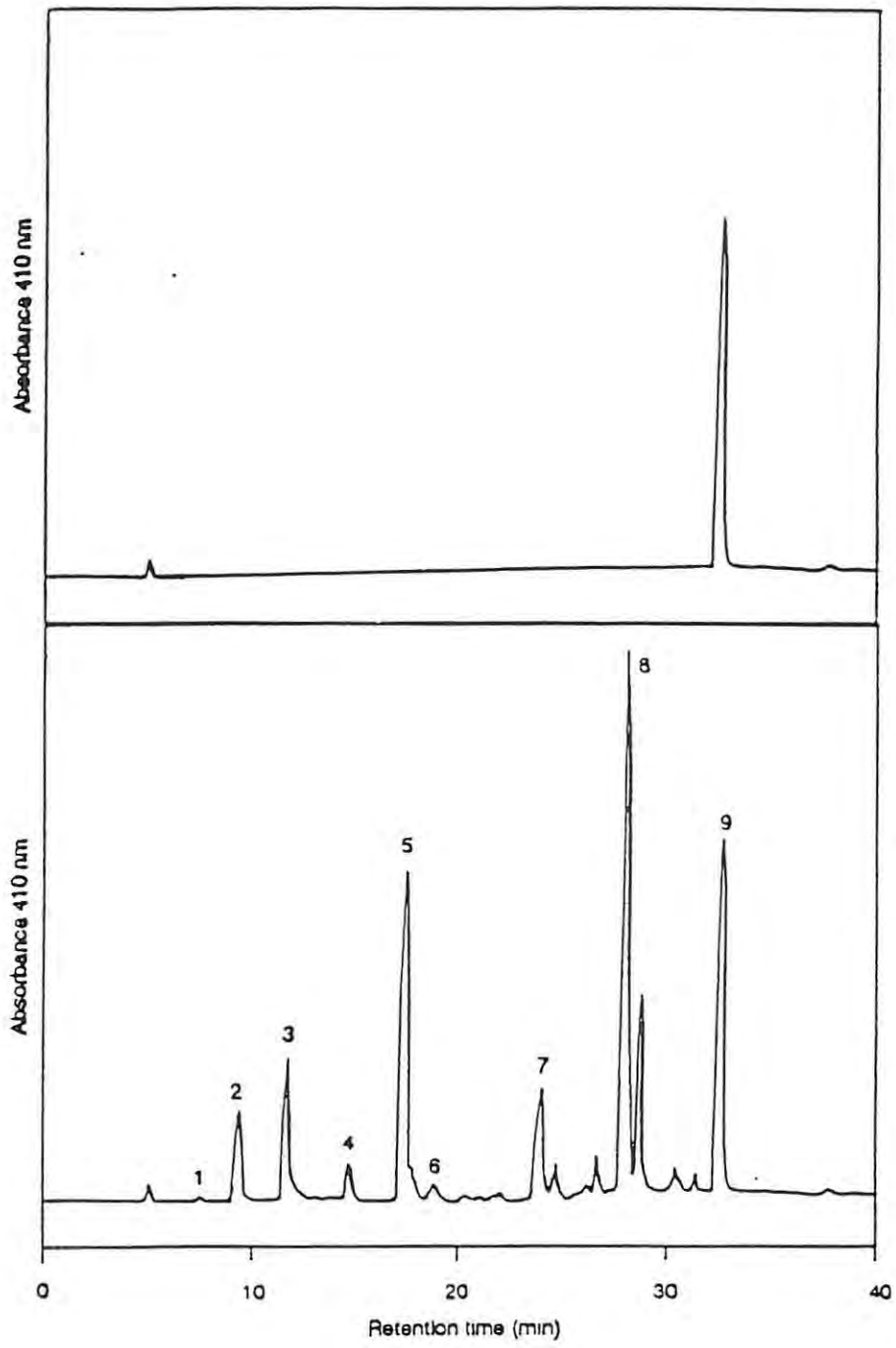


Figure 2.2. A typical HPLC trace obtained using System 2 (Hewlett Packard) showing separation of pigments of interest extracted from algal samples. (top - ß-carotene standard, bottom - algal sample). Refer to Table 2.2 for identification of peak numbers.

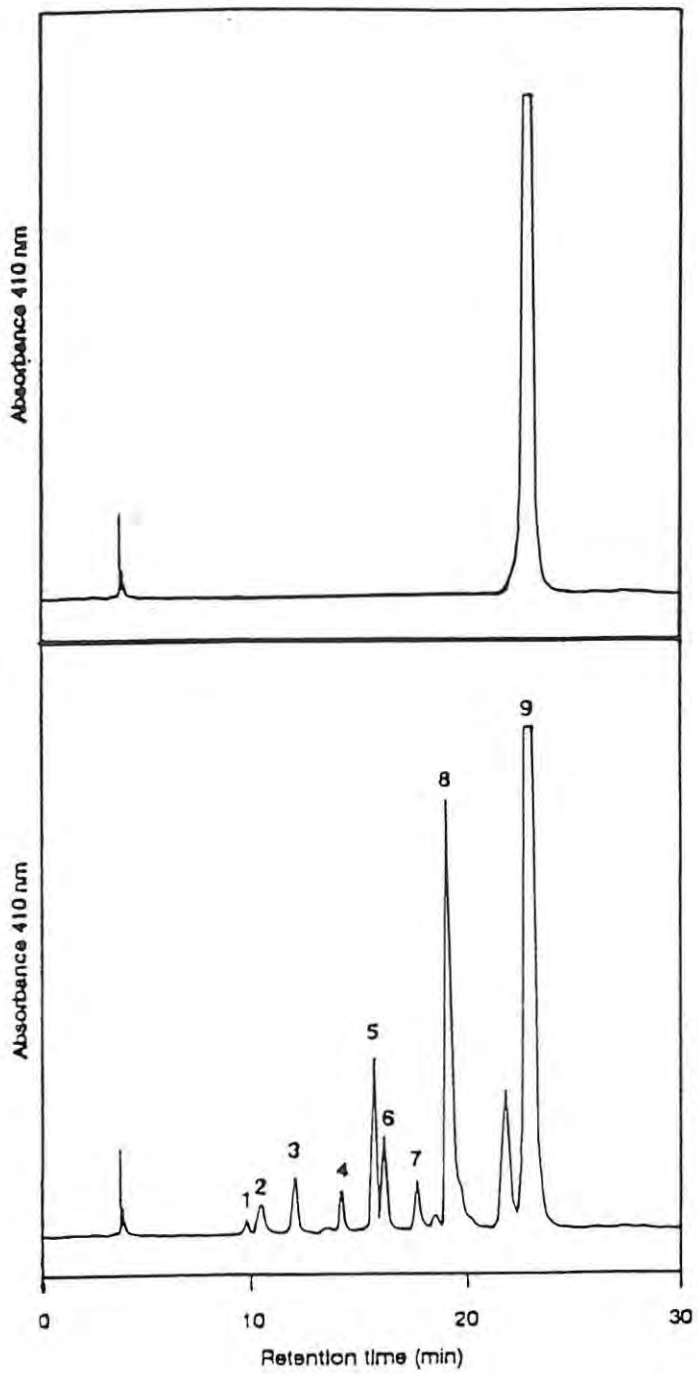


Figure 2.3. A typical HPLC trace obtained using System 3 (Beckman Gold) showing separation of pigments of interest extracted from algal samples. (top - ß-carotene standard, bottom - algal sample). Refer to Table 2.2 for identification of peak numbers.

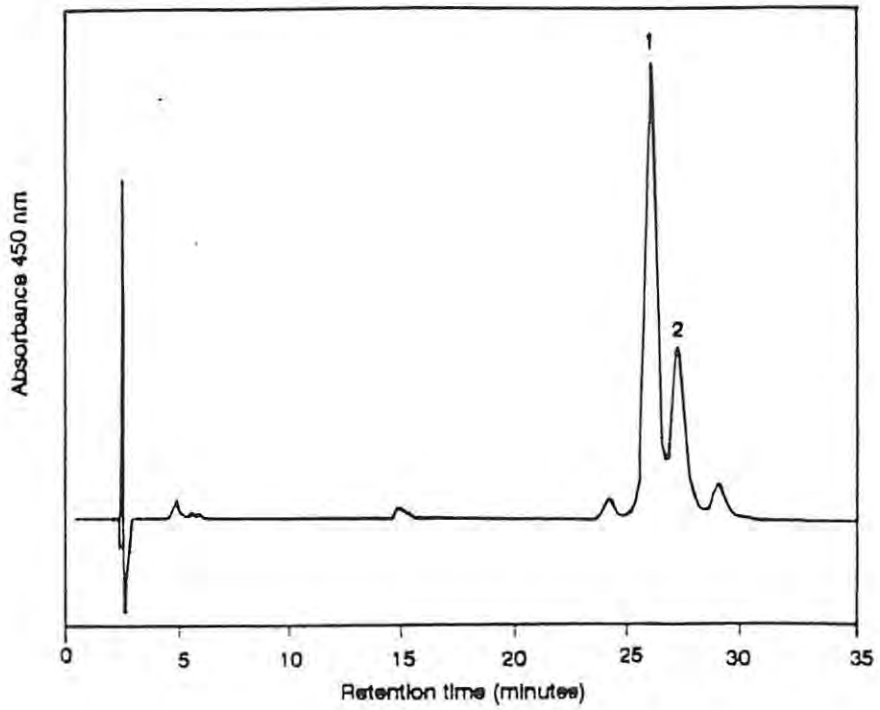


Figure 2.4. A typical HPLC trace of the separation of the ß-carotene isomers. Peak 1 represents the all-*trans* isomer and peak 2 the 9-*cis* isomer.

Identification of Carotenoids.

Identification of the carotenoids was achieved by fractionating the pigment extracts resolved by reverse phase HPLC (**System 3**). A LKB Bromma 2112 Redirac fraction collector was used to collect the pigment fractions which were subsequently dried under nitrogen and resuspended in hexane and ethanol. The absorbance spectra of each component in both hexane and ethanol was obtained using a Shimadzu UV-160A dual beam scanning spectrophotometer. The presence of 5,6-epoxide groups was confirmed by spectral shifts following addition of 1.0 M HCl to an ethanolic solution of the purified pigments (Davies,

1965). Pigments were identified by their spectral characteristics. The absorption maxima of the pigments in hexane, ethanol and ethanol/HCl are represented in Table 2.2.

Table 2.2. HPLC retention times and spectral maximum of pigments in acetone extracts of *D. salina*.

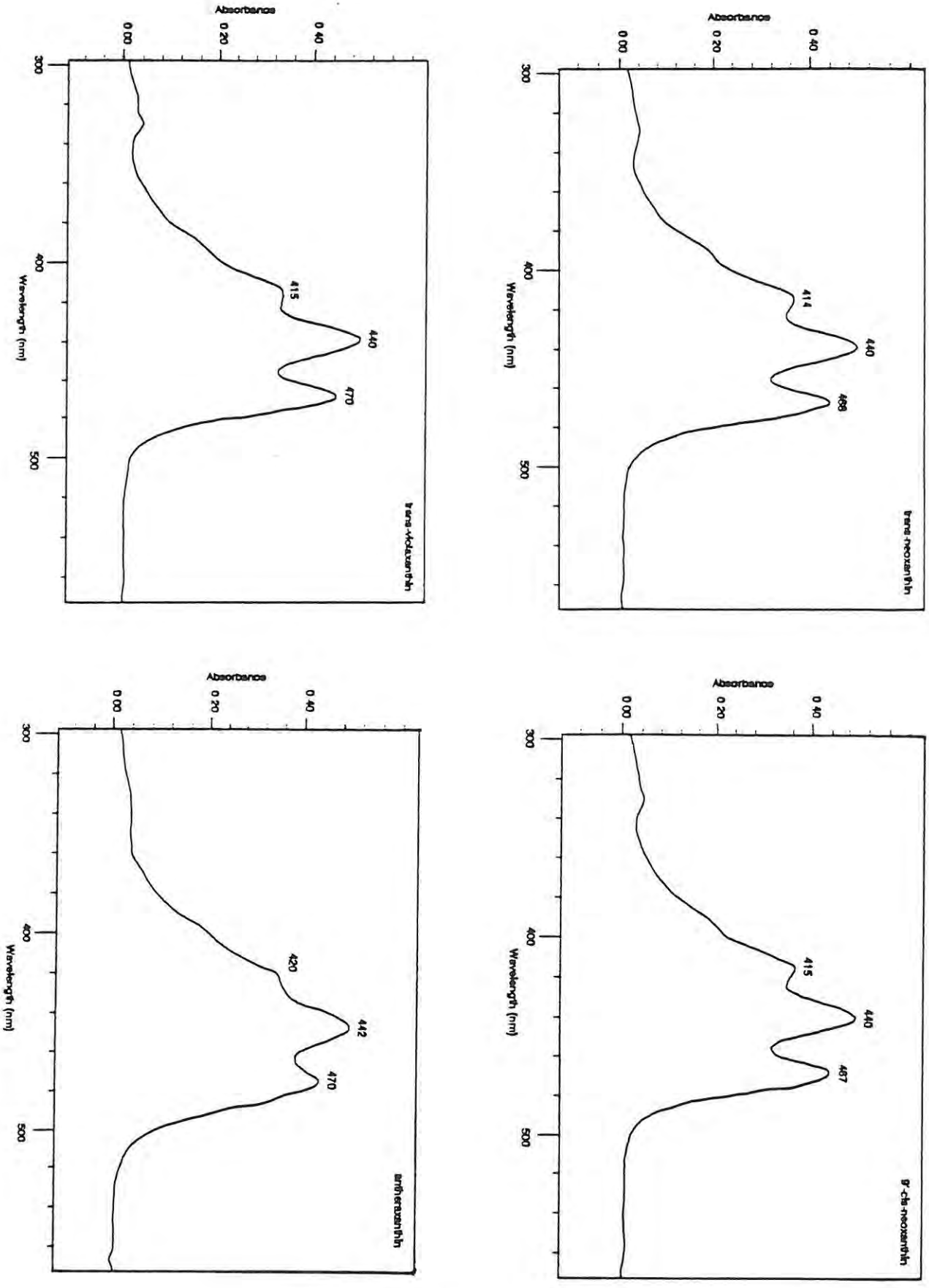
Peak No.	Pigment	Retention Time (min)	Hexane			Ethanol			Ethanol + HCl		
1	trans -neoxanthin	9.6	415	440	467	419	446	469	402	420	450
	Reference 1		412	436	468	422	441	470	402	422	476
2	9'-cis -neoxanthin	10.1	414	440	468	416	437	464	400	412	450
	Reference 2		412	436	465	412	438	464		443	
3	trans -violaxanthin	11.6	415	440	470	412	436	464	380	404	426
	Reference 2		416	440	470	417	441	470	380	402	423
4	antheraxanthin	13.7	420	442	470	422	445	475	402	425	452
	Reference 2		421	445	470	422	444			422	
5	lutein	14.6	420	442	472	423	447	474		NS	
	Reference 2			445	473		444			NS	
6	zeaxanthin	15.2	424	448	478	427	450	475		NS	
	Reference 2		422	445	475					NS	
9	ß-carotene	21.5	425	449	474	426	452	474		NS	
	Reference 3		420	449	476	429	451	477		NS	

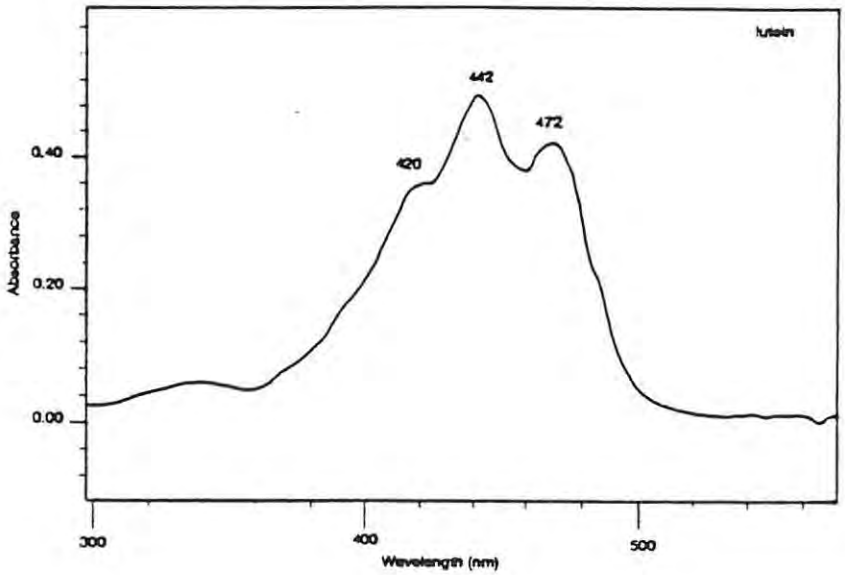
References 1, Parry and Horgan, 1990; 2, Duckham, et al, 1991; 3, Val, et al., 1986.

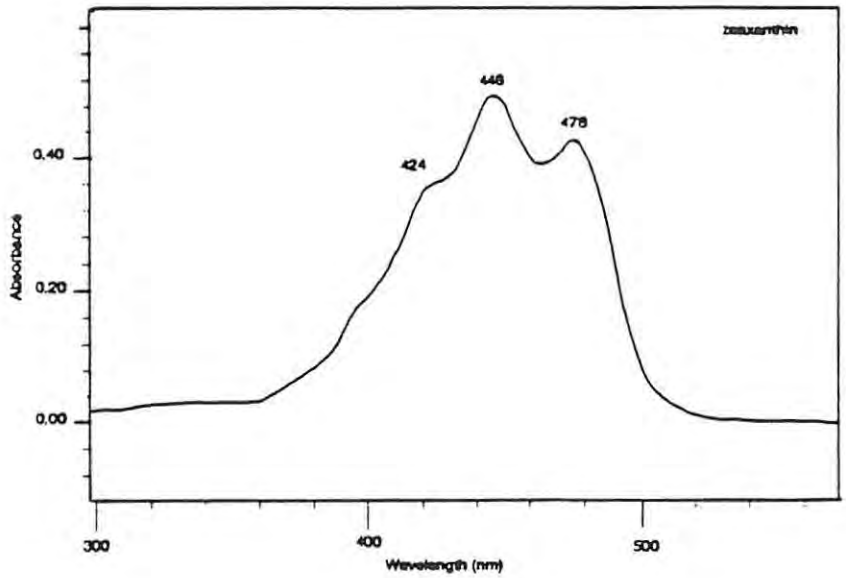
NS : no shift

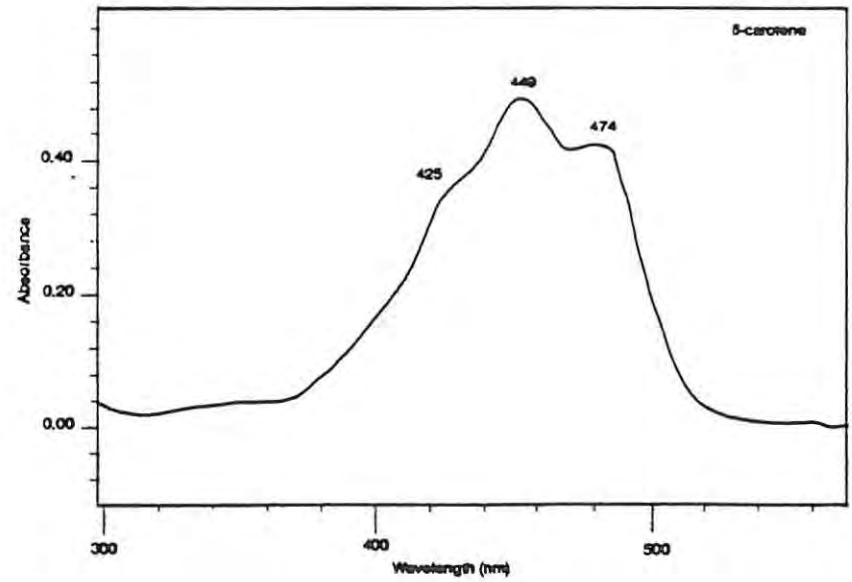
Peaks 7 and 8 were identified as chlorophyll *b* & *a* respectively.

Figures on page 41 and 42 represent the absorption spectra of the xanthophylls and ß-carotene in hexane as indicated in Table 2.2.


Absorbance
0.00
0.20
0.40
300
400
500
Wavelength (nm)
414
440
468
trans-neoxanthin
Absorbance
0.00
0.20
0.40
300
400
500
Wavelength (nm)
415
440
470
trans-violaxanthin
Absorbance
0.00
0.20
0.40
300
400
500
Wavelength (nm)
415
440
467
9'-cis-neoxanthin
Absorbance
0.00
0.20
0.40
300
400
500
Wavelength (nm)
420
442
470
antheraxanthin


lutein
442
472
420
0.40
0.20
0.00
Absorbance
300
400
500
Wavelength (nm)


zeaxanthin
448
478
424
0.40
0.20
0.00
Absorbance
300
400
500
Wavelength (nm)


β-carotene
449
474
425
0.40
0.20
0.00
Absorbance
300
400
500
Wavelength (nm)

Derivation of Normalized Coefficients for Pigment Quantification.

Samples were fractionated as described above using **System 1** and the individual pigments isolated. These isolated fractions were dried under nitrogen, resuspended in hexane, and the pigment concentrations were determined using the coefficients of extinction indicated in Val *et al.*, (1986). The method was calibrated by injecting known amounts of pure pigments, and plotting peak area (integrator counts) versus mass of pigment injected. This value was termed $C_{pigment}$. The coefficient of ß-carotene ($C_{ß\text{-carotene}}$) was then used to derive normalized coefficients ($C_{pigment}/C_{ß\text{-carotene}}$) for each compound of interest (de las Rivas, *et al.*, 1989). Since the method remained the same, (the only variation between these systems was a different detector for each of the three HPLC systems used), the coefficient of ß-carotene ($C_{ß\text{-carotene}}$) was used to calculate system specific normalized coefficients. The normalized coefficients for the specific pigments as calculated for each HPLC system are listed in Table 2.3.

Table 2.3. System specific normalized coefficients.

Pigment	System 1	System 2	System 3
neoxanthin	1.71	1.89	1.48
violaxanthin	1.53	1.68	1.33
antheraxanthin	1.41	1.50	1.18
lutein	1.41	1.50	1.18
zeaxanthin	1.34	0.72	1.16
ß-carotene	1.00	1.00	1.00
chlorophyll *a*	10.8	11.8	9.40
chlorophyll *b*	1.71	1.80	1.50

Extraction, purification and analysis of ABA and related compounds.

Extracts containing a known amount of high specific activity [^{3}H]-ABA (added to correct for recovery) and ABA-methyl ester, as an internal standard, were filtered through Whatman No. 1 filter paper. Filtrates were reduced to dryness *in vacuo* at 35 °C and resuspended in 0.5M

potassium phosphate buffer (pH 8.5). The aqueous phase was partitioned three times against equal volumes of diethyl ether to remove neutral and basic impurities. Aqueous extracts were then acidified to pH 2.5 and partitioned three times against equal volumes of ethyl acetate to extract the acids. Water was removed by freezing and filtration, and the ethyl acetate-soluble acids resuspended in 1% acetic acid were applied to sep-pak C_{18} cartridges, as described by Pierce and Raschke (1981). ABA and related compounds were eluted with 60% methanol/1% acetic acid and eluates dried under nitrogen. Aliquots of the incubation media were similarly purified following acidification.

HPLC analysis of ABA and related compounds.

Extracts were analyzed by reverse-phase HPLC using **System 1** and **System 3.** Separation was achieved on a 10 μM Phenomenex column using a 0-100% methanol gradient containing 0.5% acetic acid throughout. A flow rate of 1.0 mL.min^{-1} was maintained over 45 min and peaks were monitored at 254 nm. Peaks were quantified by integration and calibrated with standard compounds ($\pm$ *cis,trans* ABA). A typical HPLC trace of the separation achieved on these systems can be seen in Figure 2.5.

Separation of the *cis* and *trans* ABA isomers was achieved using the method outlined above. The isomers were identified by co-chromatography with authentic standards. Typical separation of the ABA isomers can be seen in Figure 2.6.

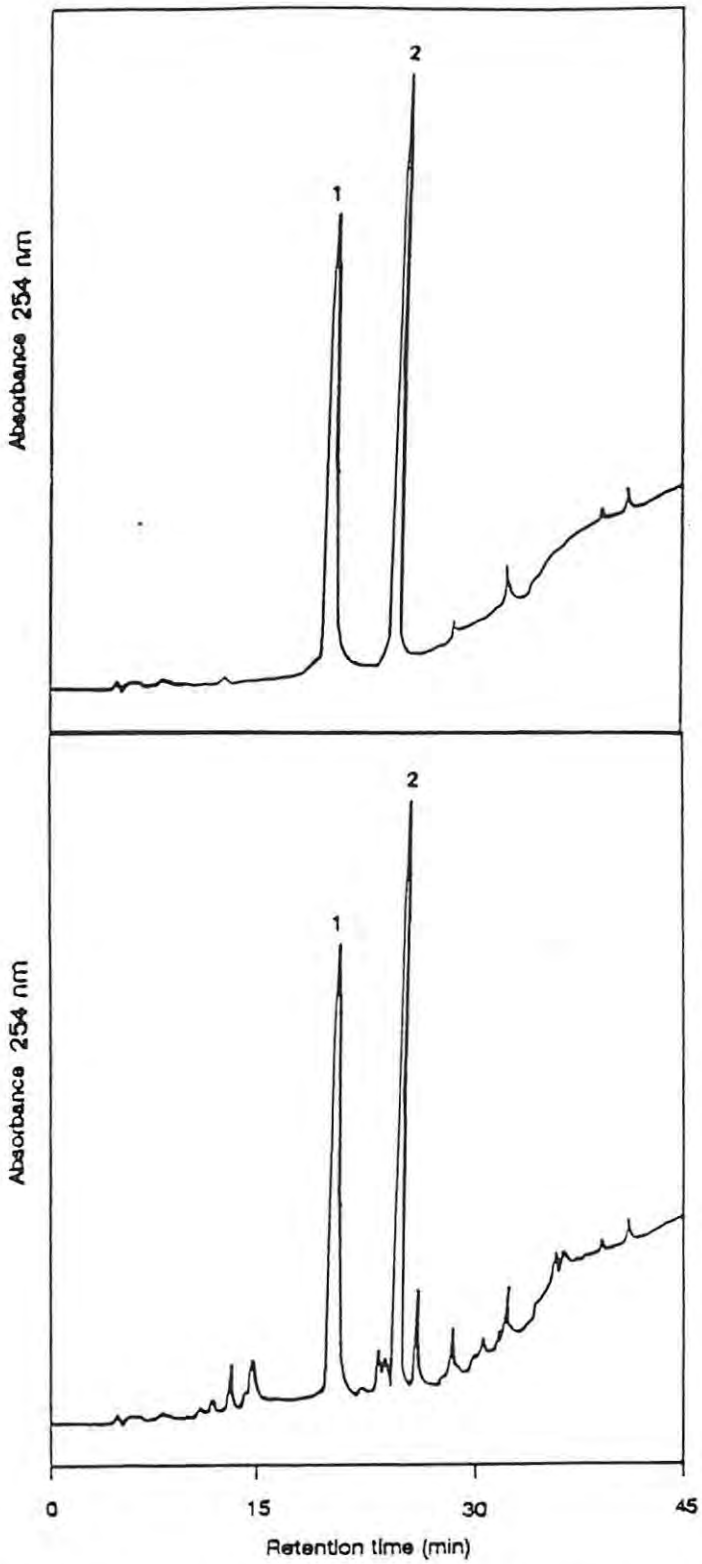


Figure 2.5. A typical HPLC trace showing the separation of ABA and ABAMe extracted from an algal sample. Separation was achieved using the conditions described above. Peak 1 represents ABA and peak 2 ABAMe. (top - ABA and ABAme standards, bottom - algal sample)

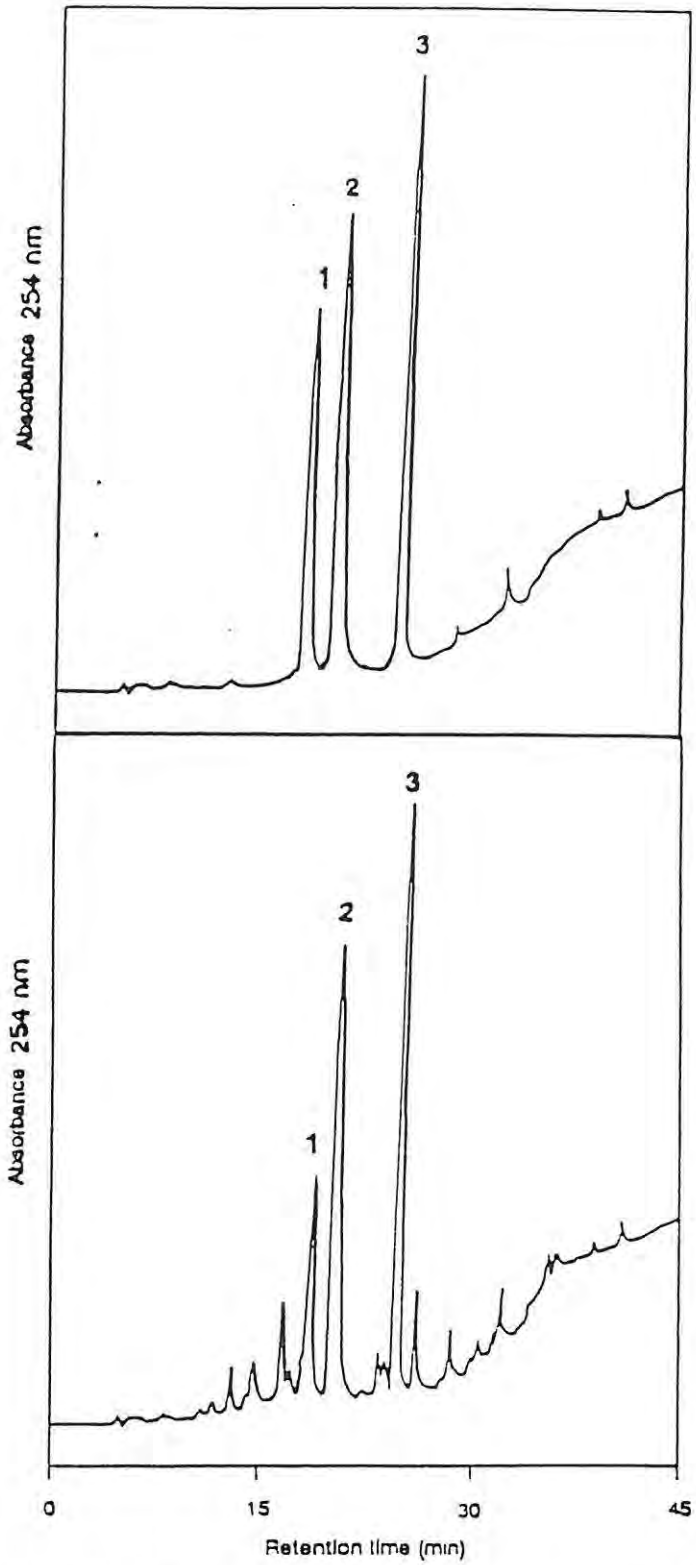


Figure 2.6. A typical HPLC trace showing the separation of ABA isomers and ABAMe extracted from a high light treated algal culture. Separation was achieved using the conditions described above. Peak 1 represents ABA (*trans*), peak 2 ABA (*cis*) and peak 3 ABAMe. (top - ABA standards, bottom - algal sample).

Identification of Abscisic Acid.

Extracts containing ABA were separated on HPLC as described above. Fractions which eluted at time points corresponding to authentic ABA standard peaks, were collected and those exhibiting maximum radioactivity, were pooled, reduced to dryness, and methylated with excess ethereal diazomethane.

Unequivocal identification was established by co-chromatography using both gas chromatography fitted with an electron capture detector (GC-ECD) and combined capillary gas chromatography-mass spectrometry (GC-MS).

GC-ECD

Following methylation, compounds for analysis by GC were dissolved in a small volume of redistilled acetone. GC was performed on a Perkin-Elmer 8310 instrument fitted with an electron capture detector (^{63}Ni source). Compounds of interest were resolved on a capillary column (15 mm x 0.53 mm i.d.) of SPB-1 programmed from 120 °C at 5 °C per minute with N_2 as a carrier gas at a flow rate of 17 mL.min^{-1}. ABA methyl ester from the algal extracts was identified by co-chromatography with authentic ABAMe standards.

A typical GC-ECD trace of the separation of methylated ABA is presented in Figure 2.7.

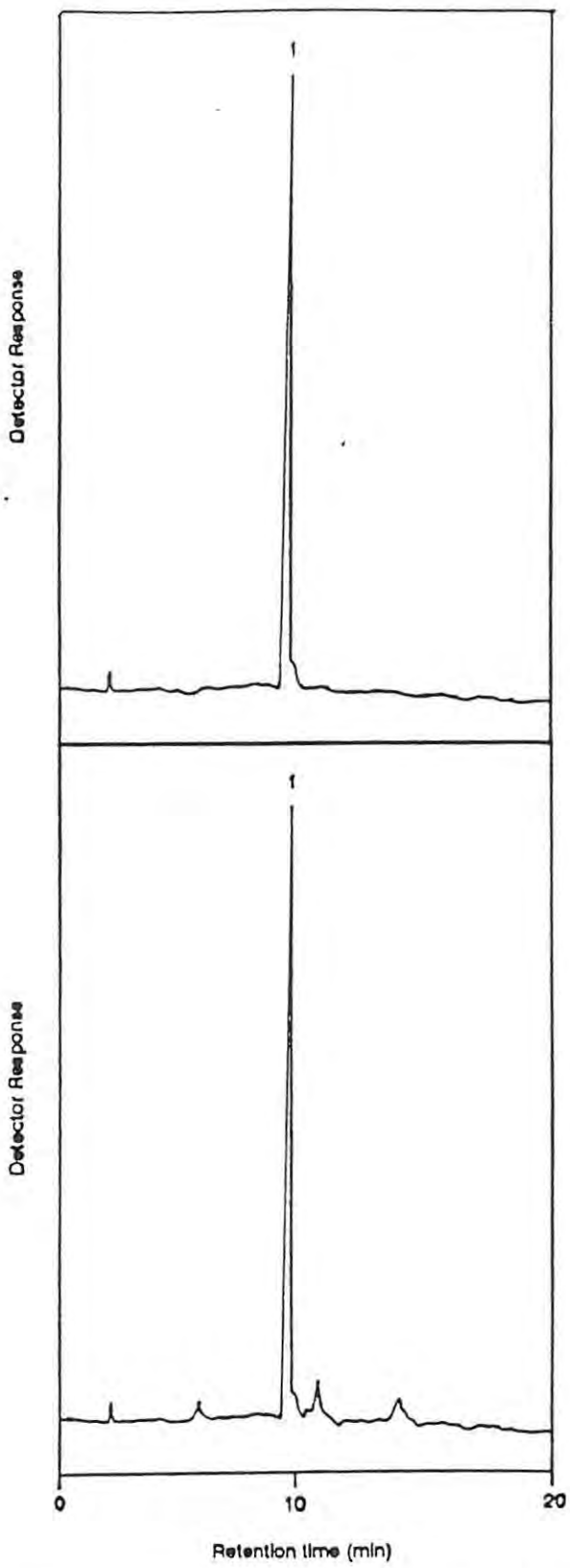


Figure 2.7. A typical GC-ECD trace showing the separation of methylated ABA from an extracted algal sample. Peak 1 represents methylated ABA which co-chromatographed with an authentic ABAMe standard.

GC-MS

GC was performed on a Hewlett-Packard 5890 gas chromatogram fitted with a flame ionization detector. Separation was achieved using a fused-silica capillary column (12 m x 0.32 mm i.d.) of HP-1 and the instrument was programmed from 160 °C at 5 °C per minute with Helium as the carrier gas (1.5 - 2 $mL.min^{-1}$).

Combined capillary GC-MS was carried out using a Hewlett-Packard 5890 instrument coupled to an HP 5988A MS system with GC conditions as described above. Electron impact mass spectra were recorded at 70 eV and a source temperature of 250°C. Identification of the compounds as methylated ABA was established using a Hewlett-Packard data processing station. Mass spectra are depicted in Figure 2.8.

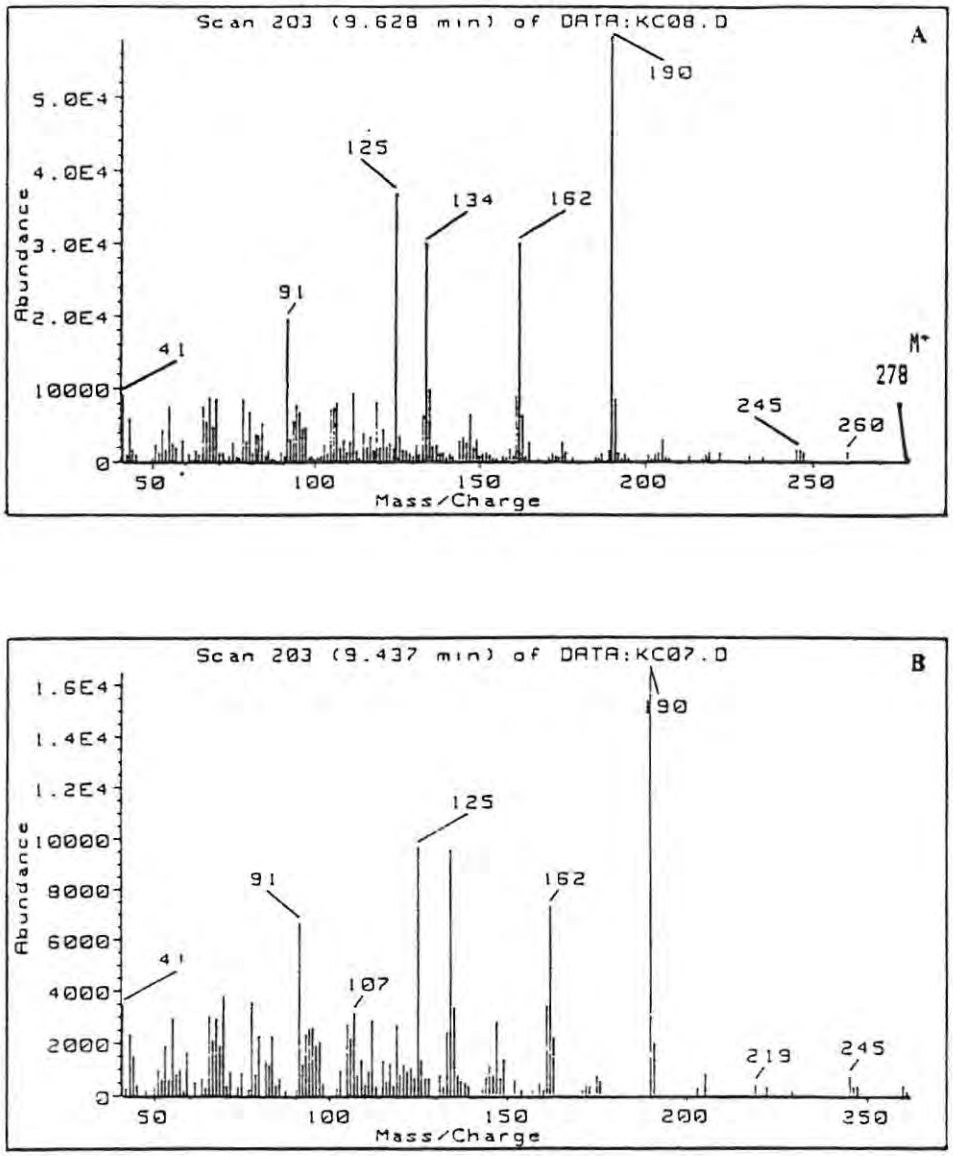


Figure 2.8. Mass spectra of an authentic methylated ABA standard (A) and methylated ABA extracted from an algal sample (B).

CHAPTER 3

Physiological and Biochemical Aspects of the Response of *D. salina* to Stress: Optimization of ß-carotene Production.

3.1. Introduction.

There is substantial evidence to support the idea that plants respond to a variety of stresses in basically the same way (Levitt, 1980a, 1980b; Alscher and Cumming, 1990; Mooney, *et al.*, 1991; Cowan, 1994). This response has been termed, "the plant stress syndrome", which involves a centralised system of physiological responses to stress. These responses are characterised by a reduction in the growth rate, lowering of photosynthetic carbon assimilation, and changes in both the capacity for nutrient uptake and hormone metabolism (Chapin, 1991; Cowan, 1994). The ß-carotene accumulating alga *D. salina* is no exception. For example, these algae show alterations in the pattern of carbon flux between starch and glycerol, accumulation of ß-carotene and increased capacity for ABA metabolism in response to various stresses (Bental, *et al.*, 1990; Cowan and Rose, 1991). These alterations in metabolism occur coincidentally with, or are preceded by changes in cell volume, lipid order and composition of the plasma-membrane, compartmental pH, and cationic membrane pump activity. All of these impact on photosynthesis and productivity.

In order to maximise biotechnological production of ß-carotene in *D. salina*, it is important to understand all aspects which are associated with the stress-induced accumulation of this commercially important carotenoid. Separation of the growth phase from the ß-carotene accumulating stress phase in dual-stage production allows for greater control and manipulation of the factors which might contribute to enhanced yield of ß-carotene.

Experiments described in this chapter were carried out to determine the general response of *D. salina* cells in terms of photosynthesis, productivity, ß-carotene accumulation and ABA production to various stress conditions. The effects of single and multiple stresses were also

evaluated in order to optimise stress treatment for maximum ß-carotene production per unit time.

3.2. Results.

3.2.1. The effect of nitrate concentration on pigment content in *D. salina* cells.

It has been demonstrated that nitrate limitation rather than complete nitrate deficiency is required for optimal ß-carotene accumulation in cells of *D. salina* (Ben-Amotz and Avron, 1983).

An experiment was conducted to determine the optimum nitrate concentration required for maximum ß-carotene accumulation in cells transferred from growth medium (5 mM NO_3^-) to nitrate limited medium. *D. salina* cells were exposed to a range (0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.5 mM, 1 mM, 2 mM and 5 mM) of nitrate concentrations for five days (Figure 3.1.) and ß-carotene, chlorophyll content and the ß-carotene:chlorophyll ratio were determined.

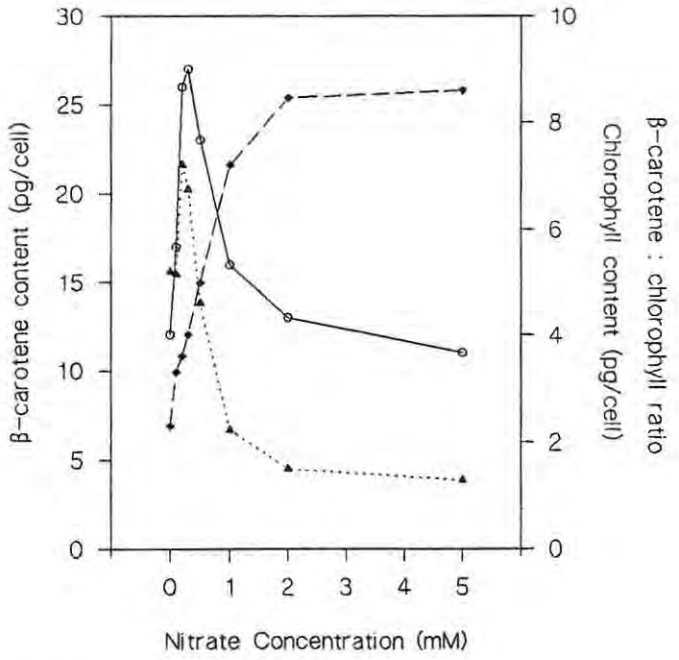


Figure 3.1. Changes in ß-carotene, chlorophyll and ß-carotene-to-chlorophyllratio of *D. salina* cells exposed to a range of nitrate concentrations under laboratory conditions. (○) ß- carotene; (♦) chlorophyll; (▲) ß-carotene:chlorophyllratio. (Results reflect Day 5 values of and a single experiment).

ß-Carotene accumulation was not stimulated by complete nitrate deficiency. The results depicted in Figure 3.1. clearly show that maximum ß-carotene content was achieved at a concentration of 0.2 mM nitrate. Chlorophyll content per cell decreased as nitrate concentration was reduced. The ß-carotene:chlorophyll ratio was consequently at its highest at approximately 0.2 mM nitrate. A level of 0.2 mM nitrate was subsequently identified for the optimum stress medium.

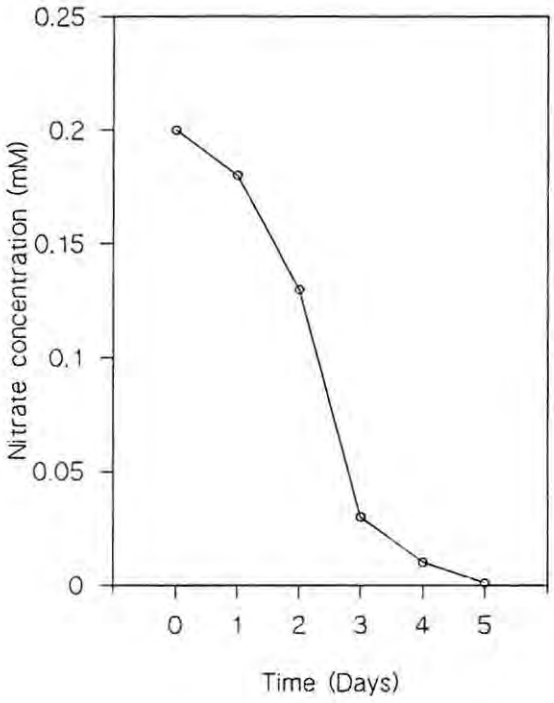


Figure 3.2. Time course changes in nitrate concentration in the culture medium illustrating nitrate utilization by *D. salina* cells grown in media containing 0.2 mM nitrate. Cells grown outdoors were harvested from growth media by flocculation and resuspended in 20 l outdoor ponds. (Results reflect a single experiment).

Nitrate utilisation by *D. salina* cells in the optimum stress medium (Figure 3.2.), revealed rapid depletion of the available nitrate. By Day 5 nitrate within the medium was no longer detectable.

3.2.2. Determination of optimal cell density for ß-carotene production in *D. salina*.

As it is nearly impossible to regulate light intensity in large-scale outdoor cultures, an attempt to optimise light interception by each algal cell was made by varying the cell density of outdoor cultures. Starting cell densities of between 10 x 10^4 cells.ml^{-1} and 80 x 10^4 cells.ml^{-1} were evaluated to determine the optimal cell density for ß-carotene accumulation under outdoor conditions.

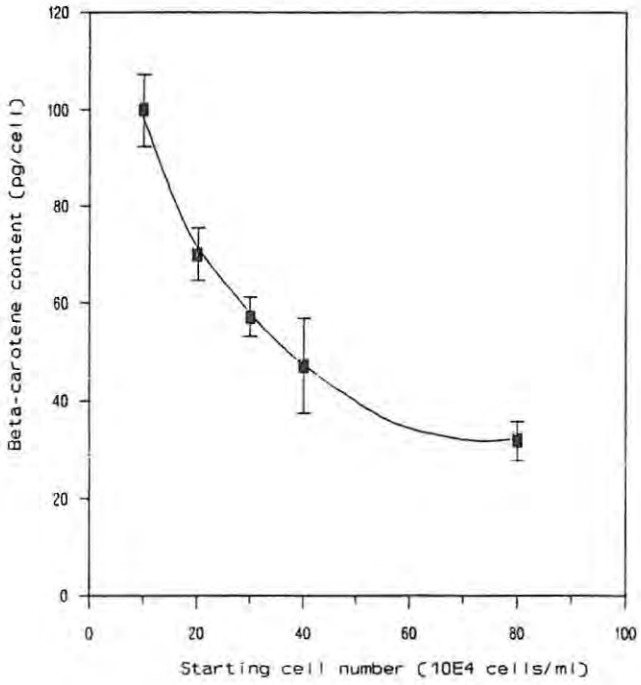


Figure 3.3. Relationship between starting cell density and cellular ß-carotene accumulation in *D. salina*. Results reflect day 5 values. Starting ß-carotene content 18 ±1 pg.cell^{-1}. Vertical bars represent Standard Deviation (SD).

It is apparent from Figure 3.3 that below a starting cell density of approximately 40 x 10^4 cells.ml^{-1} there was a sharp increase in the ß-carotene content of the cells from about 50 pg.cell^{-1} at 40 x 10^4 cells.ml^{-1} to about 100 pg.cell^{-1} at 10 x 10^4 cells.ml^{-1}. These results clearly demonstrate that as light intensity increases, due to a decrease in the culture cell density, ß-carotene accumulation is stimulated. This sharp increase coincided with a rapid decrease in cell growth over this starting cell density range (Figure 3.4).

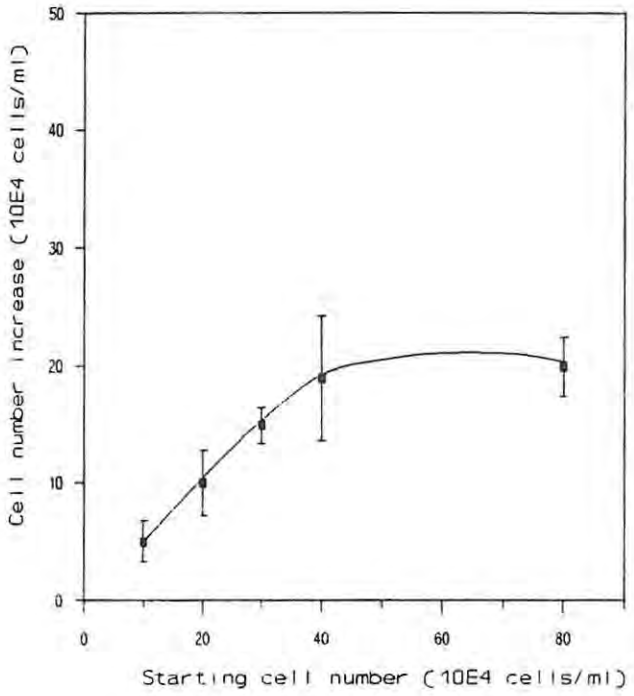


Figure 3.4. Relationship between starting cell density and *D. salina* growth. Results reflect day 5 values. Vertical bars represent SD.

A complex relationship exists between the cell density of a culture and ß-carotene accumulation. At low cell densities growth was limited, however, maximum ß-carotene accumulation occurred. This relationship has previously been quantified by Phillips (1993) and it was demonstrated that a near-perfect inverse relationship exists between algal growth and ß-carotene accumulation at starting cell densities below 80 x 10^4 cells.ml^{-1}. Therefore, over this cell density range similar volumetric ß-carotene yields are achieved in outdoor mass cultures irrespective of the starting cell density. Based on these findings, all further experiments presented in this thesis were conducted at cell densities of between 30 x 10^4 to 40 x 10^4 cells.ml^{-1} since they fall within the optimal ß-carotene production range identified by Phillips (1993).

3.2.3. Comparison of the effect of different stresses on photosynthesis, cell growth, and ß-carotene and chlorophyll content of *D. salina* cells under laboratory conditions.

The implementation of a dual-stage ß-carotene production process relies on the successful manipulation of the stress response in *D. salina*. A comparative evaluation of single and multiple stress induction of ß-carotene as well as changes in other physiological responses under laboratory conditions was necessary in order to assess the use of various stress factors which could be utilized in the scale-up of the dual-stage process.

The results of the effect of different stresses on photosynthesis, cell growth, ß-carotene and chlorophyll content in *D. salina* are presented in Table 3.1.

Table 3.1. ß-carotene and chlorophyll content, photosynthetic and cell growth data ± Standard Deviation (SD) for *D. salina* cells exposed to various stresses (data reflect Day 5 values).

Parameter	Control	Salinity stress (3 M NaCl)	High light stress	NUTRIENT STRESS			MULTIPLE STRESS		
				Nitrate limitation (0.2 mM)	Phosphate deficiency	Sulphate deficiency	High Light + nitrate limitation	High light + nitrate limitation + sulphate deficiency	High light + salinity stress + all nutrient stress
ß-carotene content (pg/cell)	2.37 ± 0.41	9.0 ± 1.0	21.0±2.5	17.0±1.0	13.7±1.3	5.25±2.3	34.0±2.0	43.0±2.3	63.5±4.5
Chlorophyll content (pg/cell)	10.5±1.5	8.0±1.6	7.16±0.9	6.1±1.1	6.8±0.93	7.0±0.89	6.4±0.4	5.16±0.2	4.5±0.35
ß-carotene/ chlorophyll ratio	0.22	1.12	2.93	2.78	2.0	0.75	5.3	8.3	14.1
Percentage cell number increase (%)	122±2.5	55±4.8	134±4.0	35±4.0	26±1.0	30±1.0	50±7.0	34±4.0	7±2.4
Oxygen evolution rate (µmol O_2/mg Chl/hr)	379.2	373.4	383.2	375.0	N.D.	N.D.	N.D.	N.D.	367.35

N.D.- (Not Determined)

All the stresses examined increased cellular ß-carotene content compared to the control. Of the single stresses, high light stress was clearly the major factor inducing enhanced ß-carotene accumulation followed by nitrate limitation.

Exposure to multiple stresses, resulted in higher cellular ß-carotene content and lower chlorophyll content. Cumulative stresses resulted in an incremental increase in cellular ß-carotene accumulation, with cells exposed to a combined stress of high light, salinity and nutrient limitation accumulating the highest level of ß-carotene per cell.

Salinity and nutrient stress had a marked effect on *D. salina* growth. All the single stresses with the exception of high light stress resulted in a reduction in growth of more than 50%. High light intensity appeared to stimulate rather than decrease growth. Cells exposed to multiple stresses exhibited a substantial reduction in cell growth. A 93% decrease in growth was evident in cells exposed to multiple stress compared to the control.

It is noteworthy that the oxygen evolution rate on a chlorophyll basis, was similar to that of the control for all the stresses investigated, indicating that the cells had adapted their photosynthetic machinery to the stress conditions.

It is evident that all stresses had similar effects on *D. salina* physiology, and that multiple stresses elicited a more intensive response in terms of higher ß-carotene content, lower chlorophyll content and slower growth rate. These changes could be considered adaptive responses which allow the organism to sustain photosynthesis under prevailing stress conditions.

The stress media listed below were chosen for further investigations in mass culture. The media consisted of standard growth media (see Table 2.1.) except for the indicated alterations.

1. hypersalinity stress (3 M), from here on referred to as **salt stress**;

2. nitrate limitation stress (0.2 mM), from here on referred to as **nitrate stress;** and

3. media consisting of 3 M NaCl, 0.2 mM KNO_3, 50 mM $NaHCO_3$, from here on referred to as **multiple stress.**

3.2.4. Changes in photosynthetic carbon fixation and oxygen evolution rate of *D. salina* cells exposed to multiple stress.

In order to monitor the effect of multiple stress on photosynthesis, the rates of carbon fixation and oxygen evolution were measured following transition to stress media.

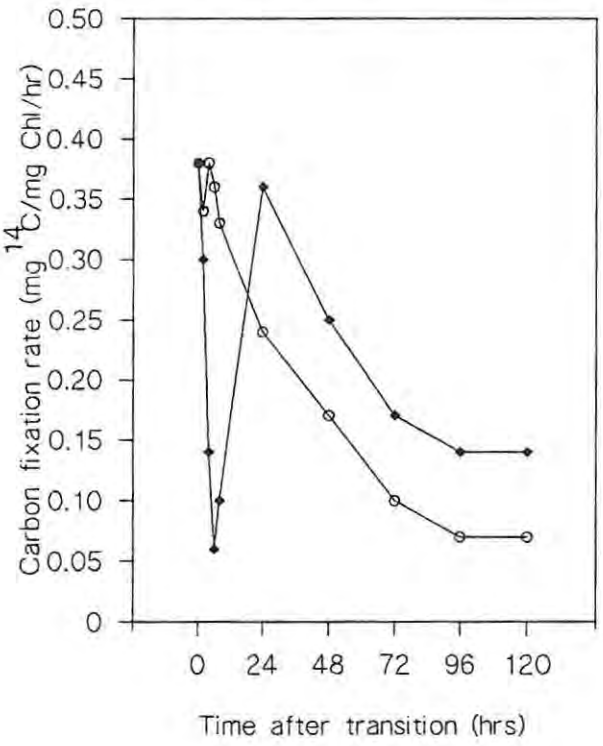


Figure 3.5. Carbon fixation rate (mg ^{14}C.mg Chl^{-1}.hr^{-1}) of stressed and non-stressed *D. salina* cells cultured in multiple stress and growth medium (control) respectively. (○) - control; (◆) - multiple stress. Results reflect a triplicate mean.

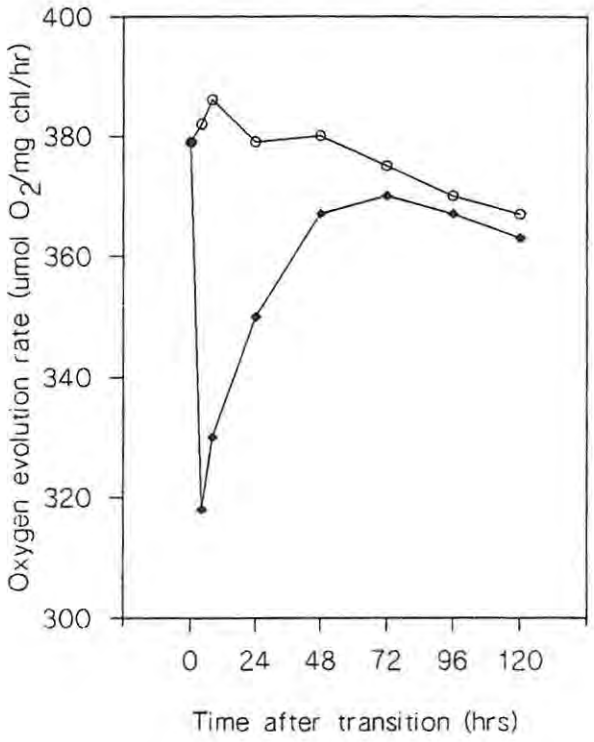


Figure 3.6. Oxygen evolution rate of stressed and non-stressed cells of *D. salina* cells cultured in multiple stress and growth medium (control) respectively. (○) - control; (◆) - multiple stress. (Results reflect a single experiment).

The results depicted in Figures 3.5. and 3.6. show that in multiple stress medium, both the carbon fixation rate and the oxygen evolution rate decreased dramatically over the first 6 hours. This decline was, however, transient. Carbon fixation rate began to return to the starting levels 24 hours after exposure to stress. Inorganic carbon acquisition appeared to be stimulated in stressed cells, since carbon fixation was higher after 24 hours in the stressed cells than in the cells cultured in growth medium. This result underlines the biosynthetic response to the stress imposed. The oxygen evolution rate also began to return to basal level about 24 hours after exposure to stress, suggesting acclimation of the cells to stress conditions.

3.2.5. Volume changes in *D. salina* cells exposed to salt, nitrate, high light and multiple stress.

All living cells comprise a soluble phase containing water and dissolved organic and inorganic solutes in membrane bound compartments. In a "non-stress" situation these compartments exist in a state of equilibrium. Any alterations brought about by elevated temperature, solar radiation, osmotic or ionic changes will alter the chemical potential of cellular water thereby shifting the equilibrium which ultimately results in volume changes (Cowan, *et al.*, 1992; Cowan, 1994). *D. salina* is a wall-less alga therefore it may be anticipated that stress conditions would impact on cell volume.

Volume changes of *D. salina* cells exposed to salt, nitrate and multiple stress were monitored for 8 hours after the onset of stress conditions. The results are presented in Figure 3.7.

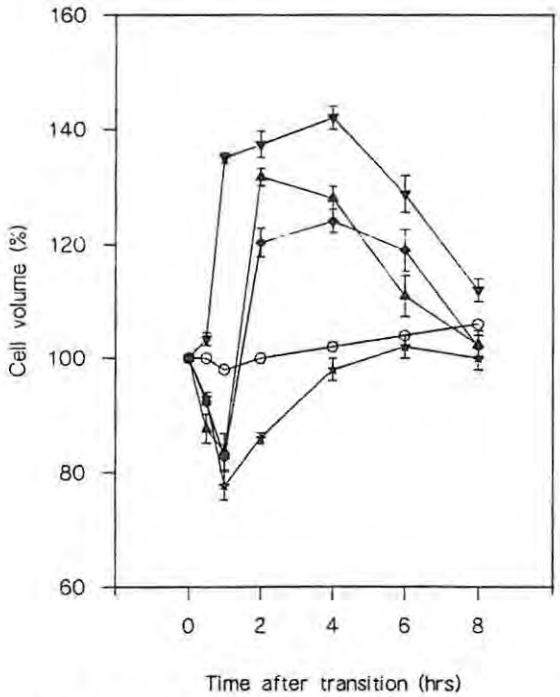


Figure 3.7. Volume changes in *D. salina* following exposure of cells to various stress conditions. (○) - control; (☆) - salt stress; (▲) - nitrate stress; (♦) - multiple stress; (▼) - high light stress. Vertical bars represent SD.

The results presented in Figure 3.7. illustrate the effect of different stresses on cell volume. As expected, transfer of cells from low to high light intensity conditions caused a substantial increase in cell volume, while exposure of cells to salt stress resulted in cell shrinkage. However, when cells were exposed to either nitrate or multiple stress (nutrient limiting conditions) cell shrinkage followed by swelling occurred. This pattern is probably indicative of the re-establishment of the cellular nitrate metabolism capacity such that turnover of the endogenous nitrogen pool was adjusted to external conditions.

These results show that all the stresses investigated impacted on cell volume. Under the conditions employed, recovery of original cell volume was achieved within 8 hours.

3.2.6. Changes in intracellular abscisic acid content in *D. salina* cells exposed to various stresses under laboratory conditions.

The plant growth regulator ABA plays a major role in the osmotic response of higher plants. It has been recognized for a number of years that guard cell turgor responds to ABA (Smith and Willmer, 1988; Hetherington and Quatrano, 1991). It is therefore not unreasonable to presume that cell volume changes may be regulated by ABA.

The production of ABA in response to stress has been demonstrated in several algal species (Tietz, *et al.*, 1989; Cowan and Rose, 1991). Changes in ABA were therefore monitored for the first 8 hours after exposure of *D. salina* cells to salt, nitrate, high light and multiple stress.

It can be seen from Figure 3.8. in which the results of the experiment are presented, that the intracellular ABA content increased in response to all the stresses investigated. This increase was however transient and declined to basal level within 12 hours. Interestingly, the ABA content appeared to increase coincidently with an increase/recovery in cell volume for each stress, indicating a possible interrelationship between ABA production and cell volume changes.

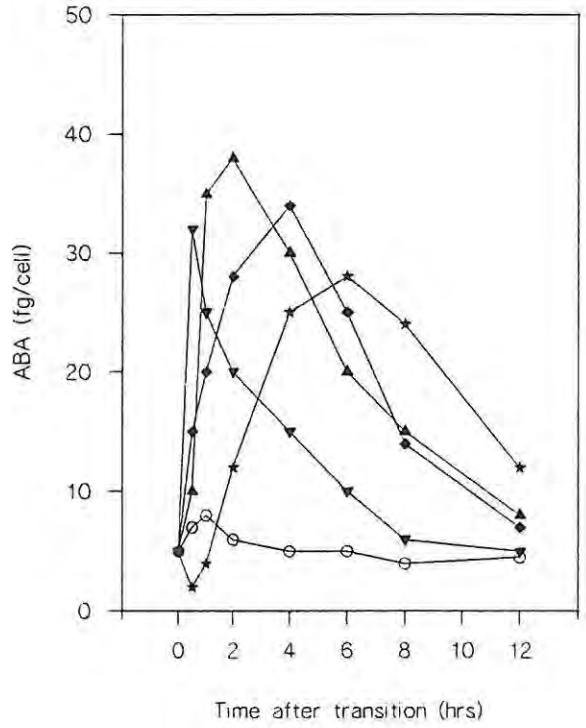


Figure 3.8. Changes in intracellular ABA content of *D. salina* cells exposed to various stresses under laboratory conditions. (○) - control; (★) - salt stress; (▲) - nitrate stress; (◆) - multiple stress; (▼) - high light stress. (Results represent the mean of three independant experiments).

3.2.7. The response of *D. salina* to stress conditions in mass culture.

Outdoor scale-up studies were undertaken to determine whether ß-carotene accumulation could be manipulated outdoors and whether similar physiological responses to stress similar to those exhibited by the cells under laboratory conditions could be obtained outdoors.

Experiments were undertaken in 20 L outdoor ponds and the ß-carotene, chlorophyll concentration and cell growth were monitored on a daily basis for 5 days after the onset of stress. Kinetic studies were carried out as more specific responses over time were required.

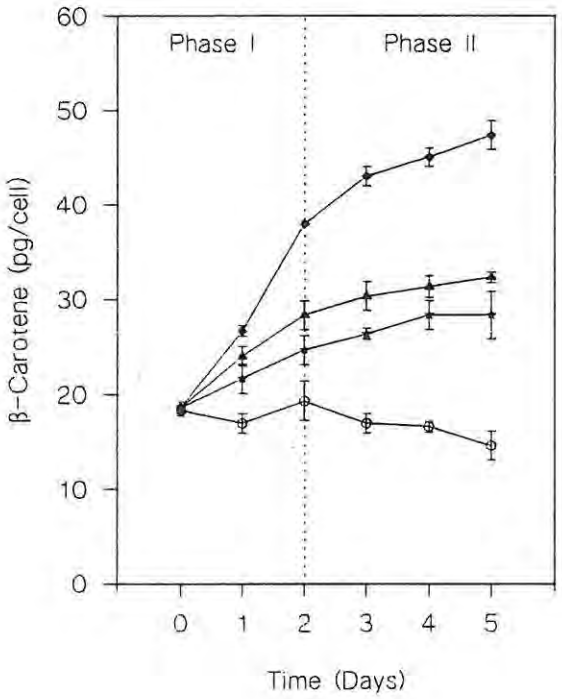


Figure 3.9. Changes in intracellular ß-carotene content of stressed and non-stressed cells of *D. salina* over a five day experimental period. (○) - control; (★) - salt stress; (▲) - nitrate limitation stress; (◆) - multiple stress. Phase I and II refer to 0-2 days and 3-5 days after transition of cells. Results reflect the mean of at least three experiments. Vertical bars represent SD.

Intracellular ß-carotene content was greatest in cells exposed to multiple stress, followed by nitrate limitation and salt stress (Figure 3.9.).

A decrease in cellular chlorophyll content was observed for all the stresses investigated with multiple stress showing the greatest decrease (Figure 3.10.). This reduction in cellular chlorophyll indicates a reduction in the size of the photosynthetic apparatus.

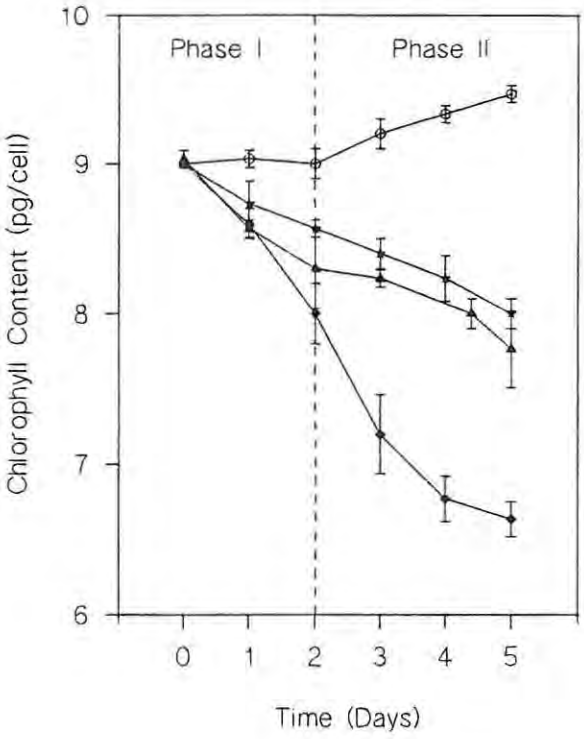


Figure 3.10. Change in chlorophyll content of stressed and non-stressed *D. salina* cells over a five day experimental period. (o) - control; (★) - salt stress; (▲) - nitrate limitation stress; (♦) - multiple stress. Phase I and II refer to 0-2 days and 3-5 days after transition of cells. Results reflect the mean of at least three experiments. Vertical bars represent SD.

The imposition of stress on *D. salina* cells had a marked effect on the growth rate. It was noted that the more severe the stress the more the growth decreased. It is apparent from Figure 3.11. that cell growth rate was less inhibited in response to nitrate limitation stress and salt stress than in the cells exposed to multiple stress.

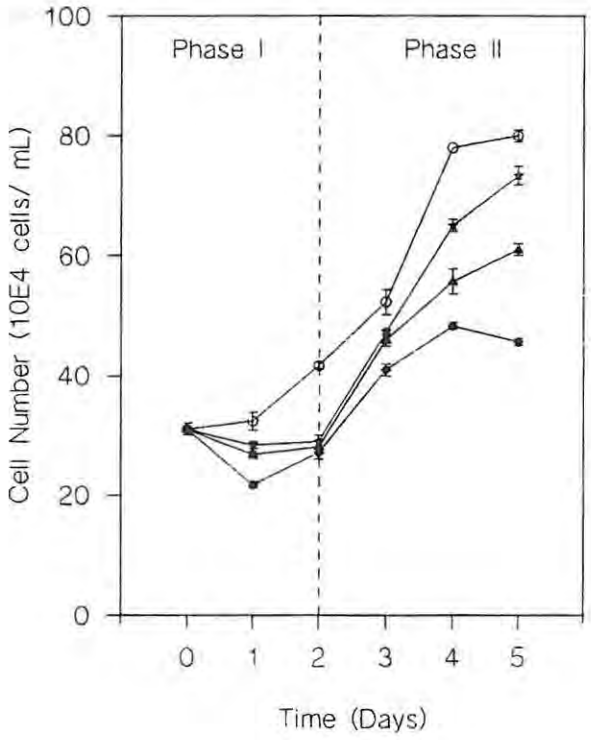


Figure 3.11. Growth curves of stressed and non-stressed *D. salina* cells over a five day experimental period. (○) - control; (★) - salt stress; (▲) - nitrate stress; (♦) - multiple stress. Phase I and II refer to 0-2 days and 3-5 days after transition of cells. Results reflect the mean of at least three experiments. Vertical bars represent SD.

Interestingly, the above results indicate that two distinct phases of ß-carotene accumulation, chlorophyll depletion and growth by *D. salina* can be observed in response to stress with an apparent transition at the end of Day 2 (rates changes for Phase I and II are shown in Figures 3.12 - 3.14.). The first occurred shortly after transition to stress and was characterised by rapid onset of ß-carotene production, chlorophyll depletion and a reduction in the growth rate. The second phase observed from 3 days onwards, was characterised by a slower rate of ß-carotene accumulation, relatively stable chlorophyll content and partial recovery of the growth rate. The initial rate of ß-carotene accumulation, chlorophyll depletion and growth rate was related to the type and magnitude of stress to which the cells were exposed. The magnitude of the stress was characterized by net ß-carotene increase after 5 days (Figure 3.15.).

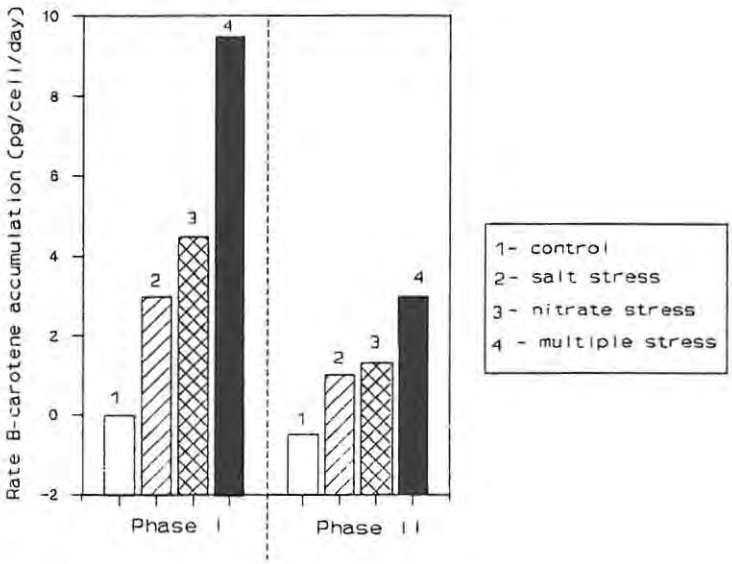


Figure 3.12. Rate changes of ß-carotene accumulation during the two distinct phases of the stress response for *D. salina* cells exposed to salt, nitrate and multiple stress. Rates were determined using the data illustrated in Figure 3.9.

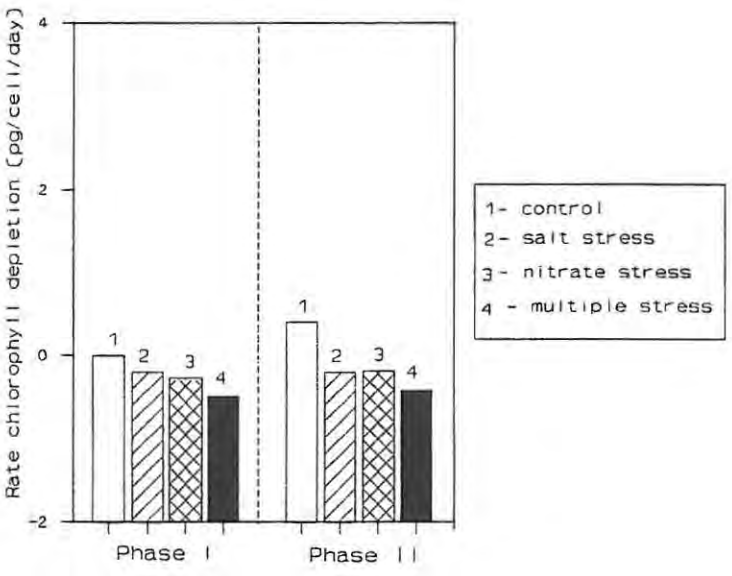


Figure 3.13. Rate changes of chlorophyll depletion during the two distinct phases of the stress response for *D. salina* cells exposed to salt, nitrate and multiple stress. Rates were determined using the data illustrated in Figure 3.10.

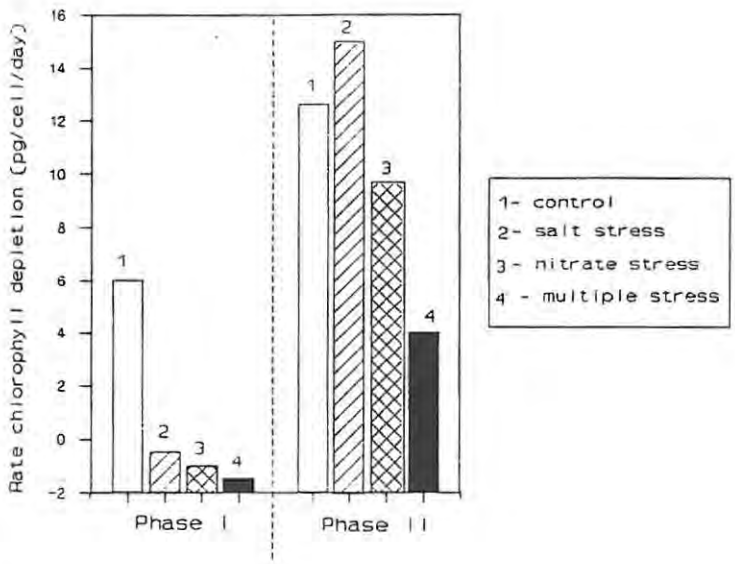


Figure 3.14. Rate changes of cell growth during the two distinct phases of the stress response for *D. salina* cells exposed to salt, nitrate and multiple stress. Rates were determined using the data illustrated in Figure 3.11.

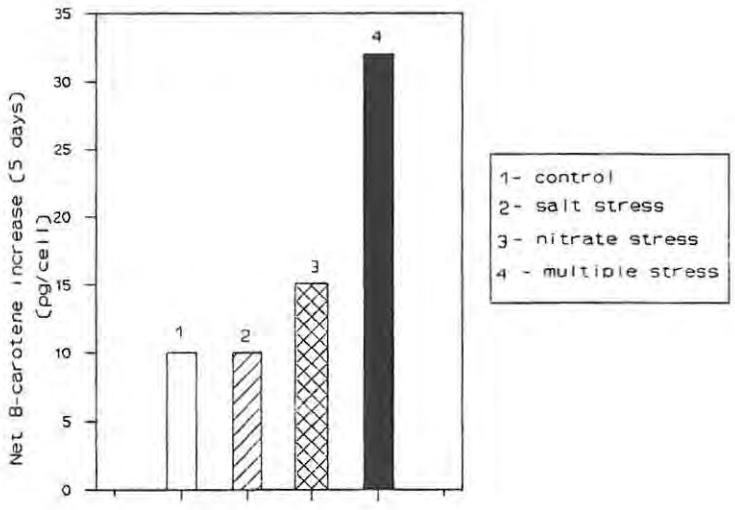


Figure 3.15. Net ß-carotene increase after 5 days in *D. salina* cells after exposure to salt, nitrate, and multiple stress. (Results refelct the mean of at least three experiments).

3.2.8. Abscisic acid content of the cells and media of stressed and non-stressed *D. salina* cells in mass culture.

An increase in the ABA content of *D. salina* cells exposed to various stresses under laboratory conditions was demonstrated in 3.2.6. Thus far all experiments which have investigated changes in the ABA content of algae exposed to stress have taken place under controlled laboratory conditions. It was therefore crucial to demonstrate the production of ABA by *D. salina* cells cultured outdoors in the stress phase of the dual-stage process. Changes in the ABA content of the cells and media were examined during the first 12 hours.

The results presented in Figure 3.16. show that the ABA content of the cells rapidly increased in response to the stresses investigated. The increase was, however, transient and within 24 hours the ABA content in the cells had declined to near basal level. A similar but less dramatic response was detected in control cultures (Figure 3.16.A), suggesting that cells may have experienced stress conditions during the harvesting procedure (see Materials and Methods, section 2.3.3.4.).

The accumulation of ABA in cells exposed to salt stress, nitrate limitation and multiple stress indicates that ABA production increased as a result of these stresses.

An increase in ABA concentration in the media was observed for all treatments, but was not observed for the control cultures (Figure 3.16.A). Moreover, the ABA content within the media of the stressed cells increased exponentially at first and then remained fairly constant throughout the incubation period (Figure 3.16 B-C), whereas the ABA level in the medium of the control cultures did not change significantly (Figure 3.16.A). These data indicate that, under the above-mentioned stress conditions, there is an immediate response in terms of increased ABA production and that the bulk of this ABA is partitioned into the culture medium.

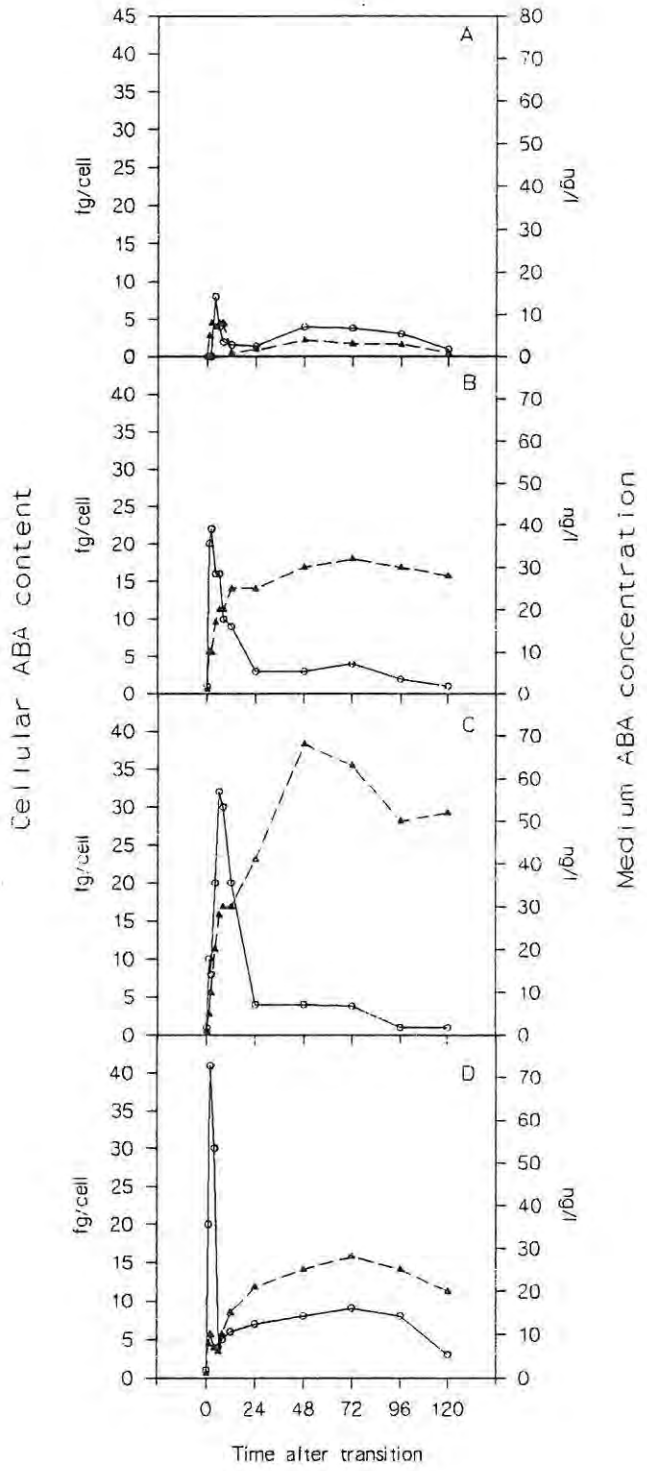


Figure 3.16. (A-D). Time course study illustrating changes in intracellular ABA content and medium ABA content of stressed and control cells of *D. salina*. A - control; B - salt stress; C - nitrate limitation; D - multiple stress. (o) - cellular ABA content; (▲) - medium ABA concentration.

Summary:

It is evident from the results described in this chapter that in response to stress, *D. salina* cells exhibit characteristic responses, including a reduced growth rate, an initial reduced rate of carbon fixation and oxygen evolution, volume changes, massive accumulation of ß-carotene, chlorophyll depletion, enhanced ABA metabolism and partitioning of ABA into the culture medium.

CHAPTER 4

ABA as a Regulator of Carotenogenesis in *D. salina*.

4.1. Introduction.

It is well established that environmental factors, such as light, temperature, oxygen, carbon, nitrogen and minerals can alter the amount and composition of carotenoids produced by organisms (Bramley, 1985). External factors such as nutrient limitation, high salinities and high light intensities are associated with the accumulation of ß-carotene in *Dunaliella* species (Ben-Amotz and Avron, 1983, 1989; Borowitzka and Borowitzka, 1988; Borowitzka, *et al.*, 1990) and this has been confirmed in the studies reported in Chapter 3. Furthermore, it has been shown that sub-lethal concentrations of copper and lead, and high temperatures can also induce ß-carotene accumulation (Pace, *et al.*, 1977; Lustigman, 1986).

Recent evidence suggests that stress-induced active oxygen species (i.e. $^1O_2$ and H_2O_2) are involved in the induction of ß-carotene accumulation in *Dunaliella* and do so by activating the transcription of ß-carotene biosynthetic genes (Shaish, *et al.*, 1993). In addition, a number of compounds are known to inhibit and/or stimulate carotenoid biosynthesis. These include trisporic acid and related compounds such as ß-ionone and ABA (Bramley, 1985). The bioinduction of carotenogenesis by ABA has been demonstrated in the fungus *Blackeslea trispora* (Dandekar, *et al.*, 1980). Norman (1991) recently demonstrated that a rapid increase in ß-carotene content in the fungus *Cercospora rosicola* occurs concurrently with rapid ABA production. In fact, ABA has been shown to stimulate the carotene biosynthetic pathway by *ca* 100% in *B. trispora* (Dandekar, *et al.*, 1980).

The ubiquitous involvement of ABA in signalling and mediating stress responses in plants has led to ABA being referred to as a "stress hormone" (Zeevaart and Creelman, 1988). It has also been thought of as a growth inhibitor due to its role in leaf abscission, and seed and bud dormancy. It is, however, now known to have multiple roles in higher plants including

regulating root growth, respiration, fruit ripening and senescence where it is associated with the accumulation of carotenoid pigments (Owen and Napier, 1988). The possibility therefore exists that the accumulation of ß-carotene in *D. salina* is hormonally mediated.

Cowan and Rose (1991) demonstrated an interrelationship between ABA production and ß-carotene accumulation in *D. salina* exposed to salinity stress. The enhanced production of ABA in cells of *D. salina* and the subsequent partitioning of ABA into the culture medium in response to salt, nitrate, and multiple stress has also been demonstrated (Chapter 3). Thus it seemed pertinent to investigate the possible role of ABA in contributing to enhanced carotenogenesis in *Dunaliella.*

4.2. Results.

4.2.1. Demonstration of the ß-carotene/ABA interrelationship.

High light stress was identified in Chapter 3 as the single most important factor influencing ß-carotene accumulation in *D. salina* cells. In order to manipulate ß-carotene in the alga, a clearer understanding of the physiological responses associated with light-induced ß-carotene accumulation is required.

In order to obtain further information regarding the proposed interrelationship between ß-carotene accumulation and ABA production in stressed cells of *D. salina*, radiolabelling studies were carried out. These involved cultivation and transfer of cells to stress conditions in the presence of $NaH[^{14}C]O_3$, whereafter the changes in labelled ß-carotene and labelled ABA were monitored. Additionally, the change in specific activity of both ß-carotene and ABA was determined following exposure of the cells to high light stress.

The results presented in Figure 4.1. show two distinct phases of enhanced ß-carotene accumulation. The first phase occurred 2 hours after transfer to high light, followed by a second more pronounced phase of ß-carotene accumulation that occurred approximately 6 hours after transfer. The identical trend was observed regarding changes in the specific

activity of ß-carotene (Figure 4.2.). This finding may indicate a switch from "stored starch" to "newly fixed carbon" as the source of accumulated ß-carotene.

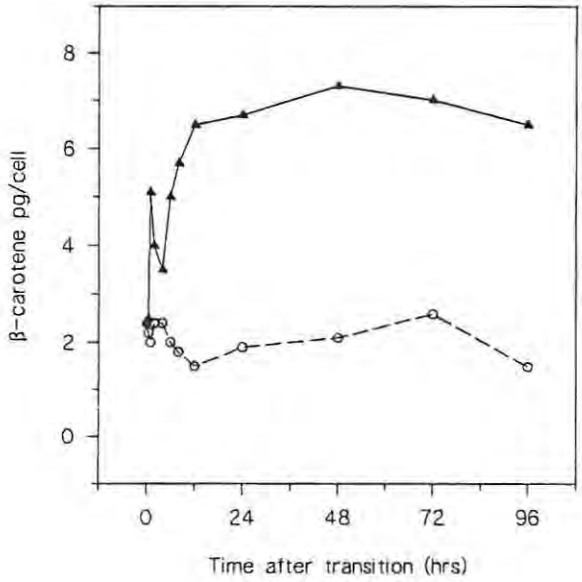


Figure 4.1. Changes in intracellular ß-carotene content of *D. salina* cells exposed to high light stress. (○)-control; (▲)-high light stress. Results reflect a single experiment.

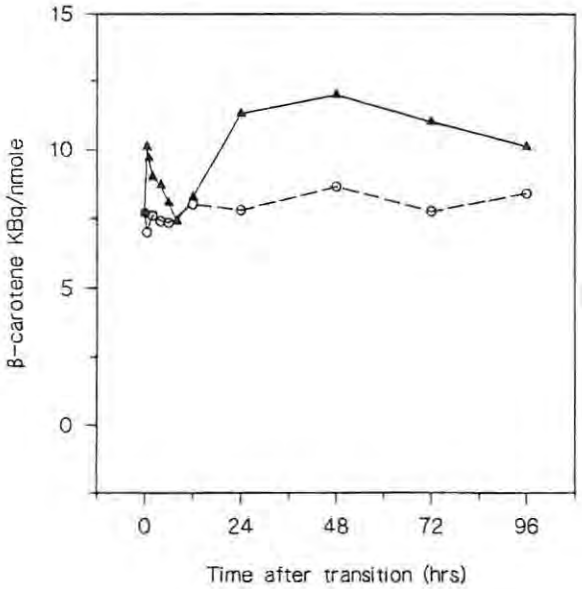


Figure 4.2. Changes in ß-carotene specific activity in *D. salina* cells exposed to high light stress. (○)-control; (▲)-high light stress. Results reflect a single experiment.

Intracellular ABA levels reached a maximum two hours after exposure to high light and then declined to basal level (Figure 4.3.). The same trend was noted in terms of changes in the

specific activity of ABA (Figure 4.4.), suggesting that enhanced ABA production occurred coincident only with the first phase of ß-carotene accumulation.

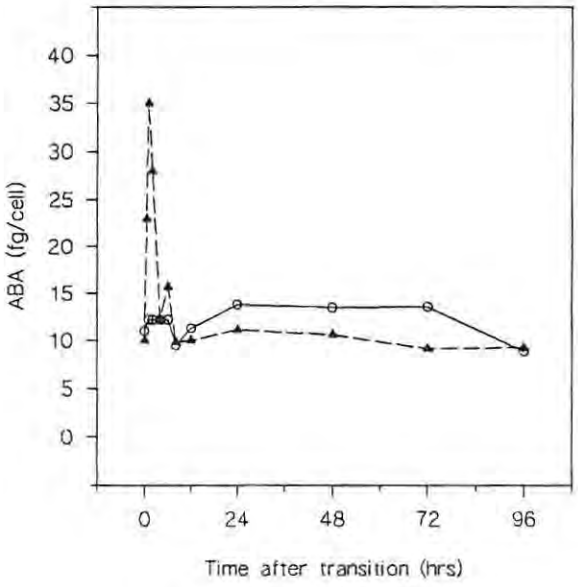


Figure 4.3. Changes in intracellular ABA content of *D. salina* cells exposed to high light stress. (○)-control; (▲)-high light stress. Results reflect a single experiment.

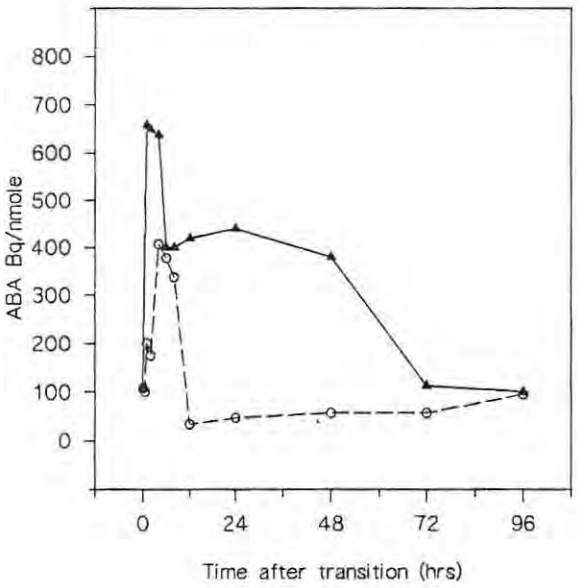


Figure 4.4. Changes in intracellular ABA specific activity in *D. salina* cells exposed to high light stress. (○)-control; (▲)-high light stress. (Results reflect the mean of three independant experiments.

In order to further elucidate this interrelationship, the rate of change of both ß-carotene and ABA production was calculated. These rates of change were only calculated for the first 8 hour period, during which time ABA production has been found to be maximal. The results, shown in Figure 4.5. indicate that the rate of ß-carotene accumulation within the first 2 hours coincided with the accelerated rate of ABA production.

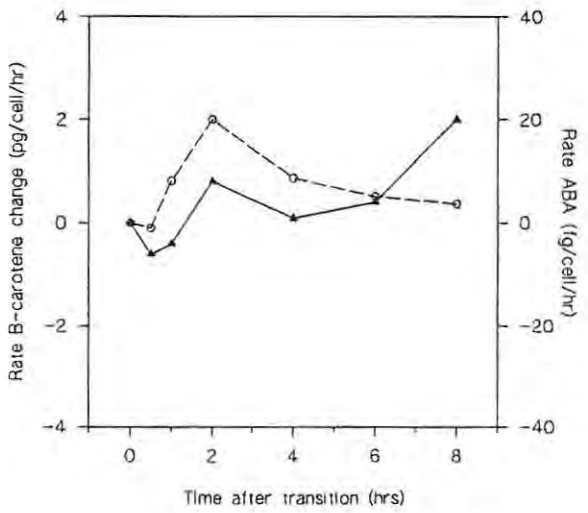


Figure 4.5. Rate of change of ß-carotene and ABA content of *D. salina* cells exposed to high light stress. (o) - ABA (fg.cell^{-1}/hr^{-1}); (▲) - ß-carotene (pg.cell^{-1}.hr^{-1}).

4.2.2. High-light-induced isomerization of ABA.

It is well known that ABA is light sensitive and is easily isomerized to the *cis* and *trans* isomers when exposed to high light intensity. When *D. salina* cells were exposed to high light both the *cis* and *trans* isomers of ABA were detected following analysis of acid extracts by HPLC. The results presented in Figure 4.6. show that in response to high light intensity, the *trans/cis* ratio of ABA in the cells increased immediately. However, this increase was transient since the ratio declined to basal level (approximately 0.2) for the remainder of the incubation period.

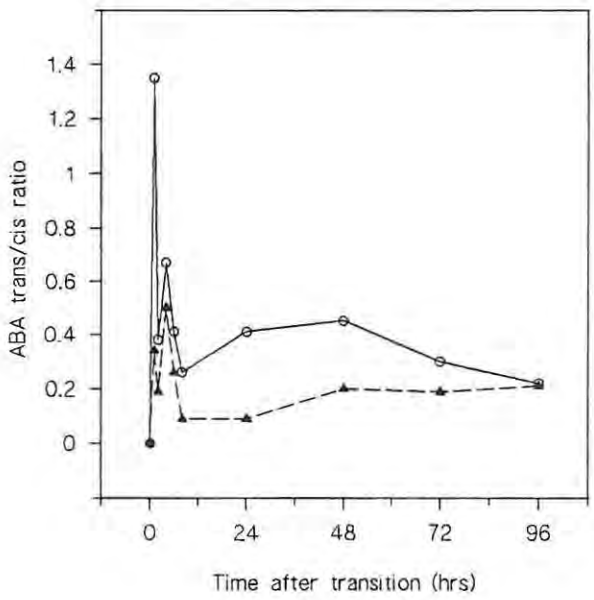


Figure 4.6. Changes in the *intracellular trans/cis* ABA ratio in *D. salina* cells exposed to high light stress. (○) - cells; (▲) - medium. (Results reflect a single experiment).

The *trans/cis* ratio in the culture medium increased only slightly during the first 8 hours, thereafter returning to near basal level. These results may indicate that a higher concentration of *trans*-ABA is found in cells and that the *cis* isomer may be preferentially partitioned into the culture medium. Furthermore, since the *trans*-isomer is purported to be biologically inactive (Kreidmann, *et al.*, 1972 cited in Stillwell and Hester, 1984), isomerization from *cis* to *trans* may represent a regulatory mechanism involved in stress-induced ß-carotene accumulation.

4.2.3. Salt stress-induced changes in intracellular ABA concentration.

In *D. salina,* hyperosmotic conditions result in cell shrinkage (Zimmerman, 1978), while hyposaline conditions cause the cells to swell (Maeda and Thompson, 1986). Clearly volume changes will impact on the intracellular ABA concentration and hence its biological activity. The impact of cell volume on changes in intracellular ABA concentrations was therefore investigated in cells exposed to hypersaline conditions. Cells were transferred from 1 M to

1.75 M, 2.5 M and 3 M NaCl and cell volume change was monitored using a Coulter Multisizer.

The results presented in Figure 4.7. illustrate rapid cell shrinkage in response to salt stress. Shrinkage was, however, transient, and cell volume was nearly re-established within 4-6 hours depending on the severity of the osmotic shock. Cells transferred from 1 M to 1.7 M NaCl regained 90% of their original cell volume within 3 hours whilst cells exposed to 3 M NaCl only regained 65% of their original cell volume during this period. The impact of these cell volume changes on the intracellular ABA concentrations is depicted in Figure 4.8.

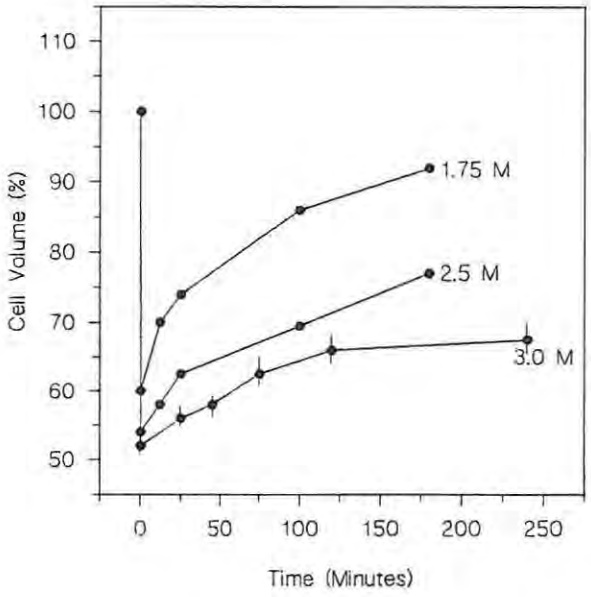


Figure 4.7. Cell volume changes of *D. salina* cells exposed to salt stress. Results represent the mean of at least three experiments for cells exposed to 3 M only. Vertical bars represent SD.

In response to salt stress, endogenous ABA concentration declined and the rate of decline was proportional to the severity of the osmotic stress. The loss in ABA concentration that was detected immediately after transfer of the cells to hypersaline conditions was mirrored by an increase in the ABA concentration of the culture (see Figure 3.8. section 3.2.5.). It should be noted that the increase in ABA concentration observed approximately 3 hours after the onset of stress was associated with the recovery of cell volume (Figure 4.8.).

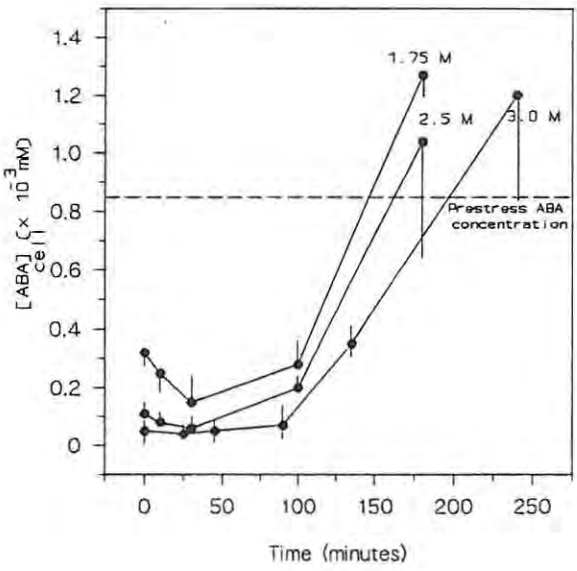


Figure 4.8. Changes in intracellular ABA concentration in response to hypersaline conditions. Prestress intracellular ABA concentration was 0.85 x 10^{-3} mM.cell^{-1}. ABA Results represent the mean of at least three experiments. Vertical bars represent SD.

4.2.4. Effect of exogenously applied ABA and related compounds on ß-carotene accumulation in *D. salina* cells.

The preferential partitioning of biologically active *cis* ABA into the culture medium in response to both high light and salt stress may suggest that this process is an integral part of the mechanism involved in the stress-induced accumulation of ß-carotene. This is particularly so given recent evidence to suggest the location of ABA receptors on the outside of higher plant cells (Anderson, *et al.*, 1994; Gilroy and Jones, 1994). Thus the effect of exogenously supplied ABA and related compounds on ß-carotene content of *D. salina* was investigated.

The results are presented in Table 4.1. and represent readings taken after 24 hours of exposure to each compound.

Table 4.1. The effect of exogenously applied ABA and related compounds (50 and 100 μmolar) on the accumulation of ß-carotene ± SD in cells of *D. salina*.*

ABA and related compounds	ß-carotene pg/cell (50 μmolar)	ß-carotene pg/cell (100 μmolar)
cis/trans ABA	5.4 ± 0.56 (112%)	5.01 ± 0.015 (no change)
trans ABA	5.5 ± 0.45 (114%)	4.78 ± 0.025 (95%)
ABA Methyl ester	5.3 ± 0.50 (110%)	5.20 ± 0.100 (104%)
1',4'-*cis* ABAdiol	5.3 ± 0.42 (110%)	4.80 ± 0.050 (96%)
1',4'-*trans* ABAdiol	5.5 ± 0.62 (115%)	5.40 ± 0.090 (108%)
control	4.8 ± 0.23 (100%)	5.00 ± 0.015 (100%)

* Starting ß-carotene concentration was 5 pg/cell.

Exogenously applied ABA and related compounds generally increased ß-carotene accumulation in *D. salina* cells. Lower ABA concentrations (50 μM) increased cellular ß-carotene content by as much as 10 - 15% whereas the higher concentration applications (100 μM) only elicited a 4 - 8% increase in ß-carotene content.

Summary:

The results of this chapter confirm the interrelationship between the production of ABA and the onset of ß-carotene accumulation in *D. salina* cells exposed to conditions of stress. It appears as though partitioning/loss of the biological active form of ABA functions in the stress response. Furthermore, exogenously applied ABA and related compounds were shown to enhance ß-carotene accumulation albeit only slightly.

CHAPTER 5

Xanthophyll Cycle Operation in *D. salina*: A Possible Correlation with Enhanced ß-carotene Accumulation.

5.1. Introduction.

Photosynthetic organisms are capable of operating a light absorbing pigment system which ensures their survival over a wide range of irradiances, including high irradiances which are potentially destructive to the system (Demmig-Adams, 1990). At low irradiance, the use of excitation energy in photosynthesis is optimised. At high irradiance, excess excitation energy is dissipated while still maintaining effective energy use for photosynthesis (Horton, *et al.*, 1988). Excess light energy cannot be stored. This is potentially hazardous because it favours the over-reduction of the electron transport system, resulting in the formation of chlorophyll triplet states. These triplet excited states readily react with oxygen, which gives rise to highly reactive, excited states of oxygen and the production of free radical species such as H_2O_2 and O_2^-. The production of these radicals leads to the inactivation of enzymes, chlorosis, loss of integrity and cell death (Foyer, *et al.*, 1989).

When light levels rise gradually over longer periods (hours/days) a gradual increase in the photosynthetic capacity and a decrease in the size of the light-harvesting chlorophyll pigment bed occurs (as demonstrated in Chapter 3). This allows the additional light energy to be utilized in photosynthesis (Anderson, 1986). However, when light levels rise rapidly or when light levels exceed the capacity for dissipation, the reaction centre of PS II is subject to photoinhibition (Powles, 1984). Photoinhibition as previously mentioned, is characterised by a decrease in the quantum efficiency of PS II and is frequently observed when plants are exposed to high intensity radiation or when environmental stress reduces their ability to utilise absorbed light (Powles, 1984; Demmig-Adams, 1990).

Processes that prevent damage to PS II and/or formation of destructive oxygen species are therefore essential for the survival of the plant. One of these processes involves the diversion of excitation energy away from the reaction centres in the form of radiationless energy dissipation. This process involves the operation of the xanthophyll cycle, and in particular, the accumulation of the xanthophyll, zeaxanthin (Rees, *et al.*, 1989; Noctor, *et al.*, 1991; Gilmore and Yamamoto, 1991, 1992, 1993; Ruban, *et al.*, 1992).

Enzymic conversions of epoxy-carotenoids occur in many eukaryotic alga groups and higher plants (Hager, 1980). These reactions have been reported to operate as a "xanthophyll cycle" and involve the de-epoxidation of violaxanthin (diepoxyzeaxanthin) via antheraxanthin (monoepoxyzeaxanthin) to zeaxanthin. Zeaxanthin is then enzymically epoxidised along an independent pathway (Yamamoto, 1979). The de-epoxidation sequence of the cycle is light driven and responds to environmental stress (Demmig-Adams, 1990). The *in vitro* rate and extent of zeaxanthin formation are functions of lumen pH, ascorbate concentration, and violaxanthin availability (Yamamoto, 1979) (see Figure 5.1.). In plants that are able to tolerate stress, superoxide dismutase converts O_2^- to H_2O_2 which then reacts with ascorbate via ascorbate peroxidase to form oxygen and water (Bowler, *et al.*, 1992). Levels of ascorbate and other free radical scavenging agents increase in response to both high light and stress (Schöner and Krauser, 1990). Ascorbate serves to reduce violaxanthin to antheraxanthin and zeaxanthin (Yamamoto, 1979). The Mehler reaction has been implicated in the induction of the development of a large transthylakoidal pH gradient that promotes zeaxanthin formation (Schreiber, *et al.*, 1991). *In vivo*, zeaxanthin is formed when photosynthesis becomes limited, i.e. at high temperature or under combined environmental stresses (Demmig-Adams, *et al.*, 1988).

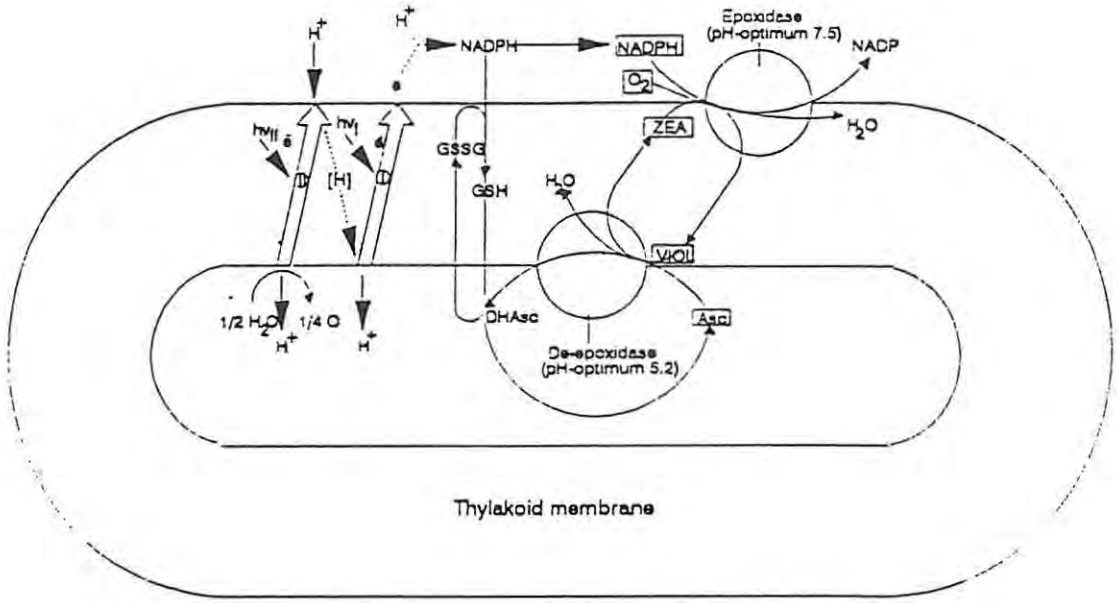


Figure 5.1. Localisation and mechanism of the xanthophyll cycle in the thylakoid membrane. The cyclic reaction can only occur because of the different pH optima of the epoxidase and de-epoxidase enzymes, when a light dependant proton gradient is formed across the thylakoid membrane.(GSH = glutathione, GSSG = oxidized glutathione). (taken from Hager, 1980).

Xanthophyll cycle operation can be characterised by calculating the percentage violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) of the (V+A+Z) pool. Epoxidation state (EPS) is a measure of the relative percentage of pigments within the (V+A+Z) pool and was derived from (0.5A+V)/(V+A+Z) as described by Thayer and Björkman (1990). This expression is an estimation of the actual number of epoxides over the maximum possible. Thus a large EPS (1.0) would indicate displacement of the cycle toward violaxanthin formation whereas a low EPS (0.001) would be indicative of increased zeaxanthin formation (de-epoxidation).

There is at present no information available concerning the operation of the xanthophyll cycle in *Dunaliella* and its possible interrelationship with enhanced ß-carotene accumulation when exposed to various stresses or changes in incident photon flux density (PFD). Experiments

described below examine the operation of the xanthophyll cycle in response to changes in diurnal PFD in outdoor cultures of *D. salina*. Experiments were carried out using *D. salina* cultured under either stressed (multiple stress) or non-stressed (growth) conditions.

5.2. Results.

5.2.1. Qualitative and quantitative changes in carotenoids in response to diurnal changes in PFD.

As shown in Figure 5.2.A, the ß-carotene content of cells grown in mass culture under stressed conditions was about four times that of non-induced (unstressed) cells. More important perhaps is the observation that the net change in ß-carotene content was similar for both treatments during the diurnal cycle (Figure 5.2.B).

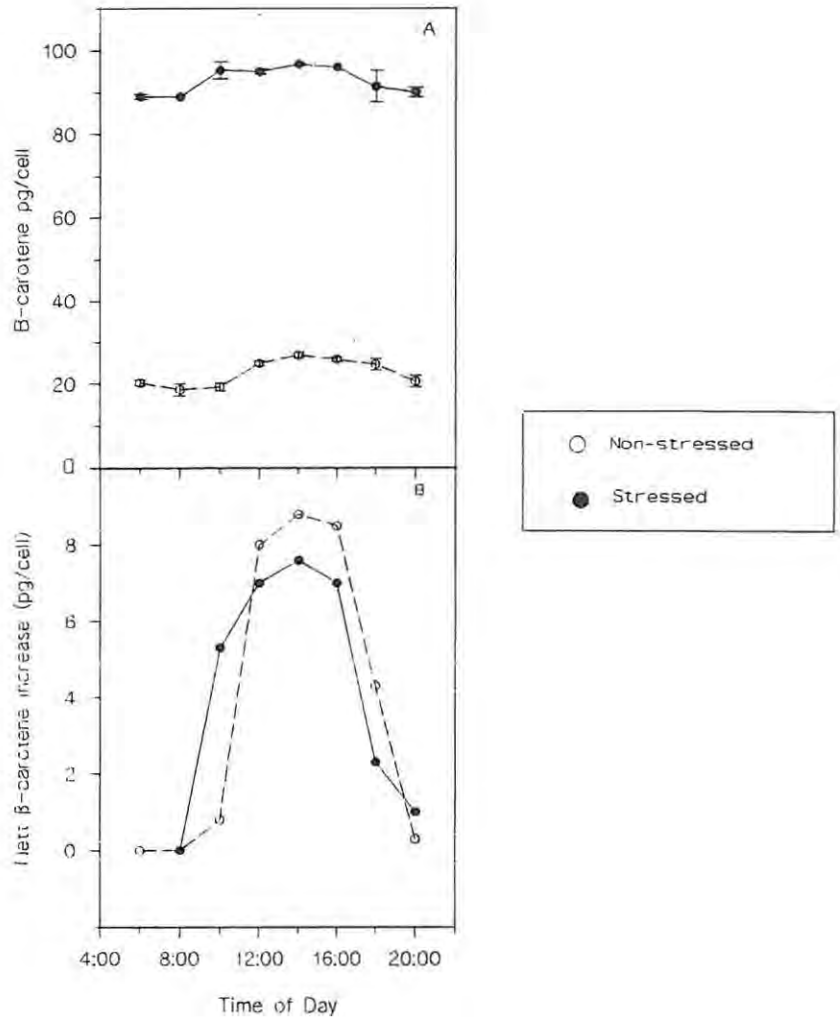


Figure 5.2.(A,B). Time course illustrating the change in intracellular ß-carotene content (A) and the net increase in ß-carotene levels (B) in stressed and non-stressed cells of *D. salina* during the diurnal cycle. Net increase in ß-carotene was calculated by subtracting the initial (T = 6:00) level from the level at the end time point of each interval. All data are representative of at least three replicates (± Standard Error Mean [SEM]).

This relationship is clearly seen in Figure 5.3. which shows that the light response curve for ß-carotene formation was biphasic for both the stressed and unstressed cells. In all cases, more ß-carotene was present at a given PFD during the descending part of the response curve. Interestingly, changes in the rate of ß-carotene formation were faster for stressed cells whereas the rate of decline in ß-carotene was identical for both treatments.

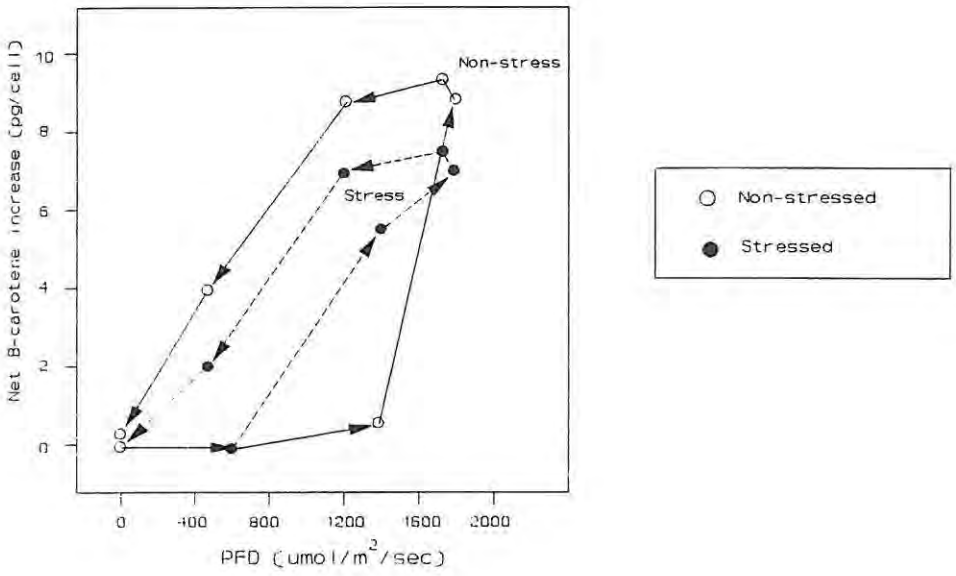


Figure 5.3. Changes in net ß-carotene content of stressed and non-stressed cells of *D. salina* in response to changes in PFD during the diurnal cycle. Data points were derived from the experiment presented in Figure 5.2.(B). Arrows indicate the course of changes in PFD and ß-carotene during the day.

Zeaxanthin and the xanthophyll cycle are considered to play a major role in photoprotection. As shown in Figure 5.4. marked differences were observed in the quantitative response of xanthophylls to diurnal changes in PFD. Firstly, levels of all major xanthophylls were higher in stressed cells suggesting that, at least for *Dunaliella*, increased irradiance was not the only factor involved in stimulating carotenoid accumulation. Secondly, xanthophyll cycle pigment (V+A+Z) content remained constant in stressed cells throughout the photoperiod whereas in non-stressed cells, xanthophyll cycle pigment content increased to a level similar to that observed for stressed cells and thereafter declined.

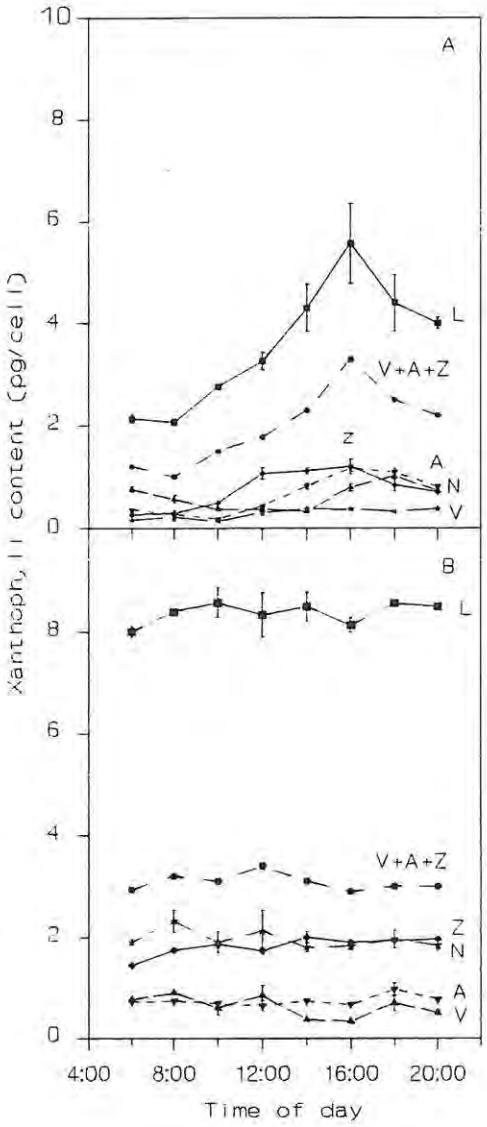


Figure 5.4.(A,B). Change in intracellular xanthophyll content of non-stressed (A) and stressed (B) cells of *D. salina* during the diurnal cycle. Xanthophylls were quantified by reversed-phase HPLC in the same acetone extracts prepared as described in Figure 5.2. All data are representative of at least three replicates (± SEM) L=lutein, Z=zeaxanthin, A=antheraxanthin, V=violaxanthin, N=neoxanthin, V+A+Z= violaxanthin + antheraxanthin + zeaxanthin. Vertical bars represent SD.

The light-response curve of zeaxanthin formation was found to be strongly biphasic in stressed and non-stressed cells (Figure 5.5.). Thus in non-stressed cells, the zeaxanthin content remained low with an initial increase in incident irradiation and only increased

strongly at PFD in excess of 1000 μmol.m^{-2}.s^{-1}. The lag phase, prior to an increase in zeaxanthin levels, may suggest either that non-stressed cells lack some factor (already present in stressed cells) or that some factor is below a critical threshold which when exceeded, facilitates rapid zeaxanthin formation and hence photoprotection.

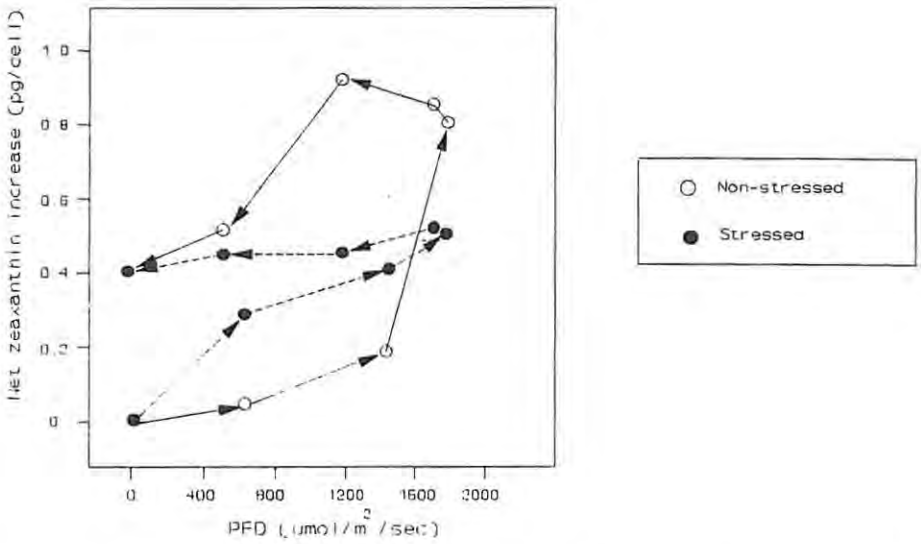


Figure 5.5. Changes in net intracellular zeaxanthin content of stressed and non-stressed cells of *D. salina* in response to changes in PFD during the diurnal cycle. Data points were derived from the experiment presented in Figure 5.4. Net zeaxanthin content was calculated by subtracting the initial (T = 6:00) level from the zeaxanthin level at the end point of each interval. Arrows indicate the course of change in PFD and zeaxanthin during the day.

5.2.2. Xanthophyll Cycle Operation.

The operation of the xanthophyll cycle in higher plants in response to diurnal changes in incident irradiation has recently been demonstrated (Demmig-Adams and Adams, 1992). In view of this, the change in xanthophyll cycle pigment composition, in response to changing PFD, was examined in more detail in stressed and non-stressed cultures of *D. salina*. When expressed as a percentage of the xanthophyll cycle pool (V+A+Z), zeaxanthin increased in both the stressed and the non-stressed cells with increasing PFD, apparently at the expense of violaxanthin (Figure 5.6.). Furthermore, it was evident that stressed cells contained substantially more zeaxanthin at the start of the photoperiod, and at all times during the photoperiod. By midpoint (peak PFD), however, cells in non-stressed cultures contained

similar amounts of zeaxanthin to those cells in stressed culture. This would seem to indicate that non-stressed cells produced more zeaxanthin than stressed cells in response to increasing irradiation. A decline in percentage zeaxanthin with decreasing PFD, occurred coincident with a considerable rise in the proportion of antheraxanthin in non-stressed cells (Figure 5.6.B) whereas in stressed cells, loss of zeaxanthin was mirrored only by an increase in violaxanthin (Figure 5.6.A).

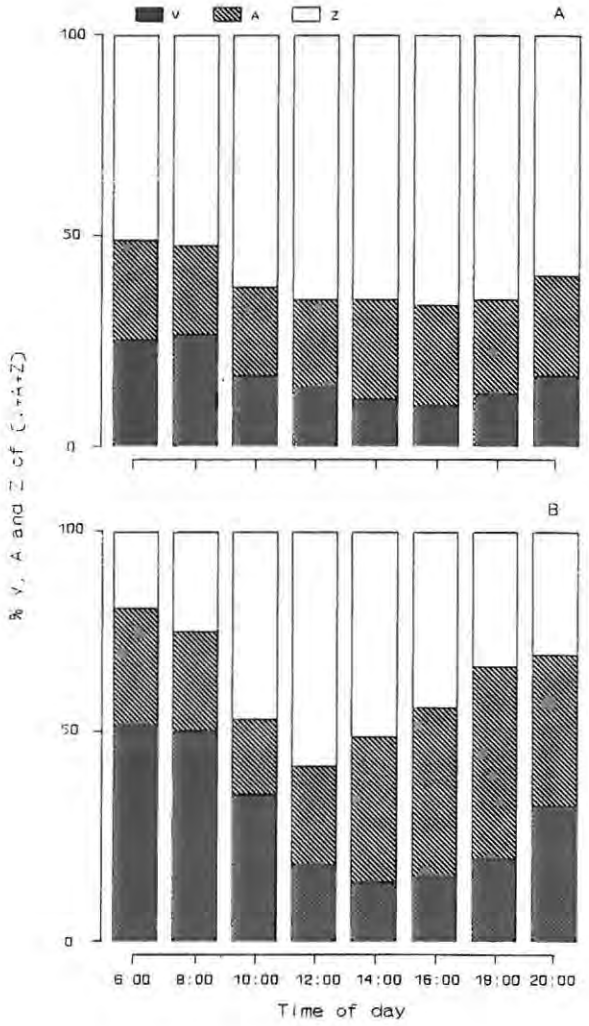


Figure 5.6. (A,B). Change in percentage violaxanthin, antheraxanthin and zeaxanthin of the xanthophyll cycle pool (V+A+Z) in stressed (A) and non-stressed (B) cells of *D. salina* during the course of the day. Data were calculated from the results presented in Figure 5.4. and represent the mean of three values.

Since non-stressed cells accumulated xanthophyll cycle pigments (V+A+Z) during the light period of the diurnal cycle (see Figure 5.4.A), the above observation suggests *de novo*

synthesis of zeaxanthin in response to increasing irradiance, some of which is subsequently transformed to antheraxanthin.

The results in Figure 5.7. summarize operation of the xanthophyll cycle in stressed and non-stressed cells of *D. salina* in mass culture under natural irradiance. Data is presented as net increase in zeaxanthin together with change in epoxidation state (EPS) in response to changing PFD on a diurnal basis.

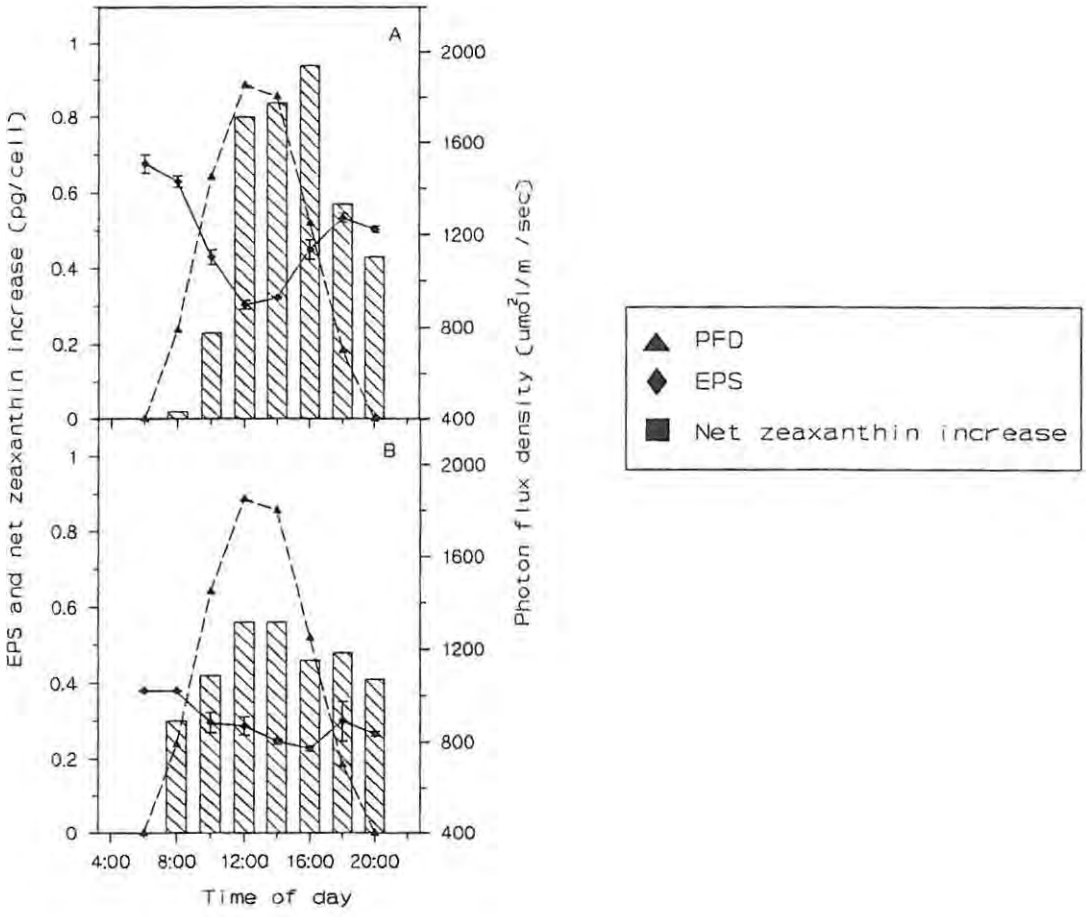


Figure 5.7. (A,B). Diurnal changes in PFD, the net increase in zeaxanthin levels, and the epoxidation state of the xanthophyll cycle (EPS = [violaxanthin + 0.5 antheraxanthin]/ [V+A+Z]) in non-stressed (A) and stressed (B) cells of *D. salina* cultured outdoors. Vertical bars represent SD.

It is clearly seen that low ß-carotene-containing cells form more zeaxanthin at high PFDs than ß-carotene-rich cells although the EPS at these PFDs is similar for both. This latter aspect probably reflects the substantial amount of zeaxanthin already present in stressed cells at the start of the photoperiod. Furthermore, these results suggest that irrespective of culture conditions (stressed or non-stressed) a given light regime induces a proportionate increase in the total xanthophyll cycle pool. Interestingly, zeaxanthin formation occurred earlier (at low PFDs) in stressed cells (Figure 5.7.B). This may be as a result of the stressed cells becoming susceptible to photoinhibition at lower PFDs, i.e. that low intensity PFD is perceived as "excess irradiation" in stressed cells.

A summary of the photosynthetic characteristics of both stressed and non-stressed *D. salina* cells at maximum PFD during the diurnal cycle is shown in Table 5.1.

Table 5.1. Photosynthetic characteristics of stressed and non-stressed cells of *D. salina* in mass culture at peak photon flux density (1830 μmol.m^{-2}.s^{-1}). Data are representative of at least three replicates ± SD. (ψi = photosynthetic energy conversion; P_{O2} = Photosynthetic rate)

Property	Non-stressed	Stressed
P_{O2}(μmol O_2.mg chl^{-1} h^{-1})	395.0±15.0	420.0±5.0
$\psi_i = P_{O2}$/PFD	0.22	0.23
Chl (*a* + *b*) (pg.cell^{-1})	13.4±2.54	5.75±1.27
ß-carotene (pg.cell^{-1})	27.0±0.82	96.0±2.16
9 *cis*/*all trans*-ß-carotene (g.g^{-1})	1.62	3.80
Chl (*a*+*b*)/ ß-carotene (g.g^{-1})	0.49	0.06
V+A+Z (pg.cell^{-1})	5.64±0.25	9.45±0.45
EPS	0.3±0.02	0.35±0.04
V+A+Z/P_{O2}	0.014	0.023

As expected both chlorophyll content and the chlorophyll (a + b)/ß-carotene ratio were markedly reduced in ß-carotene-rich cells. Photosynthetic rate (P_{O2}), determined on a chlorophyll basis, was however similar for both treatments. Likewise, the efficiency of photosynthetic energy conversion (ψi), determined at the PFD to which cells were exposed at the time of EPS and (V+A+Z) pool size measurement (Thayer and Björkman, 1990), did not differ significantly between the treatments.

The ratio $(V+A+Z)/P_{O2}$, an estimation of the proportion of energy dissipated by the xanthophyll cycle in relation to energy utilised in photosynthesis, was however, lower for non-stressed than stressed cells. The substantial zeaxanthin content of ß-carotene-rich cells, coupled with a similar EPS for both treatments suggests that zeaxanthin accumulation and operation of the xanthophyll cycle function to sustain photosynthesis in high ß-carotene-containing cells at high irradiance.

5.2.3. The violaxanthin, zeaxanthin, ß-carotene interrelationship in stressed cells of *D. salina*.

D. salina cells grown in mass culture were exposed to stress conditions and the daily changes in violaxanthin, zeaxanthin and ß-carotene were monitored over a five day period. Regression analysis of violaxanthin versus zeaxanthin at each time point for each treatment are presented in Figure 5.8. The slope of the curve as well as the correlation coefficients for this experiment are tabulated in Table 5.2.

It is evident from Figure 5.8. that an increase in zeaxanthin content results in a decrease in violaxanthin content for all the stresses investigated except salt stress. This indicates de-epoxidation of violaxanthin to zeaxanthin in response to stress in *D. salina* cells.

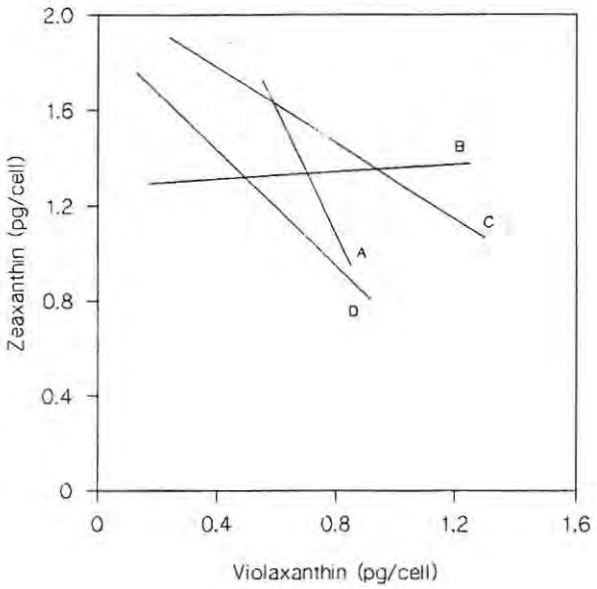


Figure 5.8. Linear regressions of violaxanthin content versus zeaxanthin content for (A)- control, (B)- salt stress (3M), (C)- nitrate limitation (0,2 mM) and (D)- multiple stressed cultures of *D. salina*. Calculations were done using daily values of zeaxanthin and violaxanthin for a five day period after transition of the cells to stress/growth media. (n) = 10. Results are tabulated in Table 5.2.

Table 5.2. Regression analysis of violaxanthin versus zeaxanthin of stressed and non-stressed cells of *D. salina* in mass culture.

Stress Type	Slope of curve	r^{2*}
No-stress (A)	-3.09	0.85
Salt stress 3M (B)	0.077	0.14
Nitrate limitation (C)	-0.83	0.80
Multiple stress (D)	-1.18	0.99

* r^2 = correlation coefficient.

The data presented in Table 5.2. indicates that for both nitrate limitation stress and multiple stress there is a near 1 to 1 relationship between changes in zeaxanthin content and changes in violaxanthin content of the cells as the slope of the curve tends towards 1. An absolute 1 to 1 stoichiometric relationship is however not anticipated because of the role played by antheraxanthin in the xanthophyll cycle.

A strong correlation between zeaxanthin and violaxanthin was not found and the slope of the curve tended towards being horizontal for salt stressed cells. This indicates that xanthophyll cycle operation and, in particular, zeaxanthin accumulation is not crucial in the protection of salt stressed cells over the long term (5 days).

A strong correlation between zeaxanthin and violaxanthin was noted for the cells grown in growth medium. The slope of the curve did not however, tend towards 1, confirming *de novo* synthesis of zeaxanthin in unstressed cells (Figure 5.8.; see also section 5.2.2.).

5.2.4. Relationship between ß-carotene and zeaxanthin content of stressed *D. salina* cells in mass culture.

In view of the results obtained from the diurnal study and recent evidence supporting the biosynthesis of zeaxanthin from ß-carotene (Sandmann, 1991), a strong interrelationship between zeaxanthin and ß-carotene accumulation in stressed *D. salina* cells was intimated. A long term study (5 days) was undertaken in order to determine the correlation between ß-carotene and zeaxanthin content of stressed cells of *D. salina*. Regression analysis of zeaxanthin versus ß-carotene at each time point for each stress treatment are presented in Figure 5.9.

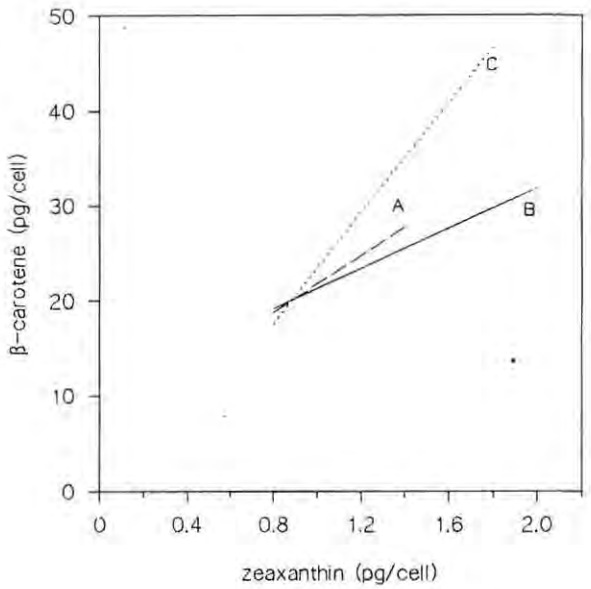


Figure 5.9. A linear regression of zeaxanthin versus ß-carotene content for (A)- salt stress (3 M) (r^2 = 0.81), (B)- nitrate limitation (0.2 mM) (r^2 = 0.83), (C)- multiple stressed (r^2 = 0.98) cultures of *D. salina*. Calculations were based on daily values of ß-carotene and zeaxanthin for a five day period after transition of the cells to stress/growth media. (n) = 10.

A strong positive correlation ($r^2 \geq 0.81$) between ß-carotene and zeaxanthin content was demonstrated for all the stress treatments. It should also be noted that a closer correlation between ß-carotene and zeaxanthin was apparent in the cells where the stress treatment was perceived as being more severe by the cells (indicated by higher ß-carotene content). This relationship was also noted for violaxanthin and zeaxanthin in cells exposed to nitrate limitation and multiple stress (see 5.2.3 above).

Examination of the (V+A+Z) pool size in the same cells used in the above experiment showed that (V+A+Z) remained basically unchanged throughout the experimental period (see Figure 5.10). It was however interesting to note that the EPS of the cells declined steadily during this time for all the stresses investigated. Regression analysis of EPS versus ß-carotene at each time point for each treatment is represented in Figure 5.11. Again a strong positive correlation ($r^2 \geq 0.78$) between ß-carotene and EPS was demonstrated for all treatments. This indicates that high zeaxanthin levels whether originating via *de novo*

synthesis or through de-epoxidation reactions is correlated to high levels of ß-carotene within the cells.

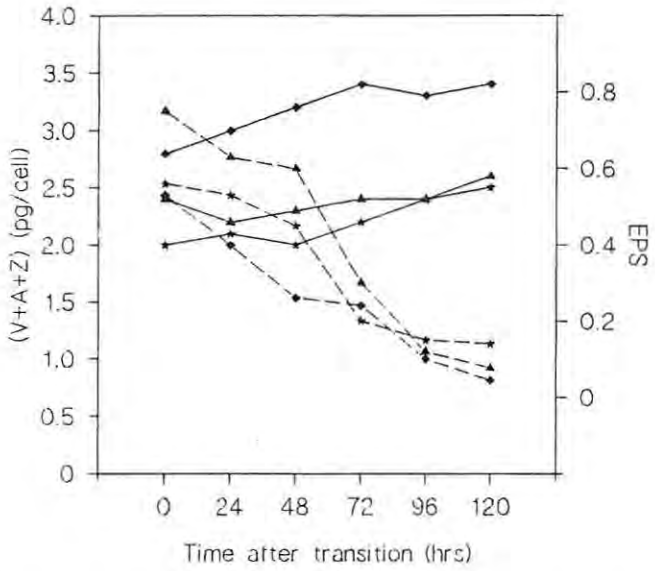


Figure 5.10. Xanthophyll cycle pool size (V+A+Z) and EPS in *D. salina* cells exposed to various stress conditions. (★) - salt stress; (▲) - nitrate stress; (◆) - multiple stress. (-) - (V+A+Z); (..) - (EPS). Data represents the mean of two independant experiments.

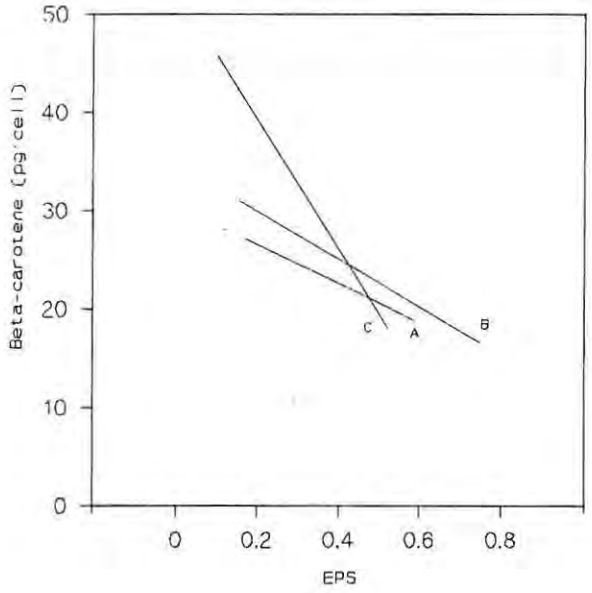


Figure 5.11. A linear regression of ß-carotene versus EPS for (A)- salt stress (3 M) (r^2 = 0.90), (B)- nitrate limitation (O.2 mM) (r^2 = 0.78), (C)- multiple stressed (r^2 = 0.99) cultures of *D. salina*. Calculations were based on daily values of EPS and ß-carotene for a five day period after transition of the cells to stress/growth media. (n) = 10.

Summary:

The results of this chapter indicate that a strong interrelationship exists between ß-carotene accumulation and zeaxanthin accumulation in stressed cells of *D. salina*. Furthermore, the biosynthesis of ß-carotene together with the operation of the xanthophyll cycle appear to act in concert to sustain photosynthetic energy conversion in stressed *D. salina* cells.

CHAPTER 6

Biosynthetic Interrelationship between Abscisic Acid and Carotenoids in Cells of *D.salina*.

6.1. Introduction.

The bulk of evidence suggests that ABA is an apocarotenoid derived along the following pathway; ß-carotene → ß-cryptoxanthin → zeaxanthin → antheraxanthin → *trans*-violaxanthin → 9-*cis*-neoxanthin → 9-*cis*-violaxanthin → xanthoxin → ABA-aldehyde → ABA (Zeevaart, *et al.*, 1989; Li and Walton, 1990; Parry, *et al.*, 1990; Rock and Zeevaart, 1990; Rock, *et al.*, 1992). Crucial to this pathway is the involvement of the xanthophyll cycle. Recent reports have shown that increased ABA biosynthesis occurs coincident with changes in levels of ß-carotene (Cowan and Rose, 1991; Norman, 1991). This was also demonstrated in Chapter 3 and 4 of this thesis.

It was demonstrated in Chapter 3 that stressed cells of *D. salina* accumulate ABA and that the bulk of the ABA is partitioned into the culture medium. In Chapter 4, the accumulation of ß-carotene coincident with enhanced ABA production was confirmed. Lastly, a good correlation between ß-carotene accumulation and xanthophyll cycle turnover was demonstrated in Chapter 5. In an attempt to elucidate the metabolic interrelationship between ABA production and stress-induced accumulation of ß-carotene, the following aspects were investigated in detail; 1) stress-induced change in xanthophyll cycle content, 2) the turnover of xanthophyll cycle pigments, and 3) the stoichiometric relationship between alterations in the xanthophylls and ABA.

6.2. Results.

6.2.1. ABA production and its relationship to xanthophyll cycle activity and ß-carotene accumulation in *D. salina* cells.

A preliminary experiment was carried out to quantify the changes of both xanthophylls and ß-carotene in cells exposed to salt stress and high light stress. The results presented in Table 6.1. indicate that shortly after imposition of salt stress (6 hours), percentage composition of the xanthophyll pool (V+A+Z) changed in favour of all-*trans*-violaxanthin production. In contrast, the percentage composition of the xanthophyll cycle of high light treated cells favoured the production of zeaxanthin, indicating de-epoxidation of violaxanthin and xanthophyll cycle turnover. Maximum ABA production was also observed by 6 hours in both high light treated and salt stressed cells. Maximum ABA production by approximately 6 hours was also previously demonstrated for both high light and salt stressed cells (see Chapter 4, Section 4.2.1 and 4.2.2.).

At 6 hours the intracellular ß-carotene content of salt-stressed cells was lower than the starting level, whilst the ß-carotene content of high light treated cells was greater than the starting ß-carotene level by this time point. Five days after exposure to both stress conditions, ß-carotene accumulation had reached a maximum for the study period and this increase was associated with increased levels of zeaxanthin and decreased levels of violaxanthin.

In order to elucidate more fully the response of the xanthophyll cycle (V+A+Z), the degree of epoxidation of the (V+A+Z) pool was calculated. The epoxidation state was highest at six hours and lowest at five days for salt-stressed cells. This increase in EPS at six hours suggested that an early response to salt stress was the formation of all-*trans*-violaxanthin, which coincided with the maximal rate of ABA production. The EPS for high light-treated cells declined progressively from 0 hours to 5 days, suggesting a faster turnover of the xanthophyll cycle.

Table 6.1. Xanthophyll cycle activity and its relation to ß-carotene and ABA production in stressed *D. salina* cells.

Algal cells were resuspended in either 3 M NaCl medium or 1.5 M NaCl medium. Incubations were carried out at 27 °C under low light illumination (90 μmol.m^{-2}.s^{-1}). For high light intensity, cells grown in 1.5 M NaCl-medium were exposed to illumination of (1000 μmol.m^{-2}.s^{-1}) at 27 °C. At 6 hours and 5 days cell number was determined, 50 mL aliquots of cells harvested by centrifugation and levels of carotenoids and ABA determined in the same acetone extracts by C_{18} reversed-phase HPLC and quantified by peak integration. Results are the mean of three replicates ± standard deviation.

TREATMENT	TIME	V+A+Z	V	A	Z	EPS[a]	N[b]	BETA-CAROTENE	ABA
		(pg/cell)	(% of V+A+Z)				(pg/cell)	(pg/cell)	(fg/cell)
1.5 M CONTROL	0 h	2.04	49	26	25	0.62	0.20±0.04	3.0±0.4	10±1.0
	6 h	1.78	50	27	23	0.63	0.20±0.04	2.9±0.3	25±1.0
	5 d	1.5	50	28	22	0.64	0.28±0.01	2.2±0.1	12±2.0
3.5 M NaCl	0 h	2.04	49	26	25	0.62	0.2 0±0.01	3.0±0.4	10±1.0
	6 h	1.98	56	21	23	0.66	0.16±0.01	2.8±0.0	74±1.5
	5 d	1.91	11	53	36	0.37	0.17±0.20	6.0±0.5	6.0±3.0
HIGH LIGHT	0 h	2.04	49	26	25	0.62	0.20±0.03	3.0±0.4	10±1.0
	6 h	2.15	25	20	45	0.35	0.18±0.01	3.2±0.2	20±4.2
	5 d	2.57	1	17	82	0.09	0.24±0.02	12.0±2.0	6.0±4.0

[a] EPS=(0.5A+V)/(V+A+Z)

[b] Expressed as sum of both 9'-cis- and trans-isomers

6.2.2. Changes in xanthophyll cycle pigment content in response to high light stress.

The results presented in Figure 6.1. show that soon after exposure of cells to high light stress, violaxanthin content increased, but the increase was transient as violaxanthin levels began to decrease and were more than 50% depleted by 12 hours. Intracellular zeaxanthin content began to increase immediately after exposure to high light stress, and continued to

accumulate up to 12 hours after the onset of stress. Antheraxanthin content remained unchanged throughout the duration of the experiment.

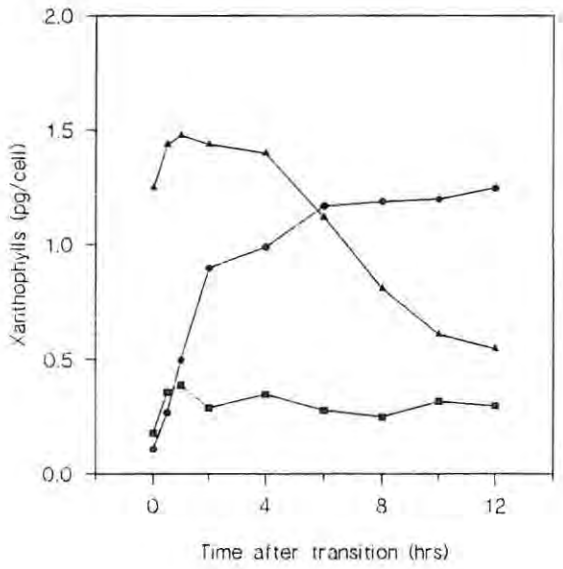


Figure 6.1. Changes in intracellular xanthophyll content of *D. salina* cells exposed to high light stress. (▲) - violaxanthin; (■) - antheraxanthin; (●) - zeaxanthin. (Results reflect the mean of three independant experiments.

When the above changes were expressed as EPS (Figure 6.2.), it was noted that EPS decreased immediately and continued to decline after exposure to high light intensity, thus indicating that the slight initial increase in violaxanthin had no effect on the epoxidation state of the xanthophyll cycle. The increase in violaxanthin reflects conversion of antheraxanthin to violaxanthin via the epoxidation reactions of the xanthophyll cycle. A decreasing EPS is indicative of net zeaxanthin accumulation, via violaxanthin de-epoxidation.

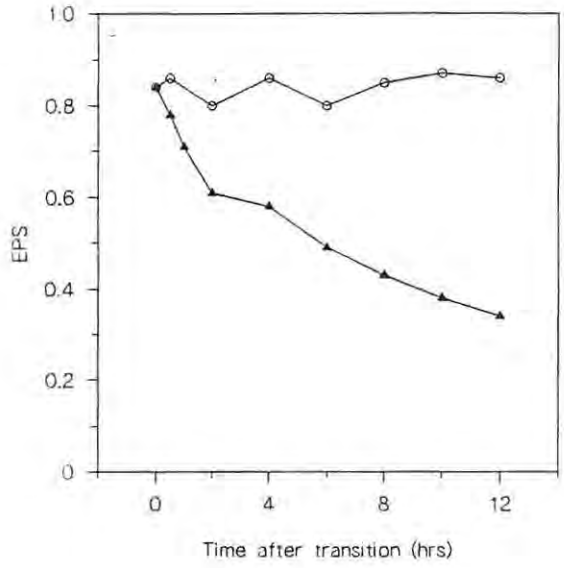


Figure 6.2. Changes in EPS of *D. salina* exposed to high light conditions. (o) - control; (▲) - high light stress. EPS was calculated using values represented in Figure 6.1.

6.2.3. Turnover of xanthophyll cycle pigments in response to high light stress.

In order to determine the effects of high light stress on the turnover of the epoxidation and de-epoxidation reactions of the xanthophyll cycle in relation to *de novo* synthesis of zeaxanthin, a pulse-chase experiment was undertaken. Cells were pre-labelled with $NaH[^{14}C]O_3$ for 24 hour prior to exposure to high light (see Materials and Methods section 2.2.2.6.) and the kinetics of the change in ^{14}C specific activity (KBq.nmol^{-1} compound) of zeaxanthin, antheraxanthin and violaxanthin were monitored. The results are presented in Figure 6.3.

The ^{14}C specific activity of the xanthophylls, violaxanthin and antheraxanthin, declined immediately after exposure to high light intensity. This decrease may have been due to a rapid dilution of the ^{14}C label caused by conversion of newly synthesised non-labelled precursors, or the loss of carbon to ABA. The subsequent increase in the ^{14}C specific activity of both violaxanthin and antheraxanthin may be explained by the continued epoxidation reactions of the xanthophyll cycle converting labelled zeaxanthin to violaxanthin, thereby

ensuring a higher radiolabel:nmole ratio for these compounds. The low ^{14}C specific activity obtained for zeaxanthin throughout the duration of the experiment may indicate *de novo* synthesis of zeaxanthin in response to high light.

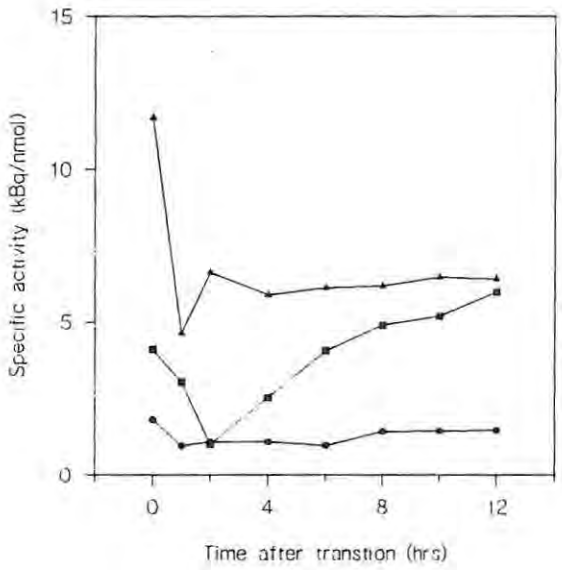


Figure 6.3. Changes in the ^{14}C specific activity of xanthophylls in *D. salina* cells exposed to high light stress. (▲) - violaxanthin; (■) - antheraxanthin; (●) - zeaxanthin. Results reflect the mean of three independant experiments.

6.2.4. Correlative changes in ABA and the xanthophylls: neoxanthin, violaxanthin, antheraxanthin and zeaxanthin .

The decrease in certain xanthophylls which accompanies ABA synthesis can be used to further investigate the interrelationship between these compounds. Table 6.2. represents an attempt to stoichiometrically link the change in zeaxanthin, antheraxanthin, violaxanthin and neoxanthin to the production of ABA in *D. salina.*

Table 6.2. Stoichiometric relationship between neoxanthin, (V+A+Z) changes and ABA synthesis.

Time	V+A+Z (mM)	Neoxanthin (mM)	Loss of xanthophyll mM	Gain in ABA (cells + medium) mM	Total ABA (cells) (mM)	ABA (medium) (mM)
0	0.47	0.100	0	0	0.012	0.007
1hr	0.42	0.050	-0.10	0.09	0.047	0.068
2hr	0.33	0.035	-0.20	0.11	0.040	0.090
4hr	0.26	0.110	-0.20	0.15	0.040	0.121
6hr	0.31	0.080	-0.18	0.16	0.340	0.155
8hr	0.33	0.025	-0.22	0.25	0.022	0.227

A regression analysis (Figure 6.4.) was performed on total neoxanthin and (V+A+Z) lost and total ABA gained as tabulated in Table 6.2. above.

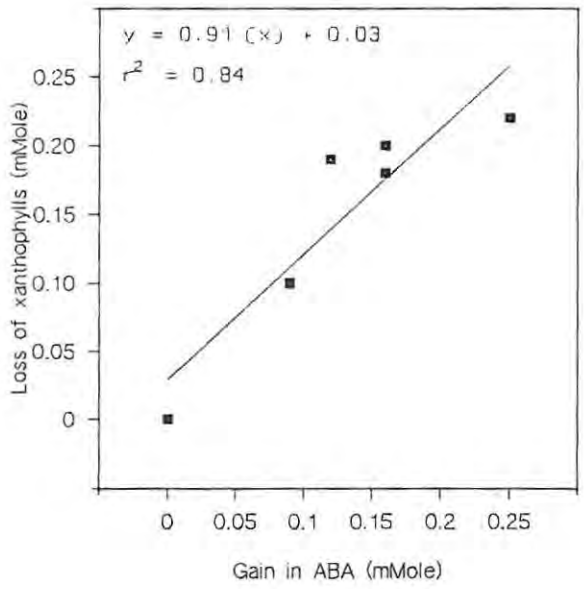


Figure 6.4. Linear regression of loss in neoxanthin and (V+A+Z) (mM) versus gain in ABA (mM). Xanthophylls refered to in the y-axis title only includes neoxanthin and (V+A+Z).

A strong correlation of ($r^2 = 0.84$) was obtained from a regression analysis of the loss of the xanthophylls, violaxanthin, antheraxanthin, zeaxanthin and neoxanthin versus gain in ABA (calculated using mM compound). The slope of the curve (0.91) reflects a near 1 to 1 stoichiometric relationship between the loss of xanthophylls and the gain in ABA.

Summary:

The results of this chapter show that on exposure to stress conditions, changes in xanthophyll cycle content and turnover of xanthophyll cycle pigments were demonstrated by cells of *D. salina*. The changes in xanthophylls were correlated to changes in endogenous ABA levels. These results support a carotenoid origin for ABA in *D. salina*.

Chapter 7

Discussion.

A dual-stage process for the production of ß-carotene from *D. salina* offers the potential to significantly increase ß-carotene yields compared to existing microalgal ß-carotene production systems (Phillips, 1993). Separation of the growth and carotenoid accumulation phases allows for greater control and manipulation of factors which maximise both cell growth and ß-carotene accumulation. However, as with most new biotechnological processes, optimization of productivity requires a fundamental understanding of the organism and the biosynthetic pathways involved in product formation.

Application of stress as a manipulative factor in the induction of secondary metabolite production in microorganisms is a well described technique in biotechnology. Induction of ß-carotene synthesis in *D. salina* is no exception. Results of the present study indicate that in response to stress, massive ß-carotene accumulation arises as a consequence of the redirection of carbon flux through the carotenoid pathway. The process is, at least in part, mediated by changes in endogenous ABA but more importantly by the impact of stress on xanthophyll cycle operation.

7.1. Factors Affecting ß-carotene Accumulation in *D. salina*.

The single stage process does not attempt to maximise ß-carotene accumulation, but instead endeavours to obtain a balance between growth and ß-carotene accumulation in order to achieve satisfactory ß-carotene yields. By comparison, the dual-stage process attempts to maximise ß-carotene accumulation to the full genetic potential of the organism. The study of factors which affect ß-carotene accumulation in *D. salina* is therefore of utmost importance.

It has been demonstrated that high light is the major factor contributing to ß-carotene accumulation in *Dunaliella*. Furthermore, a linear relationship exists between ß-carotene

accumulation and light intensity in the range of 10 to 396 μmol.m^{-2}.s^{-1} PFD (Loeblich, 1982). The relationship is further explained by the demonstration that the extent of ß-carotene accumulation is directly dependant on the integral amount of light to which the alga is exposed during one division cycle (Ben-Amotz and Avron, 1983). Of the single stress factors examined in the present study, high light stress was most effective in the induction of ß-carotene accumulation.

Salt stress and nutrient limitation also contribute to ß-carotene accumulation in *D. salina*. Hypersalinity was one of the first stresses recognised as a factor contributing to induction of carotenogenesis in *Dunaliella* spp. (Labbe, 1921; Mil'ko, 1963b). However, the present study has demonstrated that salinity stress alone cannot be considered a major ß-carotene-inducing factor. Even so, it does have the advantage of preventing invasion by competitive species in large scale outdoor cultivation processes, as very few microorganisms can withstand high salt concentrations.

Furthermore, nitrate limitation rather than total nitrate deficiency stimulated ß-carotene accumulation. A nitrate concentration of 0.2 mM was shown to be optimal for ß-carotene accumulation. This value is significantly lower than the 1 mM nitrate optimum reported by Ben-Amotz and Avron (1983), but may be explained by the fact that the cells in the present study, were transferred to stress media from nitrate sufficient growth media.

In order to further enhance ß-carotene accumulation, the effect of multiple stress factors was examined. High light together with salinity stress (3M) and nitrate limitation (0.2 mM) caused a twenty-five fold increase in ß-carotene content. Multiple stress has the added advantage of being more cost effective as fewer constituents are required in multiple stress medium make-up.

Characteristically *D. salina* responds to stress by changing both ß-carotene and chlorophyll levels. This results in an increase in the ß-carotene:chlorophyll ratio. Furthermore, ß-carotene accumulation is generally greatest under conditions which reduce the growth rate. Ben-Amotz and Avron (1983) have demonstrated an inverse relationship between the specific

growth rate and ß-carotene content of *D. bardawil*. The application of multiple stress in the present study resulted in a reduction in the growth rate and chlorophyll content of the cells.

Although chlorophyll depletion was demonstrated in the present study, a reduction in chlorophyll content did not substantially decrease the oxygen evolution rate of the cells. It has been demonstrated that ß-carotene-rich *D. salina* have a marked capability for photosynthetic adaptation under extreme environmental conditions (Ben-Amotz and Avron, 1983). When expressed on a chlorophyll basis (μmol O_2.mg $Chl^{-1}.hr^{-1}$), oxygen evolution rate was found for all stresses investigated in the present study to be very similar to control cells. High light stress actually appeared to increase oxygen evolution rate per unit mass of chlorophyll. Similarly, Gomez-Pinchetti and co-workers (1992) showed that high light stressed cells of *D. salina* which contained high levels of ß-carotene had higher photosynthetic rates on a chlorophyll basis than did cells which contained low levels of ß-carotene. However, when photosynthetic data were expressed on a per cell basis, the absolute rate of oxygen evolution (i.e. μmol $O_2.cell^{-1}.h^{-1}$) was approximately 55% lower for stressed cells. In response to the environment, cells may change either the size or number of photosynthetic units or the capacity of the photosynthetic enzyme reactions and attempts have been made to model these changes in terms of predicting photoadaptive strategies (Richardson, *et al.*, 1983). For example, a decrease in photosynthetic unit size in response to high light is predicted to result in an apparent increase in photosynthesis on a chlorophyll basis (Wihelm, 1993). A reduction in the light harvesting antenna size of both PS I and PS II in a ß-carotene non-accumulating strain of *D. salina* has been demonstrated in response to irradiance stress (Smith, *et al.*, 1990). The photosynthetic data represented in Table 3.1. might therefore be considered to be as expected.

An evaluation of the photosynthetic kinetic response of *D. salina* exposed to multiple stress demonstrated an immediate decrease in photosynthetic rate, during the first 3-4 hours. This reduction in photosynthetic capacity has been described by many authors (Ben-Amotz, *et al.*, 1989b; Gomez-Pinchetti, *et al.*, 1992; Shaish, *et al.*, 1993) and is consistent with the onset of photoinhibition. Even so, within 24 hours the photosynthetic rate was restored suggesting adaptation of the organism to the prevailing stress conditions.

7.2. The Two-Stage Response of *D. salina* Cells to Stress.

Detailed kinetic analysis of the changes in chlorophyll, ß-carotene, growth rate and photosynthesis of mass cultured *D. salina* cells exposed to salt, nitrate limitation, and multiple stress showed that the stress response could be divided into two phases. The first was characterized by a higher rate of ß-carotene accumulation, rapid chlorophyll depletion and a reduced growth rate, while the second phase was characterized by a decrease in the rate of ß-carotene accumulation, chlorophyll depletion and a partial recovery of the growth rate.

Borowitzka and co-workers, (1990) have also shown two distinct phases of ß-carotene accumulation in hypersalinity shocked cells, but ß-carotene accumulation was preceded by a lag phase, which was then followed by rapid ß-carotene accumulation. The duration of the lag phase was shown to be dependant on the final salinity and the magnitude of the salinity change. Lers and co-workers (1990) also noted two distinct stages of ß-carotene accumulation in response to high light intensity. The first occurred shortly after exposure and lasted for 24 hours, while the second stage commenced concomitant with the onset of stationary phase growth and persisted until cell death. This response was similar in timing to the response demonstrated in this study.

The initial stage (within approximately 24 hours after the onset of stress) appears to be crucial to the induction of ß-carotene accumulation. It is during this first stage of accelerated ß-carotene production that substantial changes occur in cell volume, glycerol production and endogenous ABA levels (Bental, *et al.*, 1990; Cowan and Rose, 1991). Results from the present study show that inhibition of carbon fixation and oxygen evolution, cell volume changes and an increase in endogenous ABA levels occur during the first phase. Within 24 hours, recovery is complete and cells then enter the second stage.

The recovery from stress and associated accumulation of ß-carotene is apparently dependant on both transcription and translation. For example, in the presence of either actinomycin D or cycloheximide, accumulation of ß-carotene is arrested (Lers, *et al.*, 1990). Also, recovery from salt stress occurs coincident with synthesis of a 150 kD protein located in the plasma-

membrane, which is thought to play a role in regulating cell proliferation (Sadka, *et al.*, 1991). Furthermore, recent evidence has demonstrated that *D. salina* has the ability to repair damaged reaction centres of PS II (D1 protein) and that under high irradiances chloroplasts gradually acquire a greater capacity for repair (Harrison, *et al.*, 1992). This phenomenon occurs within a time frame of about 24 hours (Kim, *et al.*, 1993). Similarly, recovery from high irradiance stress in *D. bardawil* is associated with alterations in gene expression. Kinetic studies have revealed that expression and synthesis of *cbr*, a putative zeaxanthin binding protein, is complete within 24 hours of exposure to high irradiances and that this protein associates with a minor LHC II component (Levy, *et al.*, 1993). Clearly these are mechanisms designed to protect the photosynthetic apparatus under adverse conditions. The reduced rate of chlorophyll depletion during the second phase may be explained by the demonstration that in response to irradiance stress *D. salina* adjusts the chlorophyll antennae size of each photosystem and the PS II concentration in the thylakoid membrane (Smith, *et al.*, 1990). Recovery from photoinhibition may also involve production of ß-carotene globules, since it has been proposed that these apparently act as a screen to high intensity irradiation (Ben-Amotz, *et al.*, (1987).

7.3. ABA Metabolism and Interrelationship with ß-carotene Accumulation under Stress.

It has been proposed that stress-induced ABA production causes cells to enter into a new and/or different physiological state (Hetherington and Quatrano, 1991) and that changes in intracellular ABA contribute to the adaptation of *Dunaliella* to prevailing adverse conditions (Cowan, *et al.*, 1992). Intracellular ABA content was shown to increase dramatically within 24 hours in cells exposed to various stresses both under laboratory conditions and in outdoor mass culture. These findings are in keeping with earlier reports concerning ABA production in algae under salt stress (Hirsch, *et al.*, 1989; Tietz, *et al.*, 1989; Cowan and Rose, 1991). ^{14}C labelling studies (section 4.2.1.) confirmed ABA production during the first phase of ß-carotene accumulation. The ABA/ß-carotene interrelationship was further supported by the demonstration that maximum rate of ß-carotene production and ABA occur simultaneously.

Studies using higher plants have led to the conclusion that ABA is derived from a carotenoid origin (reviewed by Parry 1993). However, nothing is known about the biosynthetic origin

of ABA in lower plants and in particular microalgae. In the present study a stress-induced decline in xanthophylls was shown to be stoichiometrically related to the accumulation of ABA. This presents strong circumstantial evidence in support of a carotenoid origin for ABA in *Dunaliella*. However, the pathway by which ABA is produced and the identity of the C_{40} carotenoid and C_{15} apocarotenoid precursors remains to be elucidated. Nevertheless, results presented here are the first indication of ABA formation from carotenoids in a microalga.

The tendency of this organism to produce and accumulate ABA in response to stress suggests that ABA may contribute to the onset of tolerance mechanisms, one of which includes massive ß-carotene accumulation. Although ß-carotene accumulation is a two-stage process, with ABA production occurring coincident with the first stage only, the initial stage of ß-carotene accumulation was distinctly biphasic (see Figure 4.1. section 4.2.1.). This biphasic response was also manifested in rate of photosynthetic CO_2 fixation and O_2 evolution (see Figures 3.3 and 3.4. section 3.2.3.). These observations may suggest that initially, ß-carotene is formed from stored carbon whereas subsequently ß-carotene accumulates directly from newly fixed carbon. A similar division of labour has been demonstrated for glycerol over-producing mutants of *Chlamydomonas* in response to osmotic shock. Since ABA accumulation coincided, temporally, with a decline in ß-carotene content during the early biphasic response it is not unreasonable to suggest that ABA inhibition of Calvin cycle activity, whether direct or indirect, contributes to the increased channelling or redirection of metabolism of newly fixed carbon into chloroplast isoprenoid synthesis and hence ß-carotene accumulation.

7.4. Regulation of ß-carotene Production at the Level of Xanthophyll Cycle Operation.

ß-carotene is the probable precursor to zeaxanthin, it was therefore proposed that in the presence of factors which promote zeaxanthin formation, an increase in both ß-carotene and zeaxanthin may be anticipated (Cowan, *et al.*, 1992). This aspect has been adequately demonstrated in the diurnal study (Figure 5.3. and 5.5.), and strong correlations ($r^2 \geq 0.81$) for the linear relationship between ß-carotene and zeaxanthin content were demonstrated in cells exposed to salt, nitrate and multiple stress on a daily basis (Figure 5.9.).

The diurnal study also demonstrated that stressed cells of *D. salina* containing substantial amounts of zeaxanthin, show continued operation of the xanthophyll cycle and sustained efficiency of photosynthetic energy conversion at maximum PFD during the diurnal cycle. Increased intracellular zeaxanthin levels were also exhibited by the non-stressed cells. This is not uncommon as levels of zeaxanthin have also been shown to increase in cells of the non-ß-carotene accumulating species *Dunaliella parva*, following exposure to high light intensity and this increase occurs concomitant with de-epoxidation of violaxanthin (Young and Britton, 1990b). Furthermore, the strong correlation but weak stoichiometric relationship between violaxanthin and zeaxanthin content in control cells in the present study would seem to indicate that zeaxanthin formation occurs firstly by rapid de-epoxidation of violaxanthin and secondly, by *de novo* synthesis of ß.ß-carotenoids resulting in an increase in the proportion of both zeaxanthin and antheraxanthin of the (V+A+Z) pool.

In contrast, zeaxanthin formation in cells exposed to changing PFD or either nitrate or multiple stress, appears to be mainly due to the de-epoxidation and epoxidation reactions of the xanthophyll cycle. The weak correlation between violaxanthin and zeaxanthin levels in salt stressed cells may be explained by the fact that salt stress induces chloroplast alkalization (Cowan, *et al.*, 1994), which consequently leads to the inhibition of violaxanthin de-epoxidase activity (Hager and Holcher, 1994).

A possible function of xanthophyll cycle operation in stressed cells could be maintenance of the transthylakoid ΔpH-induced ATP/NADPH ratio and hence photosynthesis (Young and Britton, 1990a). These authors further suggest that at high zeaxanthin levels, a small ΔpH could give rise to significant chlorophyll fluorescence quenching to allow for both high photosynthetic rates and energy dissipation to continue simultaneously since an inhibitory pH would be avoided.

Photosynthetic oxygen evolution in stressed cells, determined on a chlorophyll basis, was similar (or slightly higher) to that of non-stressed cells at maximum PFD (Table 5.1.). Chlorophyll fluorescence quenching studies have indicated formation of a large transthylakoidal ΔpH in salt-stressed cells of non-ß-carotene-accumulating *D. salina* (Canaani, 1990). Furthermore, photoacoustic quantum yield spectra of energy storage

revealed a partial disconnection of some PS II and PS I units, so that increased PS I cyclic electron flow was established upon salt stress. The substantial amount of zeaxanthin present in stressed cells at the onset of the photo-period may reflect the pH gradient induced by ionic stress. Stressed cells should, therefore, have greater capacity to dissipate excess energy at a given ΔpH (Noctor, *et al.*, 1991) and thus display higher rates of oxygen evolution. With regard to higher plants, the *aba* mutant of *A. thaliana* which is deficient in epoxy-carotenoids and accumulates zeaxanthin, shows reduced chlorophyll fluorescence yields and less PS II activity although quantum efficiency of PS II is similar to the wild-type (Rock, *et al.*, 1992). This is apparently the case in *D. salina* since photosynthetic activity is higher in stressed, carotene-rich cells at light intensities above 1500 μmol.m^{-2}s^{-1} irrespective of the basis used to calculate rates of oxygen evolution (Gomez-Pinchetti, *et al.*, 1992).

For cells experiencing changes in light intensity during the diurnal study, factors influencing nutrient status included phosphate, sulphate, nitrate and calcium availability. Deficient phosphorus supply inhibits assimilation by decreasing intermediatory metabolism. Under high light conditions, the rate of photosynthesis is limited by carbon fixation and/or carbon dioxide availability to the cells (Smith, *et al.*, 1990). This would be expected to further reduce the electron acceptor pool size of PS I (Vanselow, 1993). Under these conditions an inhibitory trans-thylakoid ΔpH might be expected since electron flow to nitrogen assimilation is not related to a strong consumption of ATP. Likewise, sulphate limitation reduces the acceptor pool of PS I further but with a concomitant decrease in utilization of ATP which might also contribute to the transthylakoid pH gradient. Furthermore, synthesis of isoprenoids requires ATP and carotenoid synthesis involves desaturation reactions in which electrons are consumed and protons liberated. In higher plants and algae these reactions are confined to granal membranes (Jones and Porter, 1985; Bramely and Mackenzie, 1988). Synthesis and accumulation of ß-carotene may therefore represent an efficient electron acceptor mechanism to counteract development of a nutrient- and/or salt-stress-induced inhibitory ΔpH in ß-carotene-accumulating strains of *Dunaliella* at high light intensity. Similar findings have recently been reported for another carotenoid accumulating alga, *Haematococcus* spp. (Hagen, *et al.*, 1993).

Another interesting result was the demonstration of an increased 9-*cis*/all-*trans* ß-carotene ratio in the cells exposed to stress in the diurnal study. Although the 9-*cis* ß-carotene isomer is destroyed and oxidized faster than the all-*trans* isomer, it has been clearly established that the 9-*cis*/all-*trans* ratio is higher under ß-carotene induction conditions (Ben-Amotz, *et al.*, 1982). It has been suggested that a natural selection of carotenoid configuration in the light harvesting complex and in the reaction centre occurs (Koyama, *et al.*, 1990). Two different reasons may be given for the preferential synthesis of the 9-*cis* isomer firstly, the all-*trans* carotenoid is located in the light harvesting complex, and functions as an accessory pigment in harvesting light energy, whereas the 9-*cis* is part of the reaction centre and functions in protection against excessive irradiation (Ben-Amotz, 1986) and secondly the 9-*cis* isomer being oily may serve as a solvating medium for the all-*trans* isomer, which has the tendency to crystallize at high concentrations and this would perturb its physical distribution within the chloroplast.

Based on the results of Chapter 5, it is apparent that the biosynthesis of ß-carotene together with operation of the xanthophyll cycle act in concert to sustain the efficiency of photosynthetic energy conversion and hence photosynthesis in stressed cells of *D. salina*. These observations suggest that in *D. salina*, ß-carotene may serve a photoprotective function similar to that proposed for zeaxanthin in higher plants (Demmig-Adams, 1990). It would also appear that zeaxanthin accumulation may retard the conversion of ß-carotene to zeaxanthin therefore causing an accumulation of both pigments.

7.5. ABA as a Regulator of Carotenogenesis.

A striking response of *D. salina* cells to either salt stress or high light intensity is a change in cell volume (Shaish, *et al.*, 1993; Cowan, *et al.*, 1994). Cell shrinkage and subsequent recovery of cell volume in response to salt stress was demonstrated in the present study and by Avron (1992). In contrast this alga was shown to swell in response to high light stress and nutrient stress. Furthermore, cell volume changes have been shown to coincide with the duration of formation of xanthophyll cycle pigments and occur concomitant with changes in compartmental pH (Cowan, *et al.*, 1994). Rapid compartmental acidification was demonstrated in *D. salina* cells following exposure to high light, but chloroplastic and

vacuolar alkalization was exhibited on exposure to salt stress (Cowan, *et al.*, 1994). Compartmental alkalization in response to hypersalinity appears to be a characteristic response of *Dunaliella* (Goyal, *et al.*, 1987; Goyal and Gimmler, 1989). Furthermore, alkalization of the chloroplast might be expected to retard violaxanthin conversion to zeaxanthin by inhibiting violaxanthin de-epoxidase hence favouring ABA production. By comparison, high light induced chloroplast acidification would favour violaxanthin de-epoxidase (pH optimum 5.2), zeaxanthin formation and redistribution of endogenous ABA into the cytoplasm.

Cell volume change results in a change in cytoplasm volume and associated plasma-membrane distortion. These are not mutually exclusive processes and are the result of dehydration due to elimination of chemically combined water (Cowan, 1994). Both the elimination of water and a change in pH result in a loss of ABA from the cell (Cowan, *et al.*, 1982). The loss of ABA from hypersalinity shocked cells has been demonstrated in the present study. In contrast, ABA isomerization and partitioning of the biologically active *cis* isomer into the culture was demonstrated, on exposure to high light. In response to both salt and high light stress, it appears that the *cis* isomer is partitioned out of the cell. This has two-fold implications. Firstly, ABA has been shown to regulate the enzyme HMG Co-reductase, which catalyses the first committed step in isoprenoid biosynthesis (Oishi and Moore, 1993). Therefore, the extrusion of *cis*-ABA may function to derepress HMG Co-A reductase, allowing isoprenoid synthesis to continue unrestricted. Secondly, the partitioning of ABA into the culture medium might suggest the existence of extracellular ABA receptors of high affinity.

Evidence for the existence of extracellular ABA reception site in *Commelina* guard cells and on the external surface of the plasma-membrane of *Hordeum vulgare* has now come to the fore (Anderson, *et al.*, 1994; Gilroy and Jones, 1994). It has also been suggested that in higher plants, ABA binds to the modulator proteins of the H^+-ATPase at the outside of the plasma-membrane of stomatal guard cells (Hartung, 1983), inhibiting H^+ extrusion which ultimately results in altered pH (Zeevaart and Creelman, 1988). Furthermore, it has been proposed that activation of the plasma-membrane ATPase may be the trigger for

osmoregulation in salt stressed *D. salina* cells (Oren-Shamir, 1989, 1990). Redistribution of intracellular ABA may therefore play a crucial role in this response.

The demonstration that exogenously applied ABA and related compounds enhanced ß-carotene levels in *D. salina* cells in the present study, almost certainly establishes a role for ABA in carotenogenesis in this alga. A more significant response in terms of ß-carotene accumulation would, however, have provided a more conclusive argument. The results do seem to indicate that lower concentrations of ABA (less than 50 μmolar) improve intracellular ß-carotene content. Perhaps exogenous ABA should be applied in the same concentration range as that detected in the culture medium after the imposition of stress. Another consideration may be that ABA concentration alone is not sufficient to control physiological responses but that cell sensitivity may also be involved. Trewavas, (1981, 1991), argues that sensitivity to growth substances, not their concentration is the limiting factor in determining the magnitude of a physiological response. In context of the stress response of *Dunaliella*, perhaps ABA receptor sites, whether internally or externally situated will only be activated (due to a change in sensitivity) after the onset of stress.

7.6. Concluding Remarks.

It has been stated that "the impact of a given type of stress on plant performance is the result of the interaction between plant, functioning as controlled system according to its genetic information, and the stress, imposing limitations of variable intensity depending on its severity and duration" (Chaves, 1991). The present study has shown that the response of *D. salina* to stress is multifaceted. These responses are clearly regulatory and allow the organism to tolerate prevailing stresses.

Recognition of the stress stimulus in *D. salina* cells appears to be at the membrane level, either as volume/pressure-induced changes or due to alterations in redox potential across (thylakoid) membranes. Occurring concomitantly with changes in cell volume are changes in compartmental pH and redistribution/efflux of ABA.

Results from the present study indicate that stress-induced efflux of ABA is required for osmoregulation and volume recovery in salt-stressed cells. This is further substantiated by the fact that ABA-deficient mutants of *D. salina* are also defective in haloadaptation (Cowan, *pers comm*). The loss of ABA from hypersalinity shocked cells and low intracellular *cis*-ABA levels following exposure to high light may shift/force the chemical equilibrium in the direction of isoprenoid synthesis, since ABA is in all probability derived from carotenoids in *D. salina*.

Furthermore, differential cell pH responses cause differential responses in terms of xanthophyll cycle operation. Alkalization of the chloroplast in response to salt-stress, favours ABA production, whereas acidification of the chloroplast in response to high light-stress favours zeaxanthin production and hence ß-carotene accumulation.

Enhanced ABA levels, occur during the early stress response and may serve to redirect cell chemistry and physiology. The late stress response which includes continued ß-carotene levels (albeit at a slower rate), a recovery of the growth rate and sustained photosynthesis clearly allows *D. salina* to withstand conditions few other organisms could tolerate.

From a biotechnological view point this study has provided an understanding of the biological pathways involved in ß-carotene accumulation. Although, the stress response is clearly complex, it may be possible to enhance ß-carotene accumulation if a more precise role for ABA could be identified. Future work should therefore centre around the effects of exogenously applied ABA, the effect of ABA on phospholipids and hence the role played by calcium as a second messenger in the induction of ß-carotene accumulation.

As one of the main features which contributes to the marketing of ß-carotene from *Dunaliella* spp. is the presence of large amounts of the 9-*cis* isomer in the product, future work should be concentrated on the effect of utilizing the information presented in the thesis to optimise the isomeric composition of the final product.

In addition, an understanding of the stress response may ultimately lead to genetic manipulation targeted at areas that would allow the organism to function above its present

genetic potential in terms of enhanced ß-carotene production levels. Furthermore, this remarkable organism may serve as a gene source for cloning into agriculturally important plant species, as it clearly has the ability to endure adverse conditions.

REFERENCES

Aaronson, S., Berner, T., Dubinsky, Z. (1980). Microalgae as a source of chemicals and natural products. *In*, Algal Biomass: Production and Use, (Eds) G. Shelef and C.J. Soeder. Elsevier/North-Holland Biomedical Press, Amsterdam.

Aasen, A.J., Eimhjellen, K.E., Liaaen-Jensen, S. (1969). An extreme source of ß-carotene. *Acta Chem. Scan.* **23**: 2544-2545.

Adams, W.W., Demmig-Adams, D. (1991). Operation of the xanthophyll cycle in higher plants in response to diurnal changes in incident sunlight. *Planta.* **186:** 390-398.

Adams, W.W., Demmig-Adams, D. (1992). Energy dissipation and photoprotection in leaves of higher plants. *In*: Photosynthetic Responses to the Environment, pp 27-30. (Eds) Yamamoto, H.Y., Smith, C.M. Rockville, Maryland.

Addicott, F.T. (1983). *In*, Abscisic Acid. (Ed) F.T. Addicott. Praeger, New York.

Alscher, R.G., Cumming, J.R. (1990). Stress responses in plants: Adaption and Acclimation mechanisms. Wiley-Liss, New York.

Anderson, J.M. (1986). Photoregulation of the composition, function and structure of thylakoid membranes. *Ann. Rev. Plant Physiol.* **37:** 97-136.

Anderson, J.M., Ward, J.M., Schroeder, J.I. (1994). Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. *Plant. Physiol.* **104:** 1177-1183.

Andrews, J.E., Ohlrogge, J. (1990). Fatty acid and lipid biosynthesis and degradation. *In*, Plant Physiology, Biochemistry and Molecular Biology. (Eds), D. Dennis and D. Turpin. Longman Scientific and Technical. U.K.

Antia, N.J., Cheng, J.Y. (1983). Evidence for anomalous xanthophyll composition in a clone of *Dunaliella tertiolecta (Chlorophyceae). Phycol.* **22:** 235-242.

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris. Plant Physiol.* **24:** 1-15.

Atkinson, B., Mavituna, F. (1991). Biochemical Engineering and Biotechnology Handbook. MacMillan Press, U.S.A.

Ausich, R.L. (1994). Production of carotenoids by recombinant DNA technology. *Pure & Appl. Chem.* **66:** 1057-1062.

Avron, M. (1992). Osmoregulation. *In. Dunaliella*: Physiology, Biochemistry and Biotechnology. (Eds) Avron, M and Ben-Amotz, A. CRC Press, Boca Raton, Florida.

Avron, M., Ben-Amotz, A. (1978). Production of glycerol from algae. US Patent 4115949.

Barnett, H.M., Hartmann, M.L., Mosher, R.C., Espoy, H.M. (1958). Carotene recovery. U.S. Patent 2848508.

Bartley, G.E., Scolnik, P.A. (1994). Molecular biology of carotenoid biosynthesis in plants. *Ann. Rev. Plant.Physiol. Plant. Mol. Biol.* **45:** 287-301.

Bartley, G., Vitanen, I., Bacot, K., Scolnik, P. (1991a). A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. *J. Biol. Chem.* **267:** 5036-5039.

Bartley, G., Vitanen, I., Pecker, I., Chamovitz, .D, Hirshberg, J., Scolnik, P. (1991b). Molecular cloning and expression in photosynthetic bacteria of a soyabean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. *Proc Natl. Acad. Sci. USA.* **88:** 6532-6536.

Beckett, J., Dibasio, D., Keogh, P.A., Chen, B.J. (1985). Algal glycerol production: initial glycerol synthesis kinetics. *Chem. Eng. Comm.* **32:** 357-367.

Ben-Amotz, A. (1980). Glycerol production in the alga *Dunaliella. In,* Biochemical and Photosynthetic Aspects of Energy Production, (Ed) A San Pietro. Academic Press, New York.

Ben-Amotz, A. (1986). ß-Carotene enhancement and its role in protecting *Dunaliella salina* against injury by high irradiance. *In,* Algal Biomass Technologies, (Eds) W.R. Barclay, R.P. McIntosh, J. Cramer. Berlin.

Ben-Amotz, A. (1987). Effect of irradiance and nutrient deficiency on the chemical composition of *Dunaliella bardawil* (Volvocales, Chlorophyta). *J. Plant Physiol.* **131:** 479-487.

Ben-Amotz, A., Avron, M. (1973). The role of glycerol in the osmotic regulation of the halophilic alga *Dunaliella parva. Plant Physiol.* **51:** 875-878.

Ben-Amotz, A., Avron, M. (1980). Glycerol, ß-carotene and dry algal meal production by commercial cultivation of *Dunaliella. In,* Algal Biomass: Production and Use, (Eds) G. Shelef and C.J. Soeder. Elsevier, Amsterdam.

Ben-Amotz, A., Avron, M. (1983). On factors which determine massive ß-carotene accumulation in the halotolerant alga *Dunaliella bardawil. Plant Physiol.* **72:** 593-597.

Ben-Amotz, A., Avron, M. (1989). The biotechnology of mass culturing *Dunaliella* for products of commercial interest. *In,* Algal and Cyanobacterial Biotechnology, (Eds) R.C. Creswell, T.A.V. Rees and N. Shah. Longmans, Harlow.

Ben-Amotz, A., Avron, M. (1990). The biotechnology of cultivating the halotolerant alga *Dunaliella. Tibtech.* **8:** 121-125.

Ben-Amotz, A., Avron, M. (1992). *Dunaliella*: Physiology, Biochemistry and Biotechnology. CRC Press, London.

Ben-Amotz, A., Edelstein, S., Avron, M. (1986). Use of the ß-carotene rich alga *Dunaliella bardawil* as a source of retinol. *Brit. Poult. Sci.* **27:** 613-619.

Ben-Amotz, A., Gressel, J., Avron, M. (1987). Massive accumulation of phytoene induced by norflurazon in *Dunaliella bardawil* (Chlorophyceae) prevents recovery from photoinhibition. *J. Phycol.* **23:** 176-181.

Ben-Amotz, A., Katz, A., Avron, M. (1982). Accumulation of ß-carotene in halotolerant algae: purification and characterization of ß-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *J. Phycol.* **18:** 529-537.

Ben-Amotz, A., Lers, A., Avron, M. (1988). Stereoisomers of ß-carotene and phytoene in the alga *Dunaliella bardawil. Plant. Physiol.* **86:** 1286-1291.

Ben-Amotz, A., Mokady, S., Edelstein, S., Avron, M. (1989a). Bioavailability of a natural isomer mixture as compared with synthetic all-*trans*-ß-carotene in rats and chicks. *Am. Inst. Nutr.* **22:** 1013-1019.

Ben-Amotz, A., Shaish, A., Avron, M. (1989b). Mode of action of the massively accumulated ß-carotene of *Dunaliella bardawil* in protecting the alga against damage by excess irradiation. *Plant Physiol.* **91:** 1040-1043.

Bendich, A., Olson, J.A. (1989). Biological actions of carotenoids. *FASEB J.* 3: 1927-1932.

Benemann, J.R. (1989). The future of microalgal biotechnology. *In*, Algal and Cyanobacterial Biotechnology, (Eds) R.C. Cresswell, T.A.V. Rees, and N. Shah. Longmans, Harlow.

Bental, M., Pick, U., Avron, M., Degani, H. (1990). The role of intracellular orthophosphate in triggering osmoregulation in the alga *Dunaliella salina. Eur. J. Biochem.* **188:** 117-122.

Beyer, P., Kronche, U., Nievelstein, V. (1991). Lycopene isomerase/cyclase reaction in *Narcissus pseudonarcissus L.* chromoplasts. *J. Biol. Chem.* **266:** 17072-17078.

Bilger, W., Bjorkman, O. (1991). Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L. *Planta* **184:** 226-234.

Borowitzka, L.J. (1991). Development of Western Biotechnology's algal ß-carotene plant. *Biores. Technol.* **38:** 251-252.

Borowitzka, L.J., Borowitzka, M.A. (1988). *Dunaliella. In*: Micro-algal Biotechnology (Eds) M.A. Borowitzka. L.A. Borowitzka, Cambridge University, Amsterdam.

Borowitzka, L.J., Borowitzka, M.A. (1990). Commercial production of ß-carotene by *Dunaliella salina* in open ponds. *Bul. Mar. Sci.* **47(1):** 244-252.

Borowitzka, L.J., Borowitzka, M.A., Moulton, T.P. (1984). The mass culture of *Dunaliella salina* for fine chemicals: from laboratory to pilot plant. *Hydrobiologica.* **116:** 115-134.

Borowitzka, L.J., Brown, A.D. (1974). The salt relations of marine and halophilic species of the unicellular green alga, *Dunaliella. Arch. Microbiol.* **96:** 37-52.

Borowitzka, L.J., Kessly, D.S., Brown, A.D. (1977). The salt relations of *Dunaliella. Arch. Microbiol.* **113:** 131-138.

Borowitzka, L.J., Moulton, T.P., Borowitzka, M.A. (1986). Salinity and the commercial production of ß-carotene from *Dunaliella salina. Nov. Hed. Bei.* **83:** 224-229.

Borowitzka, M.A. (1986). Microalgae as a source of fine chemicals. *Microbiol. Sci.* **3:** 372-375.

Borowitzka, M.A., Borowitzka, L.J., Kessly, D. (1990). Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella salina. J. Appl. Phycol.* **2**: 111-119.

Bowler, G., Van Montagu, M., Inze, D. (1992). Superoxide dismutase and stress tolerance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43:** 83-116.

Bramley, P.M. (1985). The *in vitro* biosynthesis of carotenoids. *Adv. Lip. Res.* **21**: 243-279.

Bramley, P.M., Mackenzie, A. (1988). Regulation of carotenoid biosynthesis. *Cur. Top. Cell Reg.* **29**: 291-343.

Britton, G. (1988). Biosynthesis of carotenoids. *In*, Plant Pigments. (Eds) Britton, G. and Goodwin, T. W., New York, Academic Press.

Britton, G., Powls, R. (1977). Phytoene, phytofluene and ζ-carotene isomers from *Scenedesmus obliquus* mutant. *Phytochemistry.* **16:** 1253-1255.

Brown, D., Britton, G., Goodwin, T. (1975). Carotenoid biosynthesis by cell-free preparation from a *Flavobacterium* species. *Biochem. Soc. Trans.* **3:** 740-742.

Burlew, J.D. (1953). Algal culture - from laboratory to pilot plant. Publication 600, Carnegie Institute, Washington.

Camara, B., Bardat, F., Moneger, R. (1982). Site of biosynthesis of carotenoids in *Capsium* chromoplasts. *Eur. J. Biochem.* **127:** 255-258.

Canaani, O. (1990). The role of cyclic electron flow around photosystem I and excitation energy distribution between the photosystems upon acclimation to high ionic stress in *D. salina. Photochem. Photobiol.* **56:** 591-599.

Chamovitz, D. and Hirschberg, J. (1991). The molecular basis of resistance to the herbicide Norflurazon. *Plant. Mol. Biol.* **16:** 967-974.

Chapin, F.S. (1991). Integrated response of plants to stress: A centralised system of physiological responses. *Bioscience.* **41(1):** 29-36.

Chaves, M.M. (1991). Effects of water deficits on carbon assimilation. *J. of Exp. Bot.* **42:** 1-16.

Chen, B.J., Chi, C.H. (1981). Process development and evaluation for algal glycerol production. *Biotechnol. Bioeng. Let.* **5:** 519-524.

Ciegler, A., Hall, H.H. and Nelson, G.E.N. (1962). Microbiological production of carotene in a medium comprising kerosene. U.S. Patent 3025221.

Clarke, I.E., Sandmann, G, Bramley, P.M., Boger, P. (1982). Carotene biosynthesis with isolated photosynthetic membranes. *FEBS letters.* **140:** 203-206.

Cowan, A.K. (1994). Plant Stress. *Encyc. Agric. Sci.* (in press)

Cowan, A.K., Logie, M.R.R., Rose, P.D., Phillips, L.G. (1994). Induction of zeaxanthin formation. *J. Plant Physiol. In Press.*

Cowan, A.K., Railton, I.D. (1986). Chloroplasts and the biosynthesis and catabolism of abscisic acid. *J. Plant. Gr. Reg.* **4:** 211-224.

Cowan, A.K., Rose, P.D. (1991). Abscisic acid metabolism in salt-stressed cells of *Dunaliella salina*. *Plant. Physiol.* **97:** 798-803.

Cowan, A.K., Rose, P.D., Horne, L.G. (1992). *Dunaliella salina*: a model system for studying the response of plant cells to stress. *J. Exp. Bot.* **43(257):** 1535-1547.

Cowan, I.R., Raven, J.A., Hartung, W., Farquhar, G.D. (1982). A possible role for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. *Austr. J. of Plant Physiol.* **9:** 489-498.

Creelman, R.A. (1989). Abscisic acid physiology and biosynthesis in higher plants. *Physiol. Plant.* **75:** 131-136.

Cunningham, F., Schiff. (1985). Photoisomerization of ζ-carotene steroisomers in cells of *Euglena gracillis* mutant W3BUL. *Photochem. Photobiol.* **1985:** 295-307.

Curtain, C.C., West, S.M., Schlipalius, L. (1987). Manufacture of ß-carotene from the salt lake alga *Dunaliella salina*; the scientific and technical background. *Austr. J. Biotechnol.* **1(3):** 51-57.

Dandekar, S., Modi, V.V., Jani, U.K. (1980). Chemical regulators of carotenogenesis by *Blakeslea trispora*. *Phytochem.* **19**: 795-798.

Davies, B.H. (1976). *In* TW Goodwin, ed, Chemistry and Biochemistry of Plant Pigments. Academic Press, New York. pp 489-532.

de las Rivas, J., Abadia, A., Abadia, J. (1989). A new reverse phase-HPLC method resolving all major higher plant photosynthetic pigments. *Plant. Physiol.* **91:** 190-192.

Demmig, B., Winter, K., Kruger, A., Czygan, F. (1987). Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. *Plant Physiol.* **84:** 218-224.

Demmig-Adams, B. (1990). Carotenoids and Photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochim. Biophys. Acta.* **1020:** 1-24.

Demmig-Adams, B., Adams III, W.W. (1992). Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43:** 599-626.

Demmig-Adams, B., Winter, K., Kruger, A., Cryzan, F. (1988). Zeaxanthin and the heat dissipation of excess light energy in *Nerium oleander* exposed to a combination of high light and water stress. *Plant Physiol.* **87:** 17-24.

Demmig-Adams. B., Winter. K., Winkelmann, E., Kruger, A., Czygan, F.C. (1989). Photosynthetic characteristics and the ratios of chlorophyll, ß-carotene, and the components of the xanthophyll cycle upon a sudden increase in growth light regime in several plant species. *Bot. Acta.* **102:** 319-325.

Duckham, S.C., Linforth, R.S.T., Taylor, I.B. (1991). Abscisic-acid-deficient mutants at the *aba* gene locus of *Arabidopsis thaliana* are impaired in the epoxidation of zeaxanthin. *Plant Cell Environ.* **14:** 601-606.

Ebenezer, W.J., Pattenden, G. (1993). *cis*-Stereoisomers of ß-carotene and its congeners in the alga *Dunaliella bardawil,* and their biogenetic interrelationships. *J. Chem. Soc.* Perkin Trans. **1**: 1869-1873.

Evans, L.V., Trewavas, A.J. (1991). Is algal development controlled by plant growth substances ? *J. Phycol.* **27:** 322-326.

Foyer, C.H., Dujardyn, M., Lemoine, Y. (1989). Responses of photosynthesis and the xanthophyll and ascorbate-glutathione cycles to changes in irradiance, photoinhibition and recovery. *Plant Physiol. Biochem.* **27:** 751-760.

Gage,, D.A., Fong, F., Zeevaart, J.A.D. (1989). Abscisic acid biosynthesis in isolates embryos of *Zea mays* L. *Plant Physiol.* **89:** 1039-1041.

Gamon, J.A., Field, C.B., Bilger, W., Bjorkman, O., Fredeen, A.L., Penuelas, J. (1990). Remote sensing of the xanthophyll cycle and chlorophyll fluorescence in sunflower leaves and canopies. *Oceologia.* **85:** 1-7.

Gilmore, A.M., Yamamoto, H.Y. (1991). Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificial mediated linear and cyclic electron transport. *Plant Physiol.* **96:** 635-643.

Gilmore, A.M., Yamamoto, H.Y. (1992). Dark induction of zeaxanthin-dependant non-photochemical fluorescence quenching mediated by ATP. *Proc. Natl. Acad. Sci. USA.* **89:** 1899-1903.

Gilmore, A.M., Yamamoto, H.Y. (1993). Linear models relating xanthophylls and lumen acidity to non-photochemical fluorescence quenching. Evidence that antheraxanthin explains zeaxanthin-independent quenching. *Photosynth. Res.* **35:** 67-78.

Gilmour,D.J., Hipkins, M.K., Webber, A.N. Baker, N.R. Boney, A.D. (1985). The effect of ionic stress on photosynthesis in *Dunaliella tertiolecta. Planta.* **163:** 250-260.

Gilroy, S., Jones, R.L. (1994). Perception of gibberellin and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare* L.) aleurone protoplasts. *Plant Physiol.* **104:** 1185-1192.

Ginzburg, B.Z., Ginzburg, M. (1985). Study of the comparative physiology of the genus *Dunaliella* (Chlorophyta, Volvocales). 1. Response of growth to NaCl concentration. *Br. Phyc. J.* **20:** 277.

Gocke, K., Hoppe, H.G. (1977). Determination of organic substances and respiration potential. *In,* Microbial Ecology of a Brackish-Water Environment, (Eds) G. Rheinheimer. Springer, Berlin.

Gomez-Pinchetti, J.L., Ramazanov, Z., Fontes, A., Garcia-Reina, G. (1992). Photosynthetic characteristics of *Dunaliella salina* (Chlorophyceae, Dunaliellales) in relation to ß-carotene content. *J. Appl. Phycol.* **4:** 11-15.

Goodwin, T.W. (1979). Biosynthesis of terpenoids. *Ann. Rev. Plant Physiol.* **30**: 369-404.

Goodwin, T.W. (1980a). The Biochemistry of Carotenoids. 2nd ed., vol. 1. Chapman and Hall, London.

Goodwin, T.W. (1980b). Carotenoids. *In*, Encyclopedia of Plant Physiology, (Eds) A.P. Gottingen, M.H. Zimmermamm. Springer-Verlag Berlin, Heidelburg, Germany. **Vol. 8:** 257-287.

Goyal, A., Brown, A.D., Gimmler, H. (1987). Regulation of salt-induced starch degradation in *Dunaliella tertiolecta*. *J. Plant. Physiol.* **127:** 77-96.

Goyal, A., Gimmler, H. (1989). Osmoregulation in *Dunaliella tertiolecta*: effect of salt stress, and external pH on integral pH. *Arch. Microbiol.* **152:** 138-142.

Gust, D., Moore, T.A., Bensasson, R.V., Mathis, P., Land, E.J., Chachaty, C., Moore, A.L., Liddell, P.A., Nemeth, G.A. (1985). Stereodynamics of intramolecular triplet energy transfer in carotenoporphyrins. *J. Am. Chem. Soc.* **107:** 3631-3640.

Hagen, C., Braune, W., Birkner, E., Nuske, J. (1993). Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Girod) Rostafinski (Volvocales). *New Phytol.* **125:** 625-633.

Hager, A. (1980). The reversible, light-induced conversions of xanthophylls in the chloroplast. *In*, Pigments in Plants, (Eds) F.C. Czygan. Fischer, Stutgard. 57-79.

Hager, A., Holocher, K. (1994). Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. *Planta.* **196:** 581-589.

Harding, R.W., Shropshire, W. (1980). Photocontrol of carotenoid biosynthesis. *Ann. Rev. Plant Physiol.* **31**: 217-238.

Harrison, D.M. (1986). The Biosynthesis of Carotenoids. Natural Product Report.

Harrison, M.A., Melis, A., Allen, J.F. (1992). Restoration of irradiance-stressed *Dunaliella salina* (green alga) to physiological growth conditions: changes in antenna size and composition of photosystem II. *Biochim. et Biophys. Acta.* **1100:** 83-91.

Hartung, W. (1983). The site of action of abscisic acid at the guard cell plasmalemma of *Valerianella locusta*. *Plant, Cell and Envir.* **6:** 427-428.

Hartung, W., Heilmann, B., Gimmler, H. (1981). Do chlorolplasts play a role in abscisic acid synthesis ? *Plant Sci. Let.* **22:** 235-242.

Heilmann, B., Hartung, W., Gimmler, H. (1980). The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. *Zeitschrift fur Pflanzenphysiol.* **97:** 67-78.

Hetherington, A.M., Quatrano, R.S. (1991). Mechanism of action of abscisic acid at the cellular level. *New Phytologist.* **119:** 9-32.

Hirsch, R., Hartung, W., Gimmler, H. (1989). Abscisic acid content of algae under stress. *Bot. Acta.* **41:** 21-53.

Hornby, C., Weiler, E.W. (1984). High-affinity binding sites for abscisic acid on the plasmalemma of *Vicia faba* guard cells. *Nature.* **310:** 321-324.

Horton, P., Oxborough,K., Rees, D., Scholes, J.D. (1988). Regulation of the efficiency of photosystem II. Consequences for the light response of field photosynthesis. *Plant Physiol. Biochem.* **26:** 453-460.

Howes,C. and Batra, P. (1970). Mechanism of photoinduced carotenoid synthesis , further studies on the action spectrum and other aspects of carotenogenesis. *Arch. Biochem. Biophys.* **137**: 175-180.

Jensen, A. (1978) Chlorophylls and carotenoids. *In,* Handbook of Phycological and Biochemical Methods, (Eds) J.A. Hellebust, J.S. Graigie. Cambridge University Press.

Jones, B.L., Porter, J.W. (1985). Biosynthesis of carotenes in higher plants. *CRC Critical Reviews in Plant Sciences.* **3**: 295-325.

Kawaguchi, K. (1980). Microalgae production systems in Asia. *In,* Algal Biomass: Production and Use, (Eds) G. Shelef and C.J. Soeder (eds). Elsevier/North-Holland Biomedical Press, Amsterdam.

Kim, J.H., Nemsom, J.A., Melis, A. (1993). Photosystem II reaction centre damage and repair in *Dunaliella salina* (Green alga). *Plant Physiol.* **103:** 181-189.

Kirst, G.O. (1990). Salinity tolerance of eukaryotic marine algae. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **41:** 21-53.

Koyama, Y.I., Takatsuka, M., Kanaji, K., Tomimoto, M., Kito, T., Shimamura, J., Yamashita, K., Saiki, K., Tsukida, K. (1990). Configuration of carotenoids in the reaction centre and the light-harvesting complex of *Rhodospirillum rubrum*. Natural selection of carotenoid configuration by pigment protein complexes. *Photochem. Photobiol.* **51:** 119-128.

Kramer, P.J. (1988). Changing concepts regarding plant water relations. *Plant.Cell and Environ.* **11:** 565-568.

Kreuz, K., Beyer, P., Kleinig, H. (1982). The site of carotenogenic enzymes in chromoplasts from *Narcissus pseudonarcissus*. *Planta.* **154:** 66-69.

Krinsky, N.I. (1971). Function. *In,* Carotenoids, (Ed) O. Isler. Birkhauser, Basel.

Krinsky, N.I. (1978). Non-photosynthetic functions of carotenoids. *Phil.Trans. R. Soc. Lond. B.* **284:** 581-590.

Krinsky, N.I. (1993). Actions of carotenoids in biological systems. *Annu. Rev. Nutr.* **13**: 561-87.

Labbe, A., (1921). Les cycles biologiques des *Dunaliella*. *Archives d'anatomie microscopique.* **21:** 313-399.

Lers, A., Biener, Y., Zamir, A. (1990). Photoinduction of massive ß-carotene accumulation by the alga *Dunaliella bardawil*. *Plant Physiol.* **93:** 389-395.

Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck. J., Kratzeisen, C., Rosenberger, M., Lovey,A., Grippo, J.F. (1992). 9-*cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature.* **335**: 359-361.

Levitt, J. (1980a). Responses of Plants to Environmental Stresses. Vol 1. 2nd. Edition. New York: Academic Press.

Levitt, J. (1980b). Responses of Plants to Environmental Stresses. Vol 2. 2nd. Edition. New York: Academic Press.

Levy, H., Gokham, I., Zamir, A. (1992). Regulation and light-harvesting complex II association of a *Dunaliella* protein homologous to early light-induced proteins in higher plants. *J. Biol. Chem.* **267:** 18831-18836.

Levy, H., Tal, T., Shaish., A, Zamir., A. (1994). Cbr, an algal homolog of plant early light-induced proteins (Elips), is a putative zeaxanthin binding protein. *J. Biol. Chem.* (in press).

Li, Y., Walton, D.C. (1987). Xanthophylls and abscisic acid biosynthesis in water-stressed bean leaves. *Plant Physiol.* **85:** 910-915.

Li, Y., Walton, D.C. (1990). Violaxanthin is an ABA precursor in water-stressed dark-grown bean leaves. *Plant Physiol.* **92:** 551-559.

Loeblich, L.A. (1982). Photosynthesis and pigments influenced by light intensity and salinity in the halophilic *Dunaliella salina* (Chlorophyceae). *J. Mar. Biol. Ass.* **62**: 493-508.

Lustigman, B.K. (1986). Enhancement of pigment concentrations in *Dunaliella tertiolecta. Bull. Environ. Contam. Toxicol.* **37:** 710-713.

Maeda, M., Thompson, G.A. (1986). On the mechanism of rapid plasma membrane and chloroplast envelope expansion in *Dunaliella salina* exposed to hypo-osmotic shock. *Cell Biol.* **102:** 289-297.

Milborrow, B.V. (1974). Biosynthesis of abscisic acid by cell-free system. *Phytochemistry.* **13:** 131-136.

Mil'ko, E.S. (1963a). Effect of various environmental factors on pigment production in the alga *Dunaliella salina. Mikrobiologiya.* **32**: 299-307.

Mil'ko, E.S. (1963b). Effect of illumination and temperature on pigment formation in *Dunaliella salina. Mikrobiologiya.* **32:** 590-597.

Mooney, H.A., Winner, W.E., Pell. E.J. (1991). Responses of plants to multiple stress. Academic Press, San Diego.

Moore, R., Smith, J.D. (1985). Graviresponsiveness and abscisic acid content of *Zea mays* seedlings treated with fluridone. *Planta.* **162:** 342-344.

Mordi, R.C. (1993). Carotenoids: Functions and Degradation. *Chem. Ind.* February: 79-83.

Moulton, T.P., Borowitzka, L.J., Vincent, D.J. (1987). The mass culture of *Dunaliella salina* for ß-carotene: From pilot plant to production plant. *Hydrobiologica.* **151:** 99-105.

Nagasawa, H., Fujii, Y., Yamamoto, K., Konishi, R., Ben-Amotz, A. (1989a). No deleterious side-effects on mammary growth and endocrine parameters of chronic ingestion of beta-carotene-rich alga *Dunaliella bardawil* in virgin mice in comparison with synthetic all-*trans*-beta-carotene. *Cancer Journal.* **2 (11):** 391-394.

Nagasawa, H., Konishi, R., Sensui, N., Yamamoto, K., Ben-Amotz, A. (1989b). Inhibition by ß-carotene - rich algae *Dunaliella* of spontaneous mammary tumorigenesis in mice. *Anticancer Res.* **9:** 71-76.

Noctor, G., Rees, D., Young, A., Horton, P. (1991). The relationship between zeaxanthin, energy-dependant quenching of chlorophyll fluorescence and transthylakoid pH gradient in isolated chloroplasts. *Biochim. Biophys. Acta.* **1057:** 320-330.

Noddle, S.M., Robinson, D.R. (1969). Biosynthesis of abscisic acid: Incorporation of radioactivity from (2-^{14}C) mevalonic acid by intact fruit. *Biochem. J.* **112:** 547-548.

Norman, S.M. (1991). ß-carotene from the abscisic acid producing strain of *Cercospora rosicola. Plant. Growth. Reg.* **10:** 103-108.

Oishi, K.K., Moore, K.B. (1993). The role of abscisic acid in the regulation of endosperm 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Plant Physiol. Supplem.* **102:** 1

Oren-Shamir, M., Pick, U., Avron. M. (1989). Involvment of the plasma membrane ATPase in the osmoregulatory mechanism of the alga *Dunaliella salina. Plant. Physiol.* **89:** 1258- 1263.

Oren-Shamir, M., Pick, U., Avron. M. (1990). Plasma membrane potential of the alga *Dunaliella*, and its relation to osmoregualtion. *Plant Physiol.* **93:** 403-408.

Osmond, C.B. (1981). Photorespiration and Photoinhibition: Some implications for the energetics of photosynthesis. *Biochim. Biophys Acta.* **639:** 77-98.

Oswald, W.J., Golueke, C.G., Horning, D.O. (1965). Closed ecological systems. *J. San. Eng. Div. ASCE*, **91 SA (4):** 23-45.

Owen, J.N., Napier, J.A. (1988). Abscisic acid: New ideas on its role and mode of action. *Plants Today.* March-April. 55-59.

Pace, F.,Ferrara, R., DelCarratore, G. (1977). Effect of sub-lethal doses of copper sulphate and lead nitrate on growth and pigment composition of *Dunaliella salina* Toed. *Bul. Env. Cont. Tox.* **17:** 679-685.

Parry, A.D. (1993). Abscisic acid metabolism. *In:* Methods in Plant Biochemistry. Academic Press Limited. **9:** 381-402.

Parry, A.D., Horgan, R. (1990). Carotenoids and ABA biosynthesis. *In*, News Bulletin, British Society for Plant Growth Regulation. Vol 11. **2.**

Parry, A.D., Babiano, M.J., Horgan, R. (1990). The role of *cis*-carotenoids in abscisic acid biosynthesis. *Planta.* **182:** 118-128.

Parry, A.D., Horgan, R. (1991). Carotenoid metabolism and the biosynthesis of abscisic acid. *Phytochem.* **30:** 815-821.

Parry, A.D., Neill, S.J., Horgan, R. (1988). Xanthoxin levels and metabolism in the wildtype and wilty mutants of tomato. *Planta.* **173:** 397-404.

Persoon, G., Claus, C. (1980). Mass culture of algae: a bottleneck in the nursery culturing of molluscs. *In,* Algal Biomass: Production and Use, (Eds) G. Shelef and C.J. Soeder. Elsevier/North Holland Biomedical Press, Amsterdam.

Peto, R., Doll, R., Buckley, J.D., Sporn, M.B. (1981). Can dietary ß-carotene materially reduce human cancer rates? *Nature.* **290:** 201-208.

Phillips, T.D. (1993). Stress Manipulation in *Dunaliella salina* and Dual-Stage ß-carotene Production. PhD Thesis, Rhodes University, Grahamstown.

Pick, U. (1992). ATPases and ion transport in *Dunaliella. In. Dunaliella*: Physiology, Biochemistry and Biotechnology. (Eds) Avron, M and Ben-Amotz, A. CRC Press, Boca Raton, Florida.

Pierce, M., Raschke, K. (1981). Synthesis and metabolism of ABA in detatched leaves of *Phaseolus vulgaris* L. after loss and recovery of turgor. *Planta.* **155:** 156-165.

Pötter, E., Kloppstech, K. (1993). Effects of light stress on the expression of early light-inducible proteins in barley. *Eur. J. Biochem.* **214:** 779-786.

Powles, S.B. (1984). Photoinhibition of photosynthesis induced by visible light. *Ann. Rev. Plant Physiol.* **35:** 15-44.

Rees, C.D., Young, A.J., Noctor, G., Britton, G., Horton, P. (1989). Enhancement of the pH-dependant dissipation of excitation energy in spinach chloroplasts by light-activation : Correlation with the synthesis of zeaxanthin. *FEBS Lett.* **256:** 85-95.

Richardson, K., Beardall, J., Raven, J.A. (1983). Adaption of unicellular algae to irradiance: an analysis of strategies. *New Phytol.* **93:** 157-191.

Rock, P.D., Heath, T.G., Zeevaart, J.A.D. (1992). 2-*Trans*-abscisic acid biosynthesis and metabolism of ABA-aldehyde and xanthoxin in wild type and the *aba* mutant of *Arabidopsis thaliana*. *J. Exp. Bot.* **43:** 249-56.

Rock, C.D., Zeevaart, J.A.D. (1990). Abscisic (ABA)-aldehyde is a precursor to, and 1',4'-*trans*-ABA-diol a catabolite of ABA in apple. *Plant Physiol.* **93:** 915-923.

Rock, P.D., Zeevaart, J.A.D. (1991). The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proc. Natl. Acad. Sci.* **88:** 7496-7499.

Rose, P.D. (1992). Algal biotechnology and the beneficiation of saline effluent wastes. Phd Thesis, Rhodes University, Grahamstown.

Rose, P.D., Cowan, A.K. (1991a). A growth and separation process for algae and to useful products obtained therefrom. RSA Patent Application No 91/5070.

Rose, P.D., Cowan, A.K. (1991b). A process for the production of useful products from effluents. RSA Patent Application No 91\5071.

Ruban, A.V., Ress, D., Pascal, A.A., Horton, P. (1992). Mechanism of ΔpH-dependant dissipation of absorbed excitation energy by photosynthetic membranes. II. The relationship between LHCII aggregation *in vitro* and qE in isolated thylakoids. *Biochem. Biophys. Acta.* **1102:** 39-44.

Sadka,A., Himmelhoch, S., Zamir, A. (1991). A 150 kilodalton cell surface protein is induced by salt in the halotolerant green alga *Dunaliella salina. Plant Physiol.* **95:** 822-831.

Sandmann, G. (1991). Biosynthesis of cyclic carotenoids: Biochemistry and molecular genetics of the reaction sequence. *Physiol. Plant.* **83**:186-193.

Sapozhnikov, D.J., Krasovskaya, T.A., Mayevskaya, A.N. (1957). Changes observed in the reaction between the main carotenoids in the plastids of green leaves exposed to light. *Docl. Akad. Nauk SSSR.* **113:** 465-467.

Schlipalius, L. (1991). The extensive commercial cultivation of *Dunaliella salina. Biores. Technol.* **38:** 241-243.

Schöner, S., Krause, G.H. (1990). Protective systems against oxygen species in spinach: Response to cold acclimation in excess light. *Planta.* **180:** 383-389.

Schreiber, U., Reising, H., Neubauer, C. (1991). Contrasting pH-optima of light-driven O_2^- and H_2O reduction in spinach chloroplasts as measured via chlorophyll fluorescence quenching. *Z. Naturforsch.* **46:** 635-643.

Semenenko, V.E., Abdullaev, A.A., (1980). Parametric control of ß-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation. *Fiziol. Rast.* **27:** 31-41.

Senger, H., Wagner, C., Hermsmeier, D., Hohl, N., Urbig, T., Bishop, N. (1993). The influence of light intensity and wavelength on the contents of α and ß-carotene and their xanthophylls in green algae. *J. Photochem. Photobiol. B. Biol.* **18:** 273-279.

Shaish, A., Avron, M., Ben-Amotz, A. (1990). Effect of inhibitors on the formation of stereoisomers in the biosynthesis of ß-carotene in *Dunaliella bardawil. Plant Cell Physiol.* **31(5):** 689-696.

Shaish, A., Avron, M., Pick, U., Ben-Amotz, A. (1993). Are active oxygen species involved in induction of ß-carotene in *Dunaliella bardawil* ? *Planta.* **190:** 363-368.

Shklar, G., Schwartz, J. (1988). Tumour necrosis factor in experimental cancer regression with alphatocopherol, beta-carotene, canthaxanthin and algal extract. *Eur. J. Cancer Clin. Oncol.* **5:** 839-850.

Siefermann-Harms, D., Joyard, J., Douce, R. (1978). Light-induced changes of the carotenoid levels in chloroplast envelopes. *Plant Physiol.* **61:** 530-533.

Sindhu, R.K., Walton, D.C. (1988). Xanthoxin metabolism in cell-free preparations from wild-type and wilty mutants of tomato. *Plant. Physiol.* **88:** 178-182.

Smith, B.M., Morrissey, P.J., Guenther, J.E., Nemson, J.A., Harrison, M.A., Allen, J.F., Melis, A. (1990). Response of the photosynthetic apparatus in *Dunaliella salina* (Green Algae) to irradiance stress. *Plant Physiol.* **93:** 1433-1440.

Smith, G.N., Willmer, C.M. (1988). Effects of calcium and abscisic acid on volume changes of guard cells protoplasts of *Commelina communis*. *J. Exp. Bot.* **30:** 1529-1539.

Soeder, C.J., Binsack, R. (Eds) (1978). Microalgae for food and feed: A status analysis. *Ergebn. Limnol.* **1:** 300pp.

Stillwell, W., Hester, P. (1984). Abscisic acid increases membrane permeability by interacting with phosphatidylethanolamine. *Phytochem.* **10:** 2187-2192.

Tamiya, H. (1957). Mass culture of algae. *Ann. Rev. Pl. Physiol.* **8:** 309-334.

Taylor, H.F., Burden, R.S. (1973). Preparation and metabolism of 2-[^{14}C]-*cis,trans*-xanthoxin. *J. Exper. Bot.* **24:** 873-880.

Thayer, S.S., Bjorkman, O. (1990). Leaf xanthophyll content and composition in sun and shade determined by HPLC. *Photosynth. Res.* **23:** 331-343.

Tietz, A. (1985). Characterization of a novel (±)-abscisic metabolite. *Physiol. Plant.* **65:** 171-176.

Tietz, A., Rutikowski, V., Kohler, R., Kasprik, W. (1989). Further investigations on the occurance and the effects of abscisic acid in algae. *Biochem. und Physiol. der Pflazen.* **184:** 259-66.

Trewavas, A.J. (1981). How do plant substances work?. *Plant Cell Environ.* **4:** 203-228.

Trewavas, A.J. (1991). How do plant substances work?. *Plant Cell Environ.* **14:** 1-12.

Val, J., Abadia, J., Heras, L., Monge, E. (1986). Higher plant photosynthetic pigment analysis. Determination of carotenoids and chlorophylls by HPLC. *J. Micronutr. Anal.* **2:** 305-312.

Vanselow, K.H. (1993). Some critical remarks on the suitability of the concept of the photosynthetic unit in photosynthesis research and phytoplankton ecology. *Bot.Acta.* **106:** 287-293.

Walton, D.C. (1980). Biochemistry and physiology of abscisic acid. *Ann. Rev. Plant Physiol.* **31:** 453-489.

West, C. (1990). Terpene biosynthesis and metabolism. *In,* Plant Physiology, Biochemistry and Molecular biology. (Eds) D.T. Dennis and D.H. Turpin, Longman Group, UK Limited.

Wilhelm, C. (1993). Some critical remarks on the suitability of the concept of the photosynthetic unit in photosynthesis research and phytoplankton ecology. *Bot. Acta.* **106:** 287-293.

Yamamoto, H.Y. (1979). Biochemistry of the violaxanthin cycle in higher plants. *Pure & Appl. Chem.* **51:** 639-648.

Young, A. (1991). The photoprotective role of carotenoids in higher plants. *Physiol. Plant.* **83:** 702-708.

Young, A., Barry, P., Britton, G. (1989). The occurance of ß-carotene-5,6-epoxide in the photosynthetic apparatus of higher plants. *Z. Naturfosch.* **44:** 959-965.

Young, A., Britton, G. (1990a). Carotenoids and stress. *In*, Stress Responses in Plants: Adaptation and Acclimation Mechanisms. (Eds) R.G. Alscher and J.R. Cumming, Wiley-Liss Inc.

Young, A., Britton, G. (1990b). Photobleaching in the unicellular green alga *Dunaliella parva.* 19/9. *Photosynth. Res.* **25:** 129-136.

Zeevaart, J.A.D., Creelman, R.A. (1988). Metabolism and physiology of abscisic acid. *Ann. Rev. Plant Physiol. Plant Mol. Biol. 39; 439-473.*

Zeevaart, J.A.D., Heath, T.G., Gage, D.A. (1989). Evidence for a universal pathway of abscisic acid in higher plants from ^{18}O incorporation patterns. *Plant Physiol.* **91:** 1594-1601.

Ziegler, R.G. (1991). Vegetables, fruits, and carotenoids and the risk of cancer. *Am. J. Clin. Nutr.* 53:251S-9S.

Zimmermann, U. (1978). Physics of turgor and osmoregulation. *Ann. Rev. of Plant Physiol. 29: 121-148.*

POSTSCRIPT

The following is a list of papers and reports emananting from the research presented in this study:

1. Horne, L.G., Rose, P.D., Cowan, A.K. (1992). The stress response of *Dunaliella salina*. Sasol Symposium, RAU.

2. Horne, L.G., Cowan, A.K., Rose, P.D. (1992). Abscisic acid and pigment changes in stressed cells of *Dunaliella salina*. Proc Seventh Congress SA Soc. Microbiol., Bloemfontein.

3. Cowan, A.K., Rose, P.D., Horne, L.G. (1992). *Dunaliella salina*: a model system for studying the response of plant cells to stress. *J. Exp. Bot.* **43(257):** 1535-1547.

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*. Proceedings of the 10th Carotenoid Symposium, Trondheim, Norway.

5. Phillips, L.G., Cowan, A.K., Rose, P.D. (1994). Factors affecting ß-carotene accumulation in *Dunaliella salina*. Sasol Symposium, RAU.

Submitted papers

1.Cowan, A.K., Logie, M.R.R., Rose, P.D., Phillips, L.G. (1994). Induction of zeaxanthin formation. *J. Plant Physiol.* (In press).

2.Phillips, L.G., Cowan, A.K., Rose, P.D., Logie, M.R.R. (1994). Xanthophyll operation in *Dunaliella*. *J.Plant Physiol.* (In press).