Manipulation of the glycerol content of dunaliella electroporation: response of photosynthesis, cell volume and glycerol content

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MANIPULATION OF THE GLYCEROL CONTENT OF
*DUNALIELLA* BY ELECTROPORATION.
RESPONSE OF PHOTOSYNTHESIS, CELL VOLUME AND
GLYCEROL CONTENT

A thesis submitted in fulfilment of the requirements for the
award of the degree of

DOCTOR OF PHILOSOPHY

FROM

THE UNIVERSITY OF WOLLONGONG

BY

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DEPARTMENT OF BIOLOGICAL SCIENCES
1994
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DECLARATION

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the requirements for the degree of Doctor of Philosophy.

The work described in this thesis was carried out by me and has not previously been submitted to any other University or institution.

Mansoor Shariat
March, 1994
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To my son, Masih and my Australian born daughter, Mahfam I express my heartfelt thanks and apologies for not being able to spend more time with them.

Finally I wish to dedicate this thesis to my father, mother and my wife, without whose encouragement this thesis would not have been completed.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Amp</td>
<td>2-Amino-2-Methyl-1,3-propanediol</td>
</tr>
<tr>
<td>Btp</td>
<td>1,3 bis [tris (hydroxymethyl)-methylamino] propane</td>
</tr>
<tr>
<td>chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>DAG</td>
<td>1, 2-diacylglycerol</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydroxyacetone</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>$^{14}$C-DMO</td>
<td>5,5-dimethyl-2 ($^{14}$C)-oxazolidine-2,4-dione</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>G protein</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>Hepes</td>
<td>(N-[2-hydroxyethyl] piperazine)-N'-[ethanesulfonic acid])</td>
</tr>
<tr>
<td>InsP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>Mes</td>
<td>2(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
</tr>
<tr>
<td>PGA</td>
<td>phosphoglyceric acid</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>Rubisco</td>
<td>ribulose-1,5 bisphosphate carboxylase / oxygenase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TMAO</td>
<td>trimethylamine oxide</td>
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ABSTRACT

The aim of the first part of this project, was to determine the effects of salt stress on photosynthesis and respiration in the green algae *D. tertiolecta* and *D. salina*. A previously reported burst in oxygen evolution after salt addition was investigated. Following salt stress, no burst in photosynthesis was found under any conditions for both species in contradiction to the earlier reports. This might be due to strain or species differences. High salt stress (increasing salinity by a factor of 5.88) inhibited photosynthesis in *D. tertiolecta*. Low salt stress (increasing salinity by a factor of 1.66) caused in *D. tertiolecta* a small increase in photosynthesis but had no substantial effects in *D. salina*. Salt stress decreased respiration in *D. tertiolecta*, more markedly at high salt stress. In *D. salina*, salt stress by a factor of 1.66 caused respiration to increase significantly. In general, the magnitude of salt stress was more important than the final salt concentration. No significant differences were found between phosphate and Hepes buffers.

In the second part of this project, the aim was to use electroporation to lower the intracellular glycerol concentration by a small proportion (10 - 20%) while the external osmotic pressure was kept constant. The effects of electroporation on photosynthetic O₂ evolution, mitochondrial O₂ uptake, release of soluble protein and release of intracellular glycerol to the medium were measured. Electroporation conditions were optimised to release about 10% of intracellular glycerol to the external medium with minimal apparent effects on metabolism. The results showed that glycerol release did not originate from the total rupture of a small proportion of cells. The uptake of mannitol, the major solute in the electroporation medium, was determined and found to be less than 20% of glycerol release. Following the release of glycerol by electroporation, *D. tertiolecta* cells were incubated in isotonic Hepes buffer under illumination. The intracellular glycerol content then increased to reach pre-electroporation values after about 30 minutes. The cell volume, measured on motile cells by video microscopy, reduced by 23% immediately after electroporation but returned to pre-electroporation values after about 30 minutes. Because the cells were maintained at constant external osmotic pressure throughout the procedure, it is concluded that the regulatory mechanism responsible for setting the intracellular glycerol content does not sense external osmotic pressure *per se*. These findings suggest that the intracellular glycerol content is set by a mechanism that senses cell volume or some parameter linked directly to cell volume.
CHAPTER ONE:
LITERATURE REVIEW
1.1. General biology of *Dunaliella*

The genus *Dunaliella* is a unicellular microscopic alga from the phylum Chlorophyta, order Volvocales and family Polyblepharidaceae (Butcher, 1959). *Dunaliella* has a structure similar to that of the genus *Chlamydomonas*, but differs in being able to grow in extremely euryhaline environments. The cells are ovoid, ellipsoid or spherical in shape, with two equal flagella. The cell contains one cup-shaped chloroplast which occupies about 50% of the cell volume, with a single median pyrenoid embedded in the basal portion of the chloroplast (Ben-Amotz, 1980). The single nucleus is located within the cup of the chloroplast at the centre or near the anterior end. The golgi apparatus is situated near the flagellar pole (Brown, Lilley & Marengo, 1982a). Each cell has more than one eye spot at the side of the chloroplast.

*Dunaliella* reproduces by both asexual and sexual means. Asexual reproduction normally occurs by longitudinal division of the motile cells (Marano, 1976). Sexual reproduction by isogamy is also known but is much rarer and occurs in stationary phase cultures when two motile cells fuse to form a quadriflagellate zygote (Brown & Borowitzka, 1979).

Ben-Amotz & Avron (1978); Brown & Borowitzka (1979); Ben-Amotz & Avron (1983a) made statements to the effect that *Dunaliella* cells do not have a rigid polysaccharide wall, with the cell protoplast being enclosed by a thin elastic membrane only. On the other hand, the extracellular matrix of *Dunaliella* cells has been characterised by Oliviera, Bisalputra & Antia (1980) (using ruthenium red and alcian staining) and Melkonian & Preising (1984) (using Indian ink staining). They demonstrated that *Dunaliella* cells have a cell coat of glycoprotein which is visible under light microscopy though not by electron microscopy due to the coat washing off during the preparative procedures. This coating in is composed largely of glycoproteins with some neuraminic acid residues as part of the polysaccharides. The glycocalyx is not rigid and does not interfere with flexibility of the cells (Ginzburg &
Richman, 1985). Hence the main morphological characteristic which distinguishes *Dunaliella* from *Chlamydomonas* is this extracellular matrix. *Chlamydomonas* has a thick and rigid cell wall similar to other species of the order Volvocales. *Dunaliella* has an extracellular matrix which is thin and flexible. It can shrink and expand with changes in cell volume. When *Dunaliella* was subjected to media of different osmotic pressures at 2 °C (where metabolism was slowed) the cells behaved like osmometers (Katz & Avron, 1985).

The best known species of *Dunaliella*, which are classified according to size, shape, colour and other morphological differences are *D. parva*, *D. viridis*, *D. salina*, *D. bardawil*, *D. tertiolecta*, *D. primolecta*, *D. bioculata*, *D. euchlora*, *D. minuta*, *D. media*, and *D. acidophila* (Avron & Ben-Amotz, 1992). The cell size varies from 5-25 μm in length and from 3-13 μm in width (Ben-Amotz, 1980).

*Dunaliella* species are found in a range of environments. They can be isolated from the oceans, inland salt lakes, salt marshes with various chemical compositions, with widely different salt concentrations. As a genus, *Dunaliella* comprise the only eukaryotic organisms that thrive in media ranging from brackish (less than 0.1 M NaCl) to saturated salt solutions (exceeding 5 M) (Ben-Amotz & Avron, 1983a). Different species of *Dunaliella* have different apparent optimum salt concentrations for maximum growth (Brown, 1990).

### 1.2. Halophilism and Halotolerance

Organisms living under conditions of high salinity and requiring a minimum concentration of NaCl higher than normal seawater, have been called halophytes (Ben-Amoz & Avron, 1983a). However, Brown (1978b) has used the term halophilism to describe organisms which are strictly dependent on high salt concentration for growth. Halophilism can only be applied to certain bacteria. "Halophilic bacteria have an absolute requirement for at least 2.8 M NaCl and have an
optimum in the region of 4 M (depending on species and temperature)" (Brown, 1978b). These bacteria cannot be trained by serial transfer to accept lower salt concentrations, nor can other solutes substitute for sodium chloride to any appreciable extent. According to this definition, no eukaryotic alga can be classified as a halophyte.

On the other hand, the term "halotolerant" encompasses all aquatic organisms and emergent plants which are able to grow on a wide range of salinity in the medium with optimal growth usually at the lower side of the salinity range (Ben-Amotz & Avron, 1983a). Halotolerance accordingly, refers to the growth capability of the organism and not its ability to withstand high salt concentrations.

According to the above definition, the growth requirements and salt tolerance of *Dunaliella* differ from the halophilic bacteria. *Dunaliella* spp., have an optimum very close to the bottom of their salt concentration growth range. Thus, over most of this range they are, in fact, displaying a tolerance of, rather than a requirement for, salt (Brown, 1978b; Brown & Borowitzka, 1979). In addition, while the salinity range of *Dunaliella* species is wide, their growth is not NaCl dependent, because other solutes of equivalent osmotic pressure can be substituted (Wegmann, 1971; Borowitzka, Kessly & Brown, 1977; Gilmour, Hipkins & Boney, 1984a & 1984b; Belmans & Van Laere, 1987; Fuggi et al., 1988; Weiss & Pick, 1990).

Furthermore, the tolerance range of *Dunaliella* spp can be changed by training, a capacity which further distinguishes them from bacteria. *Dunaliella* spp., can be trained to higher or lower salt concentrations, so that the apparent limits of salinity tolerance of a marine species such as *D. tertiolecta* on the one hand, and halophilic species such as *D. viridis* on the other reflect the history of the inoculum as much as intrinsic characteristics of the alga (Brown & Borowitzka, 1979). For example, when the inoculating culture of *D. viridis*, a halophilic species, was grown in 3.4 M NaCl the
apparent optimum for growth and minimum NaCl concentration coincided at 1.8 M NaCl. But when the cells were trained by serial transfer to lower salinities, the optimum was about 1 M and the minimum at less than 0.2 M NaCl (Borowitzka & Brown, 1974).

1.3. Osmoregulation

Osmoregulation and turgor regulation are frequently used to described the physiological processes behind salinity tolerance. Kesseler (1959 cited by Kirst, 1989) introduced two definitions to differentiate between turgor pressure regulation and osmoregulation: (a) turgor pressure regulation, typically observed in marine algae, that adjusts cellular osmotic potential to keep the cell turgor constant (b) osmoregulation in organisms (mostly animals) that aspire to maintain the internal osmotic potential constant.

Hellebust (1976) defined osmoregulation as: "by osmoregulation is understood the processes and mechanisms by which plants regulate the osmotic pressure in their cells". Brown & Edgley (1980) defined osmoregulation as "the maintenance of approximately constant cell volume and turgor pressure in the face of changing water potential". Brown, Mackenzie & Singh (1986) proposed the more flexible definition for osmoregulation, namely, "the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism". Reed (1984) believed that osmoregulation is an inappropriate and misleading term for the adjustment of cellular osmotic potential following changes in external salinity.

Cram (1976) has stated firmly that turgor pressure is controlled in walled cells while volume is controlled in wall-less cells. However Brown (1990) pointed out that turgor pressure in wall-less cells is not zero: "(1) when direct measurements have been possible, turgor pressure in wall-less cells has usually been below the limit of the measuring instrument; (2) wall-less cells often behave as osmometers, i.e. when they
are exposed to a range of water potentials their volume shortly after transfer is approximately proportional to the reciprocal of external osmotic pressure, if they survive and regulate, their volume returns approximately to its original level". Brown (1990) has concluded the term, osmoregulation, should not be used routinely and should be replaced by turgor/volume control.

1.4. Compatible solutes

1.4.1. General

Compatible solutes are osmolytes which stabilise the structure and function of proteins because they do not interact with the protein surface. Many algae grow in environments where components in the external medium are potentially harmful to enzymes within cells. In the brown marine algae *Fucus vesiculosus* and *Laminaria saccharina*, it has have been shown that in the presence of ion concentration exceeding 100 mM, the *in vitro* activities of most enzymes are severely inhibited, while compatible solutes (e.g. mannitol) at isosmotic concentration are not or are much less inhibitory (Davison & Reed, 1985). Proteins can be salted-out and removed from the aqueous phase by these compatible osmolytes (Withers, 1992).

The term "compatible solute" was first introduced by Brown & Simpson (1972) as follows: "a compatible solute may be loosely defined as one which, at high concentration, allows an enzyme to function effectively". This definition was later extended by replacing "an enzyme" with "all essential cell processes" (Brown, 1976). Brown (1978b) has argued that "compatible solutes must always behave as osmoregulators". However, there are protective substances that fit completely into the compatible solute definition which are not osmoregulators (Brown, 1990). In essence, compatible solutes are very soluble, low molecular weight and non-charged compounds which can be accumulated to high concentration in the cytoplasm while causing little enzyme inhibition (Borowitzka, 1985).
Cations such as ammonium (NH$_4^+$), methylated amines such as (CH$_3$)$_4$N$^+$ and (CH$_3$)$_2$NH$_2^+$ and some inorganic anions such as F$^-$, PO$_4^{3-}$ and SO$_4^{2-}$ can be compatible solutes because of their stabilising effect on proteins. Some amino acids (e.g., glycine, proline), glycerol, trehalose, trimethylamine oxide (TMAO), betaine, sarcosine, taurine, and octopine are also compatible solutes (Withers, 1992).

Other types of solute have been defined by their interactions with cellular proteins. Perturbing solutes are osmolytes which bind non-specifically with proteins and destabilize their structure by unfolding the protein to allow maximal interaction of osmolytes with the surrounding water. Perturbing solutes such as urea, arginine, Na$^+$ and Cl$^-$ have inimical effects on protein structure and function, and solubilize the protein in the aqueous phase. Counteracting solutes, such as TMAO and betaine, are osmolytes that reduce the effect of perturbing solutes (Withers, 1992).

### 1.4.2. Glycerol as a compatible solute

In addition to ions, certain low molecular weight organic solutes are accumulated or degraded in algae in response to change in salinity. These include polyols such as glycerol in *Dunaliella* (Craigie & McLachlan, 1964; Wegmann, 1971), mannitol in most Phaeophyaceae (Kirst & Bisson, 1979; Reed *et al.*, 1985), sorbitol and the amino acid proline in most diatoms and some Chlorophyceae (Liu & Hellebust, 1976; Schobert, 1980). A list of osmoregulatory solutes in bacteria, yeast, fungi, and algae has been compiled by Borowitzka (1985). Although such organic solutes are accumulated or degraded in algae in response to changes in salinity, this alone does not mean they are necessarily compatible solutes. Among osmolytes tested for their protective capacity, proline and glycerol were found to be most effective while sucrose for example, was fairly poor in this respect (Kirst, 1989). Schobert (1977 cited by Kirst, 1989) suggested that polyols, especially glycerol, may act as water-like
substances that mimic the water structure and maintain an artificial water sphere around the macromolecules.

Glycerol accounts for most of the osmoregulatory function in *Dunaliella* cells following changes in the water activity of the medium (Section 1.7.3). Additionally glycerol is a compatible solute. Glycerol seems suitable for the function of protecting enzyme activity at low water activity because it remains miscible with water but only slightly inhibits the activity of several enzymes at concentrations over 5 M, whereas NaCl at much lower concentrations has strongly inhibitory (Ben-Amotz & Avron, 1972; Borowitzka & Brown, 1974).

1.5. Cell volume and compartmentation in *Dunaliella*

1.5.1. General

When *Dunaliella* cells are subjected to changes in osmotic pressure of the medium, their volume undergoes short-term changes. Determination of the internal volume of the *Dunaliella* cell is therefore important for the study of osmotic response.

The plasma membrane of *Dunaliella* is covered by a thin glycocalyx (Section 1.1). This extracellular matrix is flexible and does not affect the shrinkage or swelling of *Dunaliella* cells. Being outside the plasma membrane, this matrix is not involved in the intracellular osmotic pressure adjustment. Any determination of the intracellular volume must measure the aqueous space inside the plasma membrane including all organelles. Chloroplast, mitochondria, golgi apparatus, endoplasmic reticulum (ER), nucleus and vacuoles have all been identified in *Dunaliella* by Oliveira, Bisalputra & Antia (1980); Brown, Lilley & Marengo (1982a); Melkonian & Preising (1984); Hajibagheri *et al.* (1985). Microscopic observation of *Dunaliella* has shown that, following osmotic stress, the chloroplast and cytoplasm respond similarly (Trezzi, Galli & Bellini, 1965 cited by Sheffer & Avron, 1986). *Dunaliella* does not have a
single large vacuole like higher plant cells. Several small vacuoles have been identified in *D. tertiolecta* by Oliveira, Bisalputra & Antia (1980); Brown, Lilley & Marengo (1982a) and in *D. parva* by Hajibagheri *et al.* (1985).

An unusual model of compartmentalisation of *Dunaliella* cells was suggested by Ginzburg (1978). He suggested a two compartment model. One compartment was the chloroplast, limited by a tight membrane and the other limited by a membrane permeable to ions and small neutral molecules such as sorbitol, mannitol or glucose. This was based on early reports of large (more than 50%) non-osmotic volume in *D. parva* (Rabinowitch, Grover & Ginzburg, 1975; Zmiri & Ginzburg, 1983). They suggested that large amounts of Na⁺ ions might be restricted to such a compartment. It seems that this hypothesis was proposed to interpret contrary findings of high concentration of intracellular Na⁺ and high Na⁺ sensitivity of *Dunaliella* cells enzymes. Later, Hajibagheri *et al.* (1985) showed by X-ray microanalysis technique that, in *D. parva*, Na⁺ concentration was high in the vacuole but low in the cytosol. They did not find any non-osmotic space or Na⁺ space in cells adapted to 0.4 M or 1.5 M NaCl. This will be discussed further in Section 1.6.1.4.

Ginzburg & Richman (1985) also proposed a model that the glycocalyx of *Dunaliella* is permeable to small neutral molecules such as sorbitol, mannitol and glucose allowing equilibration with the medium. The osmotic space was 60-80% of the total cell volume within membranes which were relatively impermeable to the small molecules. Glycerol and other metabolites were proposed to be within this space. However, their measurements of cell volume were in error (Section 1.5.2.2). Blackwell & Gilmour (1989) rejected the 'Na⁺ space' because they found internal concentrations of Na⁺ much lower than those in the medium. They argued that "even if a compartment exists within the glycocalyx of the cells which freely exchanges with the external medium, it should properly be regarded as extracellular". Katz & Avron (1985) calculated in *D. salina* the non-osmotic space as 10% of the determined total
volume and remaining 90% as effective osmotic volume. There is no evidence from
direct observation for the existence or location of such non-osmotic space in
*Dunaliella*.

### 1.5.2. Cell volume measurement

Many methods have been introduced for measuring the cell volume specially for
*Dunaliella*. These methods can be classified into two groups, direct and indirect
measurement.

#### 1.5.2.1. Direct methods.

Direct measurement involves photographing or video tape recording live cells under
the microscope and measuring the dimensions of the cells on printed or projected
pictures (Ben-Amotz & Avron, 1980; Zmiri, Wax & Ginzburg, 1984; Ginzburg &
Ginzburg, 1985a, 1985b; Blackwell & Gilmour 1989) or TV monitor (Ehrenfeld &
Cousin, 1982). The volume is calculated from a suitable equation by approximating
the cells to a certain ellipsoid shape. Direct observation provides details of the
condition of the cells, such as the distribution of cell sizes and cells are studied under
*in vivo* conditions.

#### 1.5.2.2. Indirect methods.

Indirect measurement involves determination of the volume of a centrifuged pellet of
algal cells and measuring the extracellular volume between the cells. Cell volume is
finally calculated by subtracting the volume of extracellular space from the volume of
the pellet. Division of this by the cell number allows the volume per cell to be
calculated (see Fig. 1.1). Indirect measurement techniques give more accurate size of
Fig. 1.1: Determination of the intracellular volume of *Dunaliella* cells by measurement of extracellular space (Katz & Avron, 1985)

determination for the cells, however this is measured as a mean; the frequency distribution of cell volume is not obtained. For determination of pellet volume, $^3\text{H}_2\text{O}$ as a marker for total aqueous space, or using a cytocrit tube and measuring the length of the pellet column have been used. The more accurate and valid methods is with $^3\text{H}_2\text{O}$, which can freely pass between the inside and outside the cell. In the cytocrit tube technique, the algal suspension is centrifuged at high speed in a cytocrit tube.

The volume of pellet is calculated by measuring the length of cell column. This technique is not widely used and is not accurate because it assumes all extracellular space is eliminated.
Measuring the extracellular volume between the cells needs an appropriate extracellular marker which must fully equilibrate within the extracellular space. The marker must not penetrate the plasma membrane and must not bind to the extracellular matrix. Any underestimation of extracellular space will cause overestimation of volume and vice versa. Many markers such as Li+, blue dextran (MW = 2 x 10^6), ^14^C-dextran (MW = 70,000) and ^14^C-sorbitol have been used. Provided small markers such as Li+ are not absorbed by the cells, they are better than large molecules such as ^14^C-dextran (MW = 70,000). The advantage of small markers is that they can fully equilibrate within the extracellular space. Li+ does not penetrate Dunaliella cells (Katz & Avron, 1985; Blackwell & Gilmour, 1989). Hence Li+ is the best marker for accurate measuring of the extracellular space. The disadvantage of large molecules such as ^14^C-dextran (MW = 70,000) is that the marker may be unable to permeate fully the extracellular spaces in the glycocalyx of Dunaliella cells (Blackwell & Gilmour, 1989), while Li+ does. As a result, the use of large molecular size markers underestimates the extracellular space, resulting in overestimation of the cell volume.

Consequently, different cell volumes have been reported for the same species of Dunaliella (Table 1.1). When Dunalliela cells are adapted to different osmotic pressures, the volume remains constant (an average of 90 μm³ for D. salina and 600 μm³ for D. bardawil, Katz & Avron, 1985). In contrast, others found different sizes for Dunaliella cells when adapted to different salinities. Ehrenfeld & Cousin (1982) reported, for D. tertiolecta adapted to medium containing 0.02 M, 0.41 M and 1.64 M NaCl, mean cell volumes of 274, 179 and 283 μm³ respectively, measured by light microscopy. The volumes were 106, 89, and 111 μm³ respectively when indirect measurement was used with ^14^C-sorbitol as extracellular marker. With Dextran 70,000 as marker, the volumes estimated were 227, 211, and 259 μm³ respectively.
Table 1.1: **Cell volume measurement of Dunaliella cells by different methods.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Method of measurement</th>
<th>External NaCl (M)</th>
<th>Pellet volume determination</th>
<th>Extracellular volume determination</th>
<th>Cell Volume (μm³)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. salina</em></td>
<td>microscopy</td>
<td>4.0</td>
<td></td>
<td></td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td><em>D. bardawil</em></td>
<td>microscopy</td>
<td>4.0</td>
<td></td>
<td></td>
<td>400</td>
<td></td>
</tr>
<tr>
<td><em>D. tertiolecta</em></td>
<td>microscopy</td>
<td>0.02</td>
<td></td>
<td></td>
<td>274</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>microscopy</td>
<td>0.4</td>
<td></td>
<td></td>
<td>179</td>
<td></td>
</tr>
<tr>
<td></td>
<td>microscopy</td>
<td>1.64</td>
<td></td>
<td></td>
<td>283</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>0.02</td>
<td>Cytocrit</td>
<td></td>
<td>227</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>0.4</td>
<td>Cytocrit</td>
<td></td>
<td>211</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>1.64</td>
<td>Cytocrit</td>
<td></td>
<td>259</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>0.02</td>
<td>Cytocrit</td>
<td>14C-Dextran</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>0.4</td>
<td>Cytocrit</td>
<td>14C-Sorbitol</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td><em>D. tertiolecta</em></td>
<td>microscopy</td>
<td>0.5</td>
<td></td>
<td></td>
<td>273</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>microscopy</td>
<td>2.0</td>
<td></td>
<td></td>
<td>231</td>
<td></td>
</tr>
<tr>
<td><em>D. parva</em> (19/9)</td>
<td>microscopy</td>
<td>0.5</td>
<td></td>
<td></td>
<td>188</td>
<td></td>
</tr>
<tr>
<td></td>
<td>microscopy</td>
<td>2.0</td>
<td></td>
<td></td>
<td>231</td>
<td></td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>indirect</td>
<td>0.5</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>87</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>1.5</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>2.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>3.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>4.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td><em>D. bardawil</em></td>
<td>indirect</td>
<td>1.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>1.5</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>730</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>2.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>520</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>3.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>580</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>4.0</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td><em>D. parva</em> (19/9)</td>
<td>microscopy</td>
<td>0.4</td>
<td></td>
<td></td>
<td>120</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>microscopy</td>
<td>1.5</td>
<td></td>
<td></td>
<td>186</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indirect</td>
<td>0.4</td>
<td>3H₂O</td>
<td>Li⁺</td>
<td>131</td>
<td></td>
</tr>
</tbody>
</table>

Hence the light microscopic and large marker measurements were similar, but $^{14}\text{C}$-sorbitol gave about half the volume. Ginzburg & Richman (1985) also reported cell volumes for *D. tertiolecta* adapted to 0.5 M and 2 M NaCl, using the light microscope, of 273 and 231 $\mu m^3$ respectively. For *D. parva* adapted to 0.4 M and 1.5 M NaCl, mean cell volumes of 120 and 186 $\mu m^3$ respectively, measured by light microscope, were reported by Blackwell & Gilmour (1989). They also reported that the cell volume was smaller, 65 and 131 $\mu m^3$ for cells adapted to 0.4 M and 1.5 M NaCl respectively, when indirect measurement was used. They used Li$^+$ as the extracellular marker.

**1.5.2.3. Conclusion**

In general, cell volumes measured by direct observation were much higher than when measured by indirect methods. The direct measurement technique includes the osmotic, non-osmotic space and glycocalyx of *Dunaliella*. On the other hand, the indirect methods, determine a volume which is much higher for high molecular weight markers than small ones. The results obtained when Li$^+$ is used as marker seems more correct (Section 1.5.2.2). The direct measurement technique is subject to errors which cause overestimation of the cell volume size such as (a) "small errors in linear measurement leads to large errors on multiplication to give cell volume estimation", (b) "the measurement will include the glycocalyx covering", (c) "living, moving cells do not come sharply enough into focus at these magnifications for accurate determination of the limits of the cells", (d) "direct measurement includes aqueous space plus volume occupied by lipids, protein, starch, and other osmotically inert component" (Blackwell & Gilmour, 1989). For the indirect measurement technique (e.g. when Li$^+$ is used for measuring the extracellular space), only the aqueous space is measured. Provided accurate linear measurements can be made an sharply focused living cells, accurate determination of the cell volume should be possible for *Dunaliella* given the thinness of the extracellular matrix.
1.6. Role of intracellular ions in the adjustment of the internal osmotic pressure of *Dunaliella*.

To understand how much ions contribute to the internal osmotic pressure of *Dunaliella*, a reliable technique to measure the internal ion concentrations, especially Na\(^+\), is necessary.

Much has been reported about the measurement of intracellular ion concentration and the role of Na\(^+\), K\(^+\) and Cl\(^-\) in the adjustment of the internal osmotic pressure of *Dunaliella* (Ben-Amoz & Avron, 1972; Latorella & Vadas, 1973; Borowitzka & Brown, 1974; Ginzburg, 1978; Gimmler & Schirling, 1978; Ginzburg, 1981; Ehrenfeld & Cousin, 1982, 1984; Gimmler, Kaden & Kirchner, 1984; Ginzburg & Ginzburg, 1985a, 1985b; Katz & Avron, 1985; Hajibagheri *et al.*, 1985; Pick, Karni & Avron, 1986; Karni & Avron, 1988; Blackwell & Gilmour, 1989). There has been considerable debate regarding the extent of influence of NaCl in the adjustment of the internal osmotic pressure of *Dunaliella*. Intracellular NaCl concentrations ranging from below 10% (Ben-Amotz & Avron, 1972; Borowitzka & Brown, 1974; Katz & Avron, 1985; Pick, Karni & Avron, 1986; Karni & Avron, 1988) up to 50% (Gimmler & Schirling, 1978; Zmiri & Ginzburg, 1983) of the Na\(^+\) external concentration have been reported. These different results are due to the different methods which were used by the researchers, as follows (see Table 1.2, 1.3)

**1.6.1. Measurement of internal Na\(^+\) concentration**

**1.6.1.1. Measurement of internal Na\(^+\) concentration by measuring the extracellular space.**

*Dunaliella* will grow in media with high NaCl concentrations. When the cells are separated from the medium, any trapping of Na\(^+\) in the extracellular space affects the measurement of intracellular Na\(^+\). Hence, as described in Section 1.6.2.2 the main factor which is important for measuring intracellular Na\(^+\) and other ion concentrations
is determining the extracellular volume between the cells in suspensions and pellets. Provided accurate measurements of extracellular volume can be made, the intracellular Na\(^+\) concentration can be calculated from the known Na\(^+\) concentration of the medium. Any underestimation of extracellular volume will cause overestimation of intracellular Na\(^+\) concentration and *vice versa*.

Many methods have been used for measuring the extracellular space to estimate the internal Na\(^+\) concentration (Section 1.5.2.2).

An appropriate extracellular marker which can fully equilibrate within the extracellular space is required. To make this measurement different kinds of markers have been used by researchers which bring different results. The large molecular weight markers such as blue dextran or \(^{14}\)C-dextran seemed to have advantages of non-uptake by *Dunaliella* cells. However small markers such as Li\(^+\) can better fully equilibrate within the extracellular space. Li\(^+\) does not penetrate into the osmotic space of *Dunaliella* cells (Katz & Avron, 1985; Blackwell & Gilmour, 1989).

### 1.6.1.1.1. Measuring the extracellular space by using large molecular weight markers.

The use of large molecular weight marker leads to underestimation of the intracellular space due to failure to fully penetrate that space (Section 1.5.2.2).

Zmiri & Ginzburg (1983) reported for *D. parva* a high intracellular Na\(^+\) of 770 mmol.kg\(^{-1}\). They used blue dextran (MW= 2.10\(^6\) ) as the marker for extracellular space. Ginzburg & Ginzburg (1985b) grew *Dunaliella* in 0.5 M and 2 M NaCl. They measured the intracellular concentration of glycerol, Na\(^+\), Cl\(^-\), K\(^+\), Mg\(^{2+}\) and Pi by using blue dextran (MW=2.10\(^6\) ) as a marker. They proposed that the osmotic pressure of *Dunaliella* cells is maintained by glycerol together with considerable
amounts of Na+, and Cl⁻ and smaller amounts of K⁺, Mg²⁺ and Pi. Also Gimmler & Schirling (1978) reported that, in *D. parva* grown at salinities between 0.75 to 1.5 M NaCl, about half of the intracellular osmotic pressure is due to NaCl. They used ¹⁴C-sorbitol as a marker.

Ehrenfeld & Cousin (1982) grew *D. tertiolecta* on 0.2 M, 0.41 M and 1.64 M NaCl and measured the intracellular Na⁺ concentration by two methods. When they used ¹⁴C-dextran 70,000 as the extracellular marker, the amount of intracellular Na⁺ was very much higher than when ¹⁴C-sorbitol was used for the cells grown on 0.41 M, 1.64 M NaCl which gave negative intracellular Na⁺ concentration. They repeated the experiment using ¹⁴C-dextran 70,000, but used rapid washings of the cells with an ice-cold solution to remove extra Na⁺ between the cells. The amount of intracellular Na⁺ was ten times lower than without washing for cells grown at 0.41 M and 1.6 M NaCl.

1.6.1.1.2. Measuring the extracellular space by using small molecular weight markers.

Katz and Avron (1985) grew *D. parva* and *D. salina* in a wide range of NaCl concentrations in the medium (0.5 M, 1 M, 2 M, 3 M, 4 M). They measured the cell volume and intracellular Na⁺ concentration using Li⁺ as marker for extracellular space volume. Their results showed that the intracellular Na⁺ concentrations were between zero and 100 mM for algae grown on 0.5 M to 4 M (Table 1.2). The amount of intracellular Na⁺ was about 2% of the external concentration where NaCl was 4 M.
Table 1.2: **Internal concentration of Na⁺ of Dunaliella cells measured by methods dependent on extracellular space determination.**

<table>
<thead>
<tr>
<th>Species</th>
<th>External NaCl (M)</th>
<th>Pellet water or volume determination</th>
<th>Extracellular volume determination</th>
<th>Internal Na⁺ concentration (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. parva</em> (19/9) 1.5</td>
<td></td>
<td>Haematocrit</td>
<td>Blue Dextran</td>
<td>623</td>
<td>(1)</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> 0.02</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran</td>
<td></td>
<td>5</td>
<td>(2)</td>
</tr>
<tr>
<td>0.4</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran</td>
<td></td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>1.64</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran</td>
<td></td>
<td>292</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran Rapid washing</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran Rapid washing</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1.64</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran Rapid washing</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>Cytocrit</td>
<td>$^{14}$C-Dextran</td>
<td></td>
<td>-19</td>
<td></td>
</tr>
<tr>
<td>1.64</td>
<td>Cytocrit</td>
<td>$^{14}$C-Sorbitol</td>
<td></td>
<td>-39</td>
<td></td>
</tr>
<tr>
<td><em>D. parva</em> (75)   1.5</td>
<td>Wet/dry weight</td>
<td>Blue Dextran</td>
<td></td>
<td>*770</td>
<td>(3)</td>
</tr>
<tr>
<td><em>D. salina</em> 0.5</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>30</td>
<td>(4)</td>
</tr>
<tr>
<td>1.5</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>-40</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>D. bardawil</em> 1.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>80</td>
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</tr>
<tr>
<td>3.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>D. parva</em> (19/9) 1.5</td>
<td>Wet/dry weight</td>
<td>Blue Dextran</td>
<td></td>
<td>500</td>
<td>(5)</td>
</tr>
<tr>
<td><em>D. parva</em> (19/9) 0.4</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>9</td>
<td>(6)</td>
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<tr>
<td>1.5</td>
<td>$^{3}$H₂O</td>
<td>Li⁺</td>
<td></td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*mmol Kg⁻¹

1.6.1.2. Ion-exchange column technique.

Pick, Karni & Avron (1986) used the ion-exchange column technique (Gasko et al., 1976) to measure the intracellular Na⁺ concentration of *D. salina* (Table 1.3). This technique reduces the extracellular Na⁺ to zero. Their results showed that in *D. salina* cells grown in 1 M to 4 M, the intracellular Na⁺ remained within 20 mM to 40 mM as the growth medium salinity was increased from 1 to 3 M. At 4 M NaCl, intracellular Na⁺ concentration increased to 100 mM, which generally confirmed the results of Katz & Avron (1985). Similar results were obtained with the ion-exchange column technique by Karni & Avron (1988).

1.6.1.3. NMR technique.

Bental, Degani & Avron (1988b) measured intracellular Na⁺ concentration by the ²³Na-NMR technique in *D. salina*. This technique directly measures the intracellular Na⁺. They grew cells in a wide range of salinity, from 0.1 M to 4M NaCl. They reported the range of intracellular Na⁺ concentrations were between 58 and 117 mM with a mean of 88 mM.

1.6.1.4. X-ray microanalysis technique.

Hajibagheri *et al.* (1985) measured the concentration of Na⁺, Cl⁻ and K⁺ in the cytoplasm and vacuole of *D. parva* which was adapted at 0.4 M and 1.05 M NaCl and after salt stress from 0.4 M to 1.05 M NaCl. Vacuoles have been identified in *Dunaliella* (Section 1.5.1). They used an X-ray microanalysis technique which measures directly the intracellular Na⁺ and other ions regardless of the extracellular Na⁺ concentration. Their results showed that Na⁺ and Cl⁻ concentrations were high in the vacuole and low in the cytoplasm. Conversely, K⁺ concentration was higher in the cytoplasm than the vacuole. No differences were observed between ion concentrations in the cytoplasm and the vacuole of cells adapted to 0.4 M and 1.05 M NaCl.
Table 1.3: Internal concentration of Na$^+$ of *Dunaliella* cells measured by direct methods.

<table>
<thead>
<tr>
<th>Species</th>
<th>External NaCl (M)</th>
<th>method</th>
<th>Internal Na$^+$ concentration (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. salina</em></td>
<td>1.0</td>
<td>ion-exchange</td>
<td>20</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>ion-exchange</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>ion-exchange</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>ion-exchange</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>D. parva</em> (19/9)</td>
<td>1.5</td>
<td>X-ray microanalysis</td>
<td><strong>105</strong></td>
<td>(2)</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>2.0</td>
<td>$^{23}$Na-NMR</td>
<td>114</td>
<td>(3)</td>
</tr>
</tbody>
</table>

** recalculated by Blackwell & Gilmour (1989)


But there was an increase in intracellular Na$^+$ and Cl$^-$ concentrations after salt stress from 0.4 M to 1.05 M. The Na$^+$ concentration then increased in vacuoles from 349 mM to 568 mM. In the cytoplasm Na$^+$ concentration increased from 37 mM to 152 mM. On this basis Hajibagheri *et al.* (1985) proposed the following model: (a) The cytoplasm is maintained low in Na$^+$ and Cl$^-$, but with a higher K$^+$ concentration. (b) The vacuoles are high in Na$^+$ and Cl$^-$ but low in K$^+$ and could rapidly exchange with the outside medium either via direct communication with the plasma membrane or via channels in the cytoplasm. They believed this model explains how *Dunaliella* maintains a constant cytoplasmic environment and keeps Na$^+$ and Cl$^-$ level sufficiently low to avoid enzyme inhibition.

Blackwell & Gilmour (1989) have recalculated the results of Hajibagheri *et al.* (1985) by using their own estimates of vacuolar and cytoplasmic volumes as a percentage of
cell volume. Their recalculation shows the internal Na\(^+\) concentration of cells grown in 1.5 M NaCl is about 105 mM.

1.6.2. Conclusion

The findings of the valid methods (e.g. Li\(^+\) as marker, ion-exchange column) concur in showing that under steady-state conditions, the intracellular Na\(^+\) is much lower than 100 mM. The possible exception is with an external Na\(^+\) concentration of 4M where the internal Na\(^+\) concentration may rise to 100 mM or more. Immediately after an increase in external NaCl concentration, the intracellular Na\(^+\) may also increase to above 100 mM (Weiss & Pick, 1990). Many of the intracellular enzymes of Dunaliella are highly sensitive to Na\(^+\) and Cl\(^-\) (Ben-Amotz & Avron, 1972; Borowitzka & Brown, 1974; Gimmler, Kaden & Kirchner, 1984) so the low intracellular Na\(^+\) is to be expected.

Weiss & Pick (1990) observed that in D. salina, when the external NaCl concentration was increased from 0.5 to 1 M, the intracellular Na\(^+\) concentration (measured by ion-exchange column technique) rapidly increased in the first minutes then returned to the original concentration of about 100 nmol per 10\(^{-8}\) cells. These results suggest that Na\(^+\) could provide a temporary osmoticum before synthesis of glycerol commences (Kirst, 1977; Ehrenfeld & Cousin, 1984).
1.7. The role of glycerol in adjustment of the internal osmotic pressure of *Dunaliella*

1.7.1. Metabolism of glycerol in *Dunaliella*

*Dunaliella* species have the ability to adjust their internal osmotic pressure in response to salt stress by changing the intracellular glycerol concentration (Craigie & McLachlan, 1964; Wegmann, 1971; Ben-Amotz & Avron, 1973b; Brown & Borowitzka, 1979). When *Dunaliella* cells are exposed to a sudden increase or decrease in salinity of the medium, a two-phase response is observed (Brown, 1978a) (see Fig. 1.2). In the first phase (event 2, Fig. 1.2) the cell rapidly loses water and shrinks or takes up water and swells respectively, balancing the osmotic pressure inside the cell with that of the medium. In the second (event 5, Fig. 1.2) which is slower, the cell produces or eliminates glycerol. As the glycerol content increases or decreases water flows through the plasma membrane to balance the internal and external water activity until the cell reaches the original volume (event 6 and 7, Fig. 1.2). Following salt stress in *Dunaliella*, the time required to achieve a new steady-state level of glycerol varies from 90 minutes to several hours depending on species and magnitude of salt stress (Ben-Amotz & Avron 1973b; Borowitzka & Brown 1974; Borowitzka & Kessly & Brown, 1977; Brown & Borowitzka, 1979).

The carbon skeleton of glycerol is drawn from photosynthetic metabolites of the Calvin cycle or from stored, non-osmotically active polysaccharides (Ben-Amotz, 1980; Gimmler & Müller, 1981b; Ben-Amotz & Avron, 1983a; Degani et al., 1985). Starch breakdown accounts for all glycerol synthesis in the dark and for 70% of glycerol synthesis in the light following an increase in medium salinity (Brown & Borowitzka 1979), thus the remainder of glycerol production in the light must be produced via photosynthesis. In general, although more rapid glycerol production is observed in the light after salt stress (Craigie & McLachlan, 1964; Wegmann, 1971;
Fig 1.2: Volume changes in *Dunaliella* in response to hyper- and hypoosmotic stress. Numbers presents the sequence of events.

1. **Hypoosmotic stress** (Decreased salinity)
   - Water influx
   - Decrease intracellular metabolites concentration
   - Glycerol dissimilation
   - Water efflux
   - 0 - 60 seconds
   - Swelling
   - 15 - 90 minutes
   - Original volume

2. **Hyperosmotic stress** (Increased salinity)
   - Water efflux
   - Increase intracellular metabolites concentration
   - Glycerol production
   - Water influx
   - 0 - 60 seconds
   - Shrinking
Goyal, Brown & Lilley, 1988), the increase in glycerol content after salt stress can be independent of photosynthesis (Ben-Amotz 1974), since it also occurs in the dark at the expense of starch (Degani et al., 1985; Goyal, Brown & Gimmler, 1987).

Glycerol synthesis and dissimilation involve two pathways, each catalysed by two enzymes (see Fig. 1.3). Glycerol-3-phosphate dehydrogenase is located in the chloroplast (Brown, Lilley & Marengo, 1982a; Gimmler & Lotter 1982) catalysing the reduction of DHAP to glycerol-3-phosphate (Haus & Wegmann 1984a, 1984b; Marengo, Lilley & Brown 1985). This reaction is reversible. Glycerol-3-phosphatase converts glycerol-3-phosphate to glycerol (Sussman & Avron, 1981). Glycerol dissimilation occurs by the conversion of glycerol to DHA and in turn to DHAP. These two steps are catalysed by glycerol dehydrogenase (NADP-specific) and dihydroxyacetone kinase.

Glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase are located in the chloroplast and cytosol respectively (Brown, Lilley & Marengo 1982a; Gimmler & Lotter, 1982). There is uncertainty about the distribution of two other enzymes. Wegmann (1979) reported that in *D. tertiolecta* grown in 0.7 M NaCl or above, half of the carbon fixed was incorporated into glycerol in steady state conditions. When the salt concentration of medium was increased to 2 M the rate of fixation and incorporation of carbon into glycerol increased linearly. They reported that during first 10 minutes after on increase in salinity, 90% of carbon was fixed into the glycerol pool. After dilution stress, the amount of intracellular glycerol decreased, but 14C fixation into glycerol continued. On the basis of this high fixation and incorporation of carbon into the glycerol pool, Wegmann (1979) called these two pathways the "glycerol cycle". The concept of a glycerol cycle and rapid turn-over of the glycerol pool were then adopted by many other authors. If true, this means that *Dunaliella*

**Sucrose**

- DHAP
- ADP
- ATP
- NADPH
- NADP+

**Starch**

- DHAP
- NAD(P)H
- NAD(P)⁺
- Glycerol-3-phosphate
- Pi

**Cytosol**

- Glycerol

**Chloroplast envelope**

**Stroma**

1 - glycerol-3-phosphate dehydrogenase
2 - glycerol phosphate phosphatase
3 - glycerol dehydrogenase
4 - dihydroxyacetone kinase
would have a fast turn-over of the glycerol pool and would reach steady-state rapidly. Additionally, after salt stress, *Dunaliella* could accumulate glycerol by retarding the dissimilation of glycerol rather than accelerating the synthesis.

Goyal, Lilley & Brown (1986a) measured the rate of synthesis and turn-over of the glycerol pool in *D. tertiolecta* at two constant salinities (0.17 and 0.7 M NaCl). They found that the half-time for turn over of the glycerol pool was 1 h in 0.17 M NaCl and about 6 h for 0.7 M NaCl. They compared this to the half-time for turn-over of triose phosphate in spinach protoplast chloroplasts, which was 0.3 seconds (Stitt, Wirtz & Heldt, 1980). Goyal, Lilley & Brown (1986a) mentioned that although the glycerol pool of *Dunaliella* under their experimental conditions was 200 times larger than the triose phosphate pool in protoplast chloroplasts, the turn-over of this pool in *Dunaliella* is relatively slow under steady-state conditions. They concluded that their result did not support the concept that glycerol metabolism in *Dunaliella* is a cycle such as the Benson-Calvin cycle. A true metabolic "cycle" has the capability of rapid turn-over and is autocatalytic in nature. This means that a rapid increase in the intracellular glycerol concentration can not be achieved by decreasing the rate of dissimilation. It can only be achieved by increasing the rate of glycerol synthesis.

### 1.7.2. Membrane permeability in *Dunaliella* to glycerol.

Despite some early reports of high permeability of the *Dunaliella* cell membrane to glycerol (Frank & Wegmann, 1974; Enhuber & Gimmler, 1980), low permeability of the membrane of *D. salina* and *D. bardawil* to glycerol has been reported by Ben-Amotz & Avron, 1973b; Ben-Amotz, 1975; Brown *et al.*, 1982b; Gimmler & Hartung, 1988 (*D. parva*). Brown *et al.* (1982b), using an NMR technique, reported very low glycerol permeability with a half-time of 400 hour at 17 °C for *D. salina*. Ben-Amotz (1975) reported that the intracellular glycerol lost from *D. parva* cells after decreasing salinity from 1.5 M to 0.6 M NaCl was the result of dissimilation, because no leakage
of the intracellular glycerol was observed. Changes to even lower osmotic pressures, however, caused the algal cell membrane to swell, stretch, and finally burst. In the process of this disruption, glycerol was then lost to the medium.

There are many different reports of low or high amounts and rate of leakage of glycerol to the medium following dilution stress. These differences are due to different magnitudes of dilution stress or final salt concentration, different experimental conditions or methods of dilution, and different species of *Dunaliella* used by researchers.

Kessly & Brown (1981) reported 1% leakage of intracellular glycerol to the medium in *D. tertiolecta* when subjected to a stress from 1.6 M to 0.53 M NaCl. On the other hand when *D. tertiolecta* (but not the same strain) was exposed to a 0.43 M to 0.09 M NaCl stress, most of the glycerol pool leaked to the medium (Gilmour, Hipkins & Boney, 1984b). Similar high leakage of glycerol to the medium has been reported for *D. tertiolecta* by Frank and Wegmann (1974), and for *D. parva* by Enhuber and Gimmler (1980). Determining the permeability of the plasma membrane to glycerol especially, following dilution stress, is very method-dependent. Following dilution stress, the cell swells and the plasma membrane is stretched. Any damage to the plasma membrane will cause the release of glycerol from the cell to the medium. This brings a large error to the calculation of permeability of the plasma membrane. For example, Zidan, Hipkins & Boney (1987) reported that, in *D. tertiolecta* under dilution stress from 1.5 M to 0.1 M NaCl, 70% of intracellular glycerol was lost via leakage. For applying dilution stress, they directly suspended the pellet of *Dunaliella* cells into the hypotonic medium. This procedure will certainly cause cell damage and release of glycerol. Goyal (1989a) re-examined the claim of Zidan, Hipkins & Boney (1987). He confirmed that when the *D. tertiolecta* suspensions were centrifuged and resuspended to dilute hypotonic medium, a large amount of glycerol was found in the medium due to cell damage. He stated that 70% and 90% of the cells were broken,
observed microscopically when dilution was done by resuspending directly the cells in 0.4 M and 0.1 M NaCl respectively. When dilution stress was applied by adding buffer without NaCl and without centrifuging the cells, the amount of glycerol which appeared in the medium was less than 10%.

It is now widely accepted that *Dunaliella* cells have an unusual plasma membrane. It prevents extensive leakage of intracellular glycerol (which provides 80% of internal osmotic pressure of the cells (Section 1.7.3) into the medium. On the other hand it prevents influx of salt from medium into cells which is deleterious for cell enzyme activity. Brown *et al.* (1982b) determined the permeability of the *Dunaliella* cell membrane to glycerol and found it to be exceptionally low.

1.7.3. The contribution of glycerol to the internal osmotic pressure of *Dunaliella*.
Degani *et al.* (1985) measured intracellular glycerol and other major soluble metabolite concentrations such as dihydroxyacetone, pyruvate, lactate, glucose, alanine, glutamate, and α-(1-4)-glucan by $^{13}$C and $^1$H-NMR in *D. salina* grown in 1.5 M NaCl. Their results showed that the cells contained approximately 1.9 M of glycerol which is osmotically equivalent to about 1.25 M NaCl. The other soluble metabolites together contributed the equivalent of 0.25 M NaCl. Blackwell & Gilmour (1989) also has reported that for *D. parva* cells, adapted to 0.4 M NaCl, internal glycerol, Na$^+$ and K$^+$ were 0.6 M, 0.009 M and 0.126 M respectively. These values for cells adapted to 1.5 M were 1.4 M, 0.01 M and 0.109 M. Thus glycerol accounts for most of the intracellular osmotic pressure of *Dunaliella* ssp.(Ben-Amotz & Avron, 1983a). Although at high temperatures (above 40 °C), *Dunaliella* accumulates considerable amounts of sucrose, this in addition to significant amounts of glycerol (Wegmann, 1969; Müller & Wegmann, 1978).
1.7.4- Role of organic solutes other than glycerol in the adjustment of the internal osmotic pressure of *Dunaliella*.

Bental *et al.* (1988a) and Bental *et al.* (1990a) stated that, in *D. salina*, there was no substantial effect of increased salinity of the growth medium on the concentration of any metabolite detectable by $^{13}$C-NMR in fully adapted organisms other than glycerol. Müller & Wegmann (1978) found that in *D. tertiolecta* growing in 0.7 M NaCl, at high temperatures (above 40 °C), 6.5 µg sucrose per $10^6$ cell was accumulated, in addition to significant amounts of glycerol (about double sucrose accumulation). They also reported that very low amounts of sucrose were accumulated in *D. tertiolecta* at 25 °C. Goyal, Lilley & Brown (1986a) reported in *D. tertiolecta* grown at 27 °C at 0.7 M NaCl, it accumulated about 10 µg glycerol per $10^6$ (recalculated by Brown, 1990) cells. Due to the fairly specific temperature relations of sucrose metabolism (Brown, 1990) it seems, in comparison with the massive role of glycerol production and dissimilation, the contribution of sucrose at normal temperature (27 °C) to intracellular osmotic pressure of *Dunaliella* is low.

1.8. Possible determinants of the intracellular concentration of glycerol in *Dunaliella*

1.8.1. Cell volume changes and internal osmotic pressure

Theoretically, changing the concentration of an extracellular solute and hence the water activity might regulate an intracellular metabolic process in one or more ways (Brown & Borowitzka 1979).

The volume of *Dunaliella* cells changes when the cells are subjected to an increase or decrease in salinity of the medium (Fig. 1.2; Section 1.7.1). After changing the external osmotic pressure, water efflux or influx through plasma membrane of the cell (event 2, Fig. 1.2) occurs to equilibrate the water potential inside the cell with that outside. This causes, initially, equal changes in the concentration of all metabolites
and solutes (event 4 Fig. 1.2). The rates of glycerol synthesis and/or dissimilation then (event 5, Fig. 1.2) changes. As the concentration of intracellular glycerol changes, water fluxes through the plasma membrane re-equilibrate the internal water potential. This continues until the cell volume returns to its original volume (event 7, Fig. 1.2). In *Dunaliella* in all these conditions (Fig. 1.2), (except first few seconds after changing the external osmotic pressure, during which the cells rapidly change the internal osmotic pressure by efflux or influx of water) during volume recovery the cells attempt to keep the water activity (osmotic pressure) inside the cells constant and about equal to the medium. Here, the turgor pressure remains low (but not zero) because the low mechanical strength of the extracellular matrix (Section 1.5.1) offers little resistance to the swelling or shrinkage (Brown, 1990). Cram (1976) stated that "the primary signal for adaptive responses must be a function of volume in wall-less cells while it is most probably a function of turgor pressure in walled cells" (see also 1.3). There is no evidence for or against such minute turgor pressure changes having a role in *Dunaliella*, but this seems unlikely. Thus the variable factors which could be involved in osmotic regulation might be: (a) Changing salinity or osmotic pressure of the environment. (b) Cell volume changes.

Cell volume changes in *Dunaliella* have been proposed theoretically as a possible signal for triggering glycerol synthesis (Brown & Borowitzka, 1979; Ben-Amotz & Avron, 1981), but so far there is no evidence to confirm this.

There have been may attempts to identify in *Dunaliella* the signal that triggers the osmoregulatory metabolic response (glycerol synthesis or dissimilation) after an increase or decrease in salt concentration of the medium. The signals so far suggested include: external and internal NaCl concentration, water potential, changes in membrane parameters (e.g. membrane tension), change in concentration of soluble metabolites as the cell volume changes (ATP, NADP+, NADPH, Pi, or H+). Polyphosphate hydrolysis, ATPase activity changes, and inositol phospholipids have
been also suggested. In *D. salina*, an increase of abscisic acid following salt stress has been reported by Cowan & Rose (1991), but their results are not sufficient to draw any conclusion about a relationship between abscisic acid metabolism in *Dunaliella* and glycerol synthesis regulation.

### 1.8.2. External and Internal NaCl concentration

Due to the presence of a high concentration of NaCl in the typical environment in which *Dunaliella* lives, the first thing that may be expected is that NaCl might act as a signal for the regulation of glycerol metabolism. As already described in Section 1.2.6 the intracellular concentration of Na⁺ in *Dunaliella* is very low and so far there is no evidence for any external signal which could interact with a specific receptor site on the surface of *Dunaliella* cells.

Borowitzka, Kessly & Brown (1977) and Gilmour, Hipkins & Boney (1984a) showed that in *Dunaliella* the stimulation of glycerol synthesis is an osmotic phenomenon, not specific to NaCl, since it is also induced by sucrose. Belmans & Van Laere (1987) applied salt stress to *D. tertiolecta* from 0.6 M to 1.0 M NaCl. Glycerol production was the same when NaCl in the medium was replaced by mannitol or sorbitol. Similar results were shown by Wegmann (1971). Fuggi *et al.* (1988) showed that in *D. acidophila* (tolerant of high H₂SO₄ and Na₂SO₄ concentrations) glycerol synthesis and recovery after increasing the external osmomolarity was the same when NaCl in the medium was replaced by Na₂SO₄.

Weiss & Pick (1990) pre-equilibrated *D. salina* for 20 min in different media in which NaCl was replaced by other solutes such as: KCl, Tris-Cl, MgSO₄, or glycerol. After increasing the concentration of these solutes, the cells in all cases, except MgSO₄ showed similar cell volume recovery kinetics to NaCl.
In summary firstly, the contribution of intracellular Na\(^+\) to the internal osmolarity of *Dunaliella* is very small and this ion does not have a specific role as an internal osmoticum. Secondly, external Na\(^+\) is not essential for triggering of glycerol synthesis and cell volume recovery after hyperosmotic stress.

Despite these finding, Weiss, Bental & Pick (1991) recently reported that in *D. salina*, the polyphosphate hydrolysis that follows salt stress is Na\(^+\) dependent. The possible involvement of polyphosphates in regulation of glycerol metabolism will be discussed in Section 1.8.7.

### 1.8.3. Plasma membrane ATPase activity

The ATPases are located in the plasma membrane and other, internal, membranes of cells such as those of mitochondria, endoplasmic reticulum and chloroplast. Many different ATPases have been isolated from cell membranes of animals, plant, fungi, algae etc. These ATPases have different properties depending on the organism and their location in the cells. In general, ATPases are involved in ATP synthesis and the active transport of ions such as H\(^+\), K\(^+\) and Na\(^+\). The ATP-generating form is called ATP-synthetase and the transport-ATPases are called H\(^+\)-ATPase, K\(^+\)-ATPase or Na\(^+\)-ATPase.

A role for plasma membrane transport-ATPases in the regulation of glycerol metabolism after osmotic stress was suggested by Gimmler, Wiedemann & Moller (1981a); Kaaden & Gimmler (1984); Pick *et al.* (1987); Oren-shamir, Pick & Avron (1989 and 1990). In the roots of higher plants, an increase in the activity of the plasma membrane ATPase was observed after an increase in salinity (Braun *et al.*, 1986). A similar effect (an increase in plasma membrane H\(^+\)-ATPase activity after hyperosmotic stress) was expected for *Dunaliella*. For study of role of plasma membrane ATPase in osmoregulation in *Dunaliella*, a wide range of H\(^+\)-ATPase,
inhibitors have been tested by Kaaden & Gimmler (1984); Gilmour, Kadden & Gimmler (1985); Pick et al. (1987); Weiss, Sekler & Pick (1989). Oren-shamir, Pick & Avron (1989) treated D. salina with two specific inhibitors of plasma membrane H+-ATPase, vanadate and diethylstilbestrol. When cells were exposed to salt stress from 1 M to 2 M NaCl, they shrank, but cell volume recovery and glycerol content were totally inhibited by 20 μM of diethylstilbestrol or vanadate in the medium. They proposed that the activity of the plasma membrane H+-ATPase is essential for recovery of Dunaliella from hypertonic stress.

Plasma membrane H+-ATPases are considered as the major electrical gradient generators across the plant plasma membrane (Oren-shamir, Pick & Avron, 1990). Oren-shamir, Pick & Avron (1990) determined the H+-ATPase activity of D. salina in vivo by measuring the membrane potential of the cells. They showed that when the osmotic pressure of the medium of D. salina cells was increased, the plasma membrane potential rapidly underwent hyperpolarization. This hyperpolarization was eliminated by vanadate, which is a specific H+-ATPase activity inhibitor. The glycerol content did not recover after salt stress from 1 M to 2 M NaCl in the cells treated with 10 μM vanadate. On this basis they suggested the activation of H+-ATPase due to hypertonic stress may be involved in the primary signal for osmoregulation. The membrane H+-ATPases are responsible for regulating many intracellular functions. If they are inhibited, the ability of the cells to respond to external osmotic pressure changes will be reduced. Despite assertions that these membrane H+-ATPase inhibitors are specific, it is probable that these inhibitors affect indirectly other intracellular functions.

So far it is not clear by what mechanism hyperosmotic stress can induce the increasing of ATPase activity. Lysophosphatidylcholine is an activator of plant plasma membrane ATPase (Serrano, Montesinoes & Sanchez, 1988). Einspahr, Maeda & Thompson (1988) reported that in D. salina the lysophosphatidylcholine level
increased after hyper osmotic stress followed by structural and biochemical changes in the plasma membrane. Oren-shamir, Pick & Avron (1990) expected this activator may be responsible for the ATPase activation.

Oren-shamir, Pick & Avron (1989 and 1990) advanced the following mechanism for a role of H+-ATPase after an increase in osmotic pressure of the medium: (a) internal alkalinization of Dunaliella cells due to activation of H+ extrusion by H+-ATPase (b) increase in Pi and decrease in ATP concentration because plasma membrane ATPase is a major consumer of ATP (c) changes in metabolism consequent on these effects.

1.8.4. Pyridine nucleotides

Nicotinamide adenine dinucleotide phosphate (NADP+) and nicotinamide adenine dinucleotide (NAD+) are coenzymes. They function in oxidation-reduction reactions catalysed by pyridine nucleotide-linked dehydrogenases (Armstrong, 1983).

In Dunaliella, the cell content of glycerol is determined by the balance of the two-step synthesis and dissimilation pathways to and from DHAP (Wegmann, 1979). The reduction of DHAP to glycerol phosphate requires NADH or NADPH, while the oxidation of glycerol to DHA requires NADP+ (Ben-Amotz & Avron, 1973a; Borowitzka & Brown, 1974; Borowitzka, Kessly & Brown, 1977; Marengo, Lilley & Brown, 1985). Hence it is possible that changes in the concentrations of pyridine nucleotides following salt stress may be involved in regulating glycerol synthesis.

Belmans & Van Laere (1987) measured the degree of reduction (reduced / total) of NAD or NADP in D. tertiolecta cells. After an increase of salinity in the medium from 0.1 M to 0.6 M NaCl, this did not change significantly. They used an analytical technique based on polarographic recycling to measure the NAD(H) and NADP(H). On the other hand Goyal, Brown & Lilley (1988) have used an enzymic cycling
method for determination of pyridine nucleotides. They reported in *D. tertiolecta* an increase in content of NADPH and NADP⁺, a decrease in NAD⁺ and an initial increase in the NADH/NAD⁺ quotient after salt stress from 0.17 M to 0.7 M and 1 M NaCl. They concluded that, although these changes were large, they were unlikely to be responsible for accelerated glycerol synthesis.

The difference between the two set of results reported by Belmans & Van Laere (1987) and Goyal, Brown & Lilley (1988) may be due to the different methods used for determination of pyridine nucleotides. The accuracy of the enzymic cycling method is well established (Zao, Hu & Ross, 1987). Secondly, Belmans & Van Laere (1987) only reported the reduced/oxidised quotient of NAD(H) or NADP(H). Goyal, Brown & Lilley (1988) reported the absolute amounts of pyridine nucleotides as well as the reduced/oxidised quotient. As a result their more complete assessment is more reliable.

1.8.5. ATP

Adenosine triphosphate (ATP) is the currency of metabolic energy in living cells. ATP furnishes the chemical free energy for energy-requiring reactions. Release of this energy occurs principally by hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi). In *Dunaliella*, ATP is an inhibitor of glycerol phosphate dehydrogenase (Marengo, Lilley & Brown, 1985) and it is necessary for conversion of dihydroxyacetone to DAHP in the reaction catalysed by dihydroxyacetone kinase.

There are many reports regarding decreasing or increasing of ATP level in *Dunaliella* after hyper- or hypo-osmotic stress respectively (Gimmler, Wiedemann & Moller, 1981a; Ehrenfeld *et al.*, 1984; Belmans & Van Laere, 1987 and 1988; Goyal, Brown & Gimmler, 1987; Bental *et al.*, 1990a). All these reports have mentioned that a drop in the level of ATP may be involved in glycerol synthesis as a secondary signal. Any
The hydrolysis of ATP due to an increase in activity of the plasma membrane ATPase will bring about an increase in intracellular Pi. The role of Pi will be discussed in Section 1.8.6. Nevertheless, a drop in ATP level has been reported by many researchers. Marengo, Brown & Lilley (1989) stated that "there is no apparent relationship between the increased rate of glycerol synthesis following salt stress and the ATP and ADP contents or their quotient". Their results showed that in *D. tertiolecta* the highest rate of glycerol synthesis following the salt stress from 0.17 M to 0.7 M NaCl in the light was not accompanied by any significant changes in ATP or ADP contents or the quotient ATP/ADP. Also during the period between 5 and 30 minutes after salt stress from 0.17 M to 1 M NaCl in both dark and light, where the rate of glycerol synthesis was constant, the ATP content and ATP/ADP quotient were changed rapidly.

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Oren-shamir, Pick & Avron (1990), treated *D. salina* cells with vanadate as an inhibitor of H⁺-ATPase, and exposed them to a hyperosmotic stress. A drop in ATP level was observed in both vanadate treated and untreated cells. The decrease in ATP level was slower in vanadate-treated cells and they did not show a glycerol synthesis response. These authors also concluded that a drop in the level of ATP following hyperosmotic stress also is not the signal for glycerol synthesis because it happened in both untreated and cells treated with H⁺-ATPase inhibitors.

### 1.8.6. Inorganic phosphate (Pi)

The important role of intracellular Pi concentration in the regulation of starch and glycerol is well established (Walker & Sivac, 1986). Because *Dunaliella* is a green alga with a chloroplast of similar basic structure to that of higher plants (with the exception of the pyrenoid), by analogy a similar role for Pi in *Dunaliella* might be expected.
The mechanism which regulates carbon flow between the starch and glycerol pools in *Dunaliella* must be integrated with the normal pathways of carbon metabolism prevalent in these cells. Triose phosphate is produced by both photosynthesis and glycolysis. Triose phosphate is produced in the chloroplast by photosynthesis and starch breakdown. In the cytosol it is produced by glycolysis and glycerol catabolism. Triose phosphate in the chloroplast is the substrate for the synthesis of starch and glycerol. In the cytosol it is the substrate for sucrose synthesis.

In plant cells, the partitioning of triose phosphate between the chloroplast and cytoplasm is controlled by the phosphate translocator, which exchanges triose phosphates (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate), 3-phosphoglycerate and Pi (Flügge & Heldt, 1986). Chloroplasts export DHAP by the phosphate translocator at the inner chloroplast envelope membrane (Heldt *et al.*, 1977; Giersch *et al.*, 1980) and import stoichiometrically equal quantities of cytoplasmic Pi in order to do so (Walker & Sivac, 1986) (see phosphate transloctor in Fig. 1.3). If there is no Pi in the cytosol, triose phosphate can not leave the chloroplast. If there is high [Pi] in the cytosol, all triose phosphate will be exchanged out of the chloroplast. Starch synthesis in the chloroplast requires a high PGA/Pi ratio, whereas low PGA/Pi ratios inhibit the formation of starch (Preiss & Levi, 1979) and stimulate the degradation. Thus, in the chloroplast of *Dunaliella*, a high Pi-concentration should enhance the breakdown of starch (Gimmler, Wiedemann & Moller, 1981a). So any increase in the level of Pi in the cytoplasm resulting from the hydrolysis of either glycerol-3 phosphate or DHAP induces an increased export of DHAP from the chloroplast via the phosphate translocator (Giersch, *et al.*, 1980) in exchange for Pi.

In *Dunaliella* changes in the intracellular Pi concentration following changes in osmotic pressure of the medium have been reported by many researchers. Most of these used acid for extraction intracellular Pi from the intact cells (Gimmler & Müller, 1981b; Belmans & Van Laere, 1987; Goyal, Brown & Gimmler, 1987; Ginzburg,
Ratcliffe & Southon, 1988). However the acid extraction method also breaks down polyphosphate chains to Pi. This causes a large error in determining intracellular Pi. Kuchitsu, Katsuhara & Miyachi (1989) and Bental et al. (1990a and 1990b) have measured the intracellular Pi directly by $^{31}$P-NMR. Notwithstanding, they also reported an increase in intracellular Pi concentration following an increase in salinity of the medium.

Bental et al. (1990b) measured intracellular Pi, polyphosphate and ATP of *D. salina* following salt stress. Their *in vivo* study of cells by NMR techniques requires very high cell densities to obtain a good signal-to-noise ratio within a reasonable time. Such conditions are not very close to normal growth conditions. To overcome this problem, Bental et al. (1990a) trapped *D. salina* cells inside 1.5% Ca$^{2+}$ alginate beads or 3% agarose. They reported that in such conditions all vital functions, photosynthesis, respiration and cell volume recovery following osmotic stress occurred at rates comparable to free cells in suspension.

Bental et al. (1990b) studied the response of two *D. salina* cultures to hyperosmotic stress. By manipulating the Pi concentration of growth and resuspension media, one culture had low and other high intracellular Pi. These changes in internal Pi were obtained within 1-2 hours and measured by $^{31}$P-NMR. The internal Pi was also measured following salt stress. The result showed that after hyperosmotic stress (0.5 M to 1 M NaCl), glycerol synthesis and recovery of the cells with high intracellular Pi was twice as fast than cells with a low Pi level. After hypo-osmotic stress from 0.5 M to 0.25 M NaCl, glycerol elimination in the cells with high intracellular Pi was twice as slow than in cells with a low Pi level. In other words, Pi stimulated glycerol production and inhibited glycerol elimination. As a result Bental et al. (1990b) have proposed that the osmotic response in *Dunaliella* is triggered by the differential movement of water into or out of the cytoplasm, and the consequent change in the cytoplasmic volume and Pi concentration. Similar schemes were proposed earlier by
Gimmler, & Moller (1981b) and by Kuchitsu, Katsuhera & Miyachi (1989). Bental et al. (1990b) stated that "the Pi movement (via the triose phosphate translocator) into or out of the chloroplast will affect the Pi-regulated enzymes of starch synthesis or degradation. These will continue to function until sufficient glycerol has been produced or eliminated to complete volume regulation and reach a new cellular equilibrium". Although Bental et al. (1990a and 1990b) clearly showed that, following salt stress, intracellular Pi concentration changes are involved in the short-term glycerol synthesis or dissimilation response of Dunaliella cells, their hypothesis does not explain how the glycerol content of cells is maintained in the long-term or steady-state condition.

1.8.7. Polyphosphate

Many microorganisms, including bacteria, yeast, algae, and cyanobacteria accumulate large amounts of polyphosphates. For example, Liss et al., 1960 (cited by Pick & Weiss, 1991b) reported that a yeast contains up to 20% of polyphosphates by dry weight. In Dunaliella, polyphosphates were detected by $^{31}$P-NMR spectroscopy techniques (Bental et al., 1988a, Bental, Degani & Avron, 1988b; Bental et al., 1990a & 1990b; Kuchitsu, Katsuhera & Miyachi, 1989; Ginzburg, Ratcliffe & Southon, 1988; Pick & Weiss, 1991b)

Bental et al. (1990a & 1990b) reported, in D. salina, a drop in ATP level and elongation of polyphosphates after salt stress from 0.5 M to 1 M NaCl. After dilution stress from 0.5 M to 0.25 M NaCl, an increase in ATP level and an increase in shorter polyphosphate chains was recorded. They proposed that polyphosphates might have a role in osmotic adaptation of Dunaliella. The location of polyphosphates in Dunaliella cells is not clear because purification of intact organelles has been achieved only with the chloroplast from this alga. Pick, Zeelon & Weiss (1991a) and Pick & Weiss (1991b) applied an alkaline stress by adding 20 mM NH$_4$Cl, to the medium
(adjusted to pH 9) to change the vacuolar pH of D. salina. They used the fluorescent amine atebrin (an indicator of pH in acidic compartments) to follow uptake and location of amine. Accumulation of this amine in the cytosol or other cellular compartments was used to follow the decrease in pH in these compartments. pH changes in cytoplasm due to adding NH₄Cl depend on the mechanism of transport of ammonia from medium to the cell. An influx of NH₃ to the cell will induce an increase in pH due to internal protonation of NH₃. Their results showed an alkalinisation of the cytoplasm (due to NH₃ uptake) and a drop in ATP level following this alkaline stress. Then cytoplasmic pH recovered by transport of accumulated ammonia from the cytoplasm to the vacuoles. This could increase the pH of the vacuoles. Also they measured polyphosphate hydrolysis after alkaline stress. Their results showed a correlation between polyphosphate hydrolysis and accumulation of amine in the vacuoles and correlation between time course of polyphosphate hydrolysis and time course of cytoplasmic pH recovery. So they suggested that in Dunaliella polyphosphates are located in the vacuoles.

Weiss, Bental & Pick (1991) measured polyphosphate hydrolysis after osmotic stress and agreed with the results of Bental et al. (1990a &1990b). Their result showed that in D. salina, after salt stress from 0.5 M to 1.25 M NaCl, a decrease in ATP level and a rapid polyphosphate hydrolysis (complete within 2 min) to Pi chains of 5-6 units occurred. When the same magnitude of osmotic stress was applied by adding glycerol, polyphosphate hydrolysis was not induced. These results indicated that to induce rapid hydrolysis of polyphosphates in vacuoles, salt stress rather than osmotic stress itself was required. Weiss, Bental & Pick (1991) also reported that in D. salina, 2.5-fold dilution stress induced an increase in ATP level. Compared with hyperosmotic stress, polyphosphate hydrolysis was slower and the Pi chains produced were shorter (tripolyphosphate).
Weiss, Bental & Pick (1991) reported a rapid increase in intracellular Na\(^+\) concentration in the first minutes after salt stress in *D. salina*. Since polyphosphate hydrolysis following osmotic stress is Na\(^+\)-dependent, they proposed that after salt stress, Na\(^+\) might be rapidly compartmentalised into the vacuoles, in exchange for H\(^+\). The change in the pH of the vacuoles then induced polyphosphate hydrolysis. Bental *et al.* (1990a) and Weiss, Bental & Pick (1991) reported that no changes in the intracellular pH of *Dunaliella* cells occurred after salt or dilution stress. In addition, in *Dunaliella*, there is still no evidence for the existence of a vacuolar Na\(^+\)/H\(^+\) antiporter, although a similar antiporter has been characterised in *D. salina* plasma membrane vesicles (Katz, Kaback & Avron, 1986. Katz *et al.* (1991) reported that in *D. salina* lowering intracellular pH (measuring by \(^{31}\)P-NMR) activates the plasma membrane Na\(^+\)/H\(^+\) antiporter. They reported that intracellular Na\(^+\) concentration (using an ion exchange column) increased following intracellular acidification.

In summary: (a) polyphosphate hydrolysis was detected following alkalisation of acidic vacuoles. (b) polyphosphate hydrolysation were reported following osmotic stress. The demonstrated relationship between intercellular pH changes, polyphosphates hydrolysis might have a role in osmotic adaptation. The role of pH in osmoregulation will be discussed in Section 1.8.8.

### 1.8.8. Intracellular pH changes

Intracellular pH changes after osmotic stress have been proposed to be a signal for cell recovery (Goyal, Brown & Gimmler, 1987; Kuchitsu, Katsuhara & Miyachi, 1989).

Ginzburg, Ratcliffe & Southon (1988) measured the internal pH of *Dunaliella* by using \(^{31}\)P-NMR spectroscopy. They showed that *D. parva* controlled the internal pH to value close to 7 over an external range of pH between 6 to 9. At an external pH of
5, the internal pH fell sharply. Bental et al. (1990a) studied the internal pH of *D. salina* cells by $^{31}$P-NMR under *in vivo* conditions (Section 1.8.6). They measured the internal pH of *D. salina* cells by $^{31}$P-NMR following salt stress from 0.5 M to 1 M NaCl or dilution stress from 0.5 M to 0.25 M NaCl. They found no change in the intracellular pH after salt or dilution stress in disagreement with the $^{31}$P-NMR observations of Kuchitsu, Katsuhara & Miyachi (1989).

Weiss, Bental & Pick (1991) measured the vacuolar pH of *D. salina* by using the fluorescent amine indicator atebrin, which accumulates in the vacuoles in response to the pH gradient across the vacuolar membrane. They reported that, within the limits of sensitivity of their methods, no major decrease of the pH gradient across the vacuolar membrane occurred during salt stress from 0.5 M to 1.5 M NaCl, nor dilution stress from 0.5 M to about 0.17 M NaCl.

On the other hand Goyal, Brown & Gimmler (1987) and Goyal & Gimmler (1989b) reported that the intracellular pH of *D. tertiolecta* increased 0.2 units from 15-60 minutes after an increase in salinity of the medium from 0.17 to 0.77 M NaCl. They calculated the internal pH of *Dunaliella* by measuring the equilibrium distribution of $^{14}$C-DMO (5,5-dimethyl-2($^{14}$C) oxazolidine-2,4-dione) between the cells and the medium. Goyal & Gimmler (1989b) acknowledged that this technique could not be used for measuring any short-term effect of alteration of metabolism or environment on the internal pH, due to long half-time ($t_{1/2}=40$ min) of equilibration of DMO between the cells and the medium.

Goyal, Brown & Gimmler (1987) measured the (*in vitro*) pH optima of the following enzymes of starch catabolism from *Dunaliella*: maltase, phosphorylase, PFK (phosphofructokinase) and amylolytic activity. They showed that maltase and phosphorylase activity were highest at pH 6.8. The optimum pH was between 7.4 to 8 for PFK activity and 7 and 8 for amylolytic activity in different buffer compositions.
They suggested that alkanisation of stroma by salt stress stimulates amylolytic starch degradation, the activity of PFK (phosphofructokinase), and the oxidative pentose phosphate pathway, but inhibits phosphorylase, maltase, and the enzymes of glycerol synthesis. The activity in vitro of glycerol phosphate dehydrogenase, which reduces DHAP to glycerol phosphate has been reported have pH optima between 6.5 and 7.5, depending on the buffer composition (Haus & Wegmann, 1984a). Borowitzka, Kessly & Brown (1977) reported pH optima between 7.0 and 7.5 for in vitro activity of glycerol phosphate dehydrogenase, and pH optima between 8.5 for in vitro activity of glycerol dehydrogenase. However, Marengo, Lilley & Brown (1985) have reported that the in vitro activity of glycerol phosphate dehydrogenase, doubled when the pH was changed from 7.5 to 6.5. Kuchitsu, Katsuhara & Miyachi (1989) measured the internal pH of Dunaliella by using 31P-NMR. They reported that in D. tertiolecta, after salt stress from 0.17 to 1 M NaCl, cytoplasmic pH increased from 7.1 to about 8. The vacuolar pH increased from 6.0 to 6.5.

Both methods (DMO and 31P-NMR) suffer from several significant disadvantages, of which an important one is the time needed to obtain a result (more than 30 minutes for NMR and about 3 hours for DMO). Another limitation of both methods is their failure to provide separate measurements of the two major cell compartments: the cytosol and the stroma. DMO accumulates in alkaline compartments and hence does not measure acidic compartments. 31P-NMR is inherently able to make the distinction, provided there is a difference in pH between the cytosol and the stroma. But to date all such measurements have succeeded in detecting only one pool of dissolved Pi in Dunaliella (Brown, 1990).

Goyal, Brown & Gimmler (1987); Goyal & Gimmler (1989b) and Kuchitsu, Katsuhara & Miyachi (1989) reported a slow alkanisation of the internal pH of Dunaliella after salt stress. Weiss & Pick (1990) reported rapid acidification of internal pH. The contradiction between these results may be due to the different

Due to very small size of *Dunaliella* cells direct electrometric determination of internal pH is not possible. Consequently, indirect methods have been used. The widely varying results from different methods do not encourage confidence in measurements of any supposed pH changes that might be induced within minutes by an osmotic pressure stress (Brown, 1990).

### 1.8.9. Inositol phospholipids

The second messenger function for inositol 1,4,5-trisphosphate (InsP$_3$) to release internal calcium was reported by Streb *et al.* (1983) and has been discussed by Berrigge (1993), Berrigge & Irvine (1989), Einspahr & Thompson (1990).

Inositol 1,4,5-trisphosphate (InsP$_3$) controls many cellular processes, including fertilisation, cell growth, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signalling in animal cells by generating an internal calcium signal (Berrigge, 1993). The level of free Ca$^{2+}$ ion in the cytosol is usually kept below 0.2 µM. A rise as small as 1 µM in the level of cytosolic Ca$^{2+}$ triggers many cellular responses (Darnell, Lodish & Baltimore, 1990).

In general, the binding of an external signal to receptors located on the surface of the plasma membrane, triggers activation of a G protein (GTP-binding protein) that, in turn, activates phospholipase C (PLC). This enzyme cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to two second messengers: (InsP$_3$) and 1,2-diacylglycerol (DAG). All these processes occur at the plasma membrane. InsP$_3$ diffuses through
the cytosol to the endoplasmic reticulum or other intracellular vesicles that store Ca\textsuperscript{2+} and promote its release. DAG remains in the membrane where, together with Ca\textsuperscript{2+} released from the ER or intracellular vesicles to the cytosol, helps to activate protein kinase C (PKC). Activation of protein kinase C, in turn, phosphorylates several cellular enzymes and receptors and alters their activities (Berrigge, 1993; Einspahr & Thompson, 1990; Darnell, Lodish & Baltimore, 1990).

Many, perhaps most of the elements involved in the PIP\textsubscript{2} signalling process, established in animal cells, are also present in plants. However, many components have not been identified. "Despite the expected participation of a plasma membrane-associated receptor in plant PIP\textsubscript{2}-mediated signal transduction, no such receptor has been identified" (Einspahr & Thompson, 1990).

In plant cells it has not yet been determined clearly whether InsP\textsubscript{3} stimulates Ca\textsuperscript{2+} release (Schroeder & Thuleau, 1991), although there is much evidence for the involvement of InsP\textsubscript{3} in plant signal transduction (Irvine, 1990). In Vicia \textit{faba}, Ca\textsuperscript{2+} has been suggested as a trigger for reducing stomatal pore apertures (Schroeder & Hagiwars, 1989). Gilory, Read & Trewaves (1990) reported that micro injection of InsP\textsubscript{3} to \textit{Commelina communis} leaf guard cells induces an elevation in the cytosolic Ca\textsuperscript{2+} concentration and stimulates stomatal closing.

Wong & Chase (1986) showed that in urinary bladder epithelial cells, the elevation of intracellular calcium following hypo-osmotic stress, is critical for cell volume regulation.

In Dunaliella, involvement of metabolism of inositol phospholipids in osmotic stress regulation is expected. Einspahr, Peeler & Thompson (1988b) showed in \textit{D. salina} that 2-4 minutes after dilution stress from 1.7 M to 0.86 M NaCl, a decrease in plasma membrane-localised PIP\textsubscript{2}, phosphatidylinositol 4-phosphate (PIP) and an increase in
phosphatidic acid (PA) level occurred, which recovered after 30 minutes. Salt stress from 1.7 M to 3.42 M NaCl rapidly elevated the PIP2, PIP and in addition the PA (50%) declined. Einspahr, Peeler & Thompson (1989) showed that the *D. salina* plasma membrane is highly enriched in a phospholipase C, capable of hydrolysing PIP2 to inositol trisphosphate and DAG.

Ha & Thompson (1991, 1992) reported in *D. salina* an increase in the level of plasma membrane-localised DAG parallel to maximal PIP2 hydrolysis after dilution stress. Also Ha & Thompson (1992) reported for the first time that dilution stress induced an increase in chloroplast membrane DAG concentration even higher than in the combined nonchloroplast membrane DAG concentration. Also their preliminary results showed dilution stress induces an activation of protein kinase in the first few minutes of stress where DAG concentration increased. However the relative sensitivity of protein kinase C to activation *in vivo* by DAG is not clear. This work confirms that in the *Dunaliella* cell, DAG acts as a second messenger for activation of protein kinase C in a manner similar to animal cells. A Ca²⁺-dependent protein kinase has been partially purified and characterised from *D. salina* by Guo & Roux (1990). Hence most of the components that activate protein kinase in animal cells have been identified in *Dunaliella*.

These results implicated the inositol phospholipids in signal transduction processes following dilution stress. Three low molecular mass GTP-binding proteins and Ca²⁺-dependent protein kinase have been identified in *D. salina* (Roriguez-Rosales, Herrin & Thompson, 1992). Activation of protein kinase associated with increasing DAG concentration following dilution stress has been observed by Ha & Thompson (1992). However the relationship between G protein, inositol phospholipids and osmotic response via Ca²⁺ or other pathway in *Dunaliella* is not yet clear.
1.9. Electroporation

If two flat parallel metal plates are positioned facing each other 1 cm apart with a potential difference of say 1000V, then any object placed in between them will experience an electric field of 1000 V cm⁻¹ (Knight, 1981). The effect of such a field on living cells was first described by Maxwell (1892 cited by Knight, 1981) and subsequently documented by many authors.

Knight (1981) has written a comprehensive review of electroporation. When cells are exposed to electrical fields a number of physical and biological changes take place in cell membrane. The effects of electrical pulses on the cell membrane were first investigated by Neumann & Rosenheck (1972) and then by Zimmermann, Pilwat & Riemann (1974). The principle of the electroporation technique is that the electric field interacts with the plasma membrane causing hyperpolarization. Hyperpolarization beyond ±200mV (Chassay, Mercenier & Flickinger, 1988), causes the formation of pores in localised areas, whilst not disturbing the integrity of the intracellular organelles (Knight, 1981). If the electric field strength and exposure time do not exceed critical values, the breakdown of the membrane is completely reversible (Kinosita & Tsong, 1977).

Above a critical voltage, the cell membrane is irreversibly damaged by the electric field. The state of disorganisation of the membrane may become too great to repair or reverse. This, combined with excessive loss of cellular contents, may cause cell death. Thus "electropermeabilization of cells will occur above a lower specific critical threshold voltage and below a critical lethal voltage" and these two parameters are genus, species and even strain dependent (Chassay, Mercenier & Flickinger, 1988).

The electroporation technique has been used widely to perturb the membranes of animal and plant cells, bacteria, algae and isolated chloroplasts.
If a cell is exposed to an electrical field a voltage will be imposed across its membrane. The magnitude of this potential difference (by assumption the cell is hollow thin-walled sphere) depends on intensity of the field, size of the cells and conductivity of the external fluid, cytosol, and the membrane (Knight 1981). In general the optimum conditions for permeablising the cell membrane varies for each kind of cell. Variable parameters include the strength and the number and duration of fluctuations of the applied electric field, conductivity of the external fluid, size of the cell (Knight 1981) and design of the electroporation chamber (Wert & Saunders, 1992). These parameters determine the number and size of the pores formed and hence the influx or efflux of solutes. Rosemberg & Korenstein (1990) showed that, for the thylakoid membrane of isolated chloroplasts of spinach, pea and tobacco, an increase in electric field strength causes an increase in total area of the vesicle undergoing electroporation. The duration of the electric field also increases the area of individual pores. They stated that their results support the theoretical model of transient aqueous pores suggested by Abidor et al. (1979), Powel, Derrick & Weaver (1986) and Glaser et al. (1988). On the other hand, the critical voltage is temperature-dependent, decreasing as the temperature is increased (Costa & Zimmermann, 1975). Large cells require a lower voltage than smaller ones. Electroporation allows the membrane of the cell to be broken down and the cytosol accessed without seemingly disturbing the intracellular organelles and hence their function (Knight, 1981).

Resealing times following pore formation have been reported from 50ms to 2h. This wide range of reports could be due to differences in the structure of the pores produced in the cell membrane or to the differences in methods used to determine pore lifetimes (Saulis, Venslauksam & Naktinis, 1991). The kinetics of resealing pores after reversible breakdown of the membrane barrier were determined by studying membrane conductance relaxation to its initial level, or by determining the time required for their barrier function to ions or other substances to be restored. Saulis,
Venslauskas & Naktinis (1991) measured pore lifetimes in human red blood cells by determining the restoration of the membrane barrier function to Na⁺, and larger molecules such as ascorbic acid and mannitol. They suggested that, depending on the electric pulse parameters, there may be three stages of the pore resealing process after electroporation: (1) A rapid decrease in pore size with the characteristic time ≤ 1s after electroporation. For this stage the pore must be sufficiently large (radius>15 Å). (2) A slow decrease in pore size with the characteristic time of few minutes. The radius of the pore in this stage is about 4-5 Å, so for example ascorbic acid and mannitol cannot penetrate into cells, whereas sodium ions can. (3) In this stage, complete pore closure occurs with life-time more than 10 minutes. They also stated that "it is natural to think that short-lived pores are formed in the lipid matrix of the cell membrane, while the membrane proteins participate in the formation of the long-lived pores".

The transient breakdown of the membrane makes it permeable to small molecules and macromolecules (Knight, 1981). Electroporation allows the introduction of DNA, RNA, proteins, drugs and dyes into cells (Wert & Saunders, 1992). Wong & Neumann (1982) established that plastid DNA could be taken up and expressed by animal cells. Formm, Taylor & Walbot (1985) also has reported the uptake of and expression of DNA in protoplast of maize, carrot and tobacco. Expression of mRNAs introduced into plant and animal cells by electroporation has been reported by Callis, Formm & Walbot (1987). Introduction of nonpermeant molecules such as Lucifer Yellow (475 Da) and PAP (30,000 Da) directly into the cytoplasm of living cells was reported by Mir, Banoun & Paoletti (1988).

Electroporation of cells in a medium containing only low molecular weight solutes e.g. Na⁺, K⁺, Cl⁻ may allow the solute to equilibrate across the membrane. If the pore sizes are smaller than the intracellular protein, an osmotic imbalance is set up (Knight, 1981). Red blood cells swell as result of such an osmotic imbalance (Zimmermann,
Riemann & Pilwat, 1975; Kinosita & Tsong, 1977), whereas adrenal medulla cells do not swell and seem to be able to withstand electroporation (Knight, 1981).

1.10. Photosynthesis and respiration in *Dunaliella*

Most of the biochemistry of carbon metabolism during photosynthesis and photorespiration is similar in green algae and higher plants (Tolbert, 1974). Although relatively little work has been done on the biochemistry of *Dunaliella*, the major metabolic pathways and cycles in this alga are thus likely to be similar to those of higher plants.

1.10.1. Effect of salt stress on O₂ evolution and O₂ uptake in *Dunaliella*.

There are many reports on the effects of salt stress and dilution stress on photosynthetic O₂ evolution in *Dunaliella* (Kaplan, Shreiber & Avron, 1980; Kessly & Brown, 1981; Gilmour, Hipkins & Boney, 1982, 1984a; 1984b; Lilley et al., 1987; Goyal, Brown & Lilley, 1988). Most of these found that after salt stress, especially for high salt stress, the O₂ evolution was inhibited. The extent of salt stress required to stimulate or inhibit photosynthesis varies from species to species and is also influenced by the sodium chloride concentration in the medium in which the algae was grown or adapted.

When *D. tertiolecta* was adapted to 0.4 M NaCl and transferred to 0.75 M, 1.2 M, 1.5 M, or 1.75 M, O₂ evolution decreased (Gilmour, Hipkins & Boney, 1982). When the salinity of the external medium was increased beyond 1.2 M, photosynthesis was inhibited significantly and did not recover fully within 100 minutes. The respiration rate (dark O₂ uptake) was inhibited during this period, but not to the same extent as net photosynthesis (Gilmour, Hipkins & Boney, 1982). When 0.17 M NaCl adapted *D. tertiolecta* was transferred to 0.7 M NaCl, photosynthetic O₂ evolution was initially inhibited but after a few minutes recovered to rates slightly higher than before.
However, after transfer to 1 M NaCl the organism took longer to recover and the ultimate rate of O₂ evolution was about half that of control suspension in 0.17 M NaCl (Lilley et al., 1987; Goyal, Brown & Lilley, 1988).

In contrast, when *D. salina* was transferred from 1.5 M NaCl adapted to 2.1 M (Kaplan, Schreiber & Avron, 1980), and from 1.5 M to 2.5 M (M. Avron, personal communication to R.McC. Lilley), O₂ evolution increased rapidly and after minutes remained at a constant rate higher than control.

In *D. tertiolecta*, dilution stress caused a reduction in the rate of O₂ evolution (Lilley et al., 1987; Goyal, Brown & Lilley, 1988). Decreasing the external salinity was reported to have a marked inhibitory effect on the rate of photosynthesis similar to that of an increase in external salinity (Gilmour, Hipkins & Boney, 1984a), but in addition there was stimulation of the rate of dark O₂ uptake after 5 minutes at all the lower salinities but not in the absence of salt (Gilmour, Hipkins & Boney, 1984b). For example, dilution stress from 1.53 to 1.06 M NaCl inhibited photosynthetic O₂ evolution of *D. tertiolecta* and stimulated respiration by about 30% in each case. A 1.53 M to 0.53 M NaCl stress inhibited O₂ evolution by about 60% and stimulated respiration by about 50% (Gilmour, Hipkins & Boney, 1984b). The inhibition of photosynthesis caused by dilution stress is not substantially the result of impaired electron transport in either Photosystem I or II but can attributed to inhibition of the carbon dioxide fixation cycle (Brown, 1990). When *D. salina* was transferred from 1.5 M to 0.75 M, the O₂ evolution decreased rapidly but after few minutes increased for about 20 minutes, then remained at a constant rate lower than the control (M. Avron, personal communication to R.McC. Lilley).
1.10.2. Light saturation and photoinhibition in *Dunaliella*

Green algal cells can utilise only a limited amount of light energy at a time via photosynthesis (Soeder & Stengel, 1974; Owens & Esaias, 1976). This phenomenon is widely known as light saturation. The algal chloroplast is capable of working only at low energy levels, whereas the incident energy is at high level in many environments where *Dunaliella* grows (Ben-Amotz, 1980). The occurrence of this saturation effect imposes a serious limitation on the efficiency with which high intensity solar energy can be utilised by algae.

Many higher plants and algae show reduced rates of photosynthesis after a period of time of illumination. This is called photoinhibition and usually occurs at an illumination of a few thousand lux above saturation point (Yentsch, 1962 cited by Ben-Amotz, 1980). Some plants show photoinhibition at surprisingly low light intensities, viz 200 μE.m⁻².sec⁻¹. Photoinhibition can usually be induced by exposing photosynthetic organisms or isolated organelles to substantial light in the absence of photosynthetic carbon metabolism (Powles, 1984). Experiments in which intact chloroplasts have been exposed to light in the absence of photosynthetic carbon metabolism show an inhibition of electron transport (Cornic et al., 1982) and damage to PS-II reaction centres following treatment (Giersch & Robinson, 1987). Photoinhibition also has specific inhibitory effects on photosynthetic carbon metabolism. Photoinhibition results in the production of H₂O₂ and other toxic oxygen species, which may contribute to enzyme inactivation such as oxidation of thioredoxin-reduced enzymes (Giersch & Robinson, 1987). Rubisco activity was also found to be lowered by a photoinhibition treatment of intact leaves in wheat (Boyle & Keys, 1982).

The saturation point and the point at which photoinhibition begins to occur in algae, depends on the light intensity, light quality, temperature and culture conditions. Some
species of *Dunaliella* (*salina* and *bardawil*) accumulate high amounts of pigment in the form of β-carotene as a defence against high light intensity illumination and under unfavourable culture conditions such as high salinity or nitrogen deficiency (Ben-Amotz, 1980).

Borowitzka, Borowitzka & Moulton (1984) measured in *D. salina* the maximum rate of net photosynthetic O₂ evolution for two cultures of greatly differing β-carotene content (approximately 10 μmol O₂ . mg⁻¹ chl⁻¹.min⁻¹). The green, low carotenoid culture achieved light saturation at 1300 μmol m⁻².sec⁻¹ and showed marked inhibition of photosynthetic O₂ evolution at higher light intensities. The red, carotenoid-rich culture saturated at about 2300 μE m⁻².sec⁻¹. They also showed that the β-carotene content of the alga increased with growth medium salinity.

Cultures of *D. salina* were grown under low light intensity (23 μE .m⁻².sec⁻¹) and with 4.92 μg β-carotene . ml⁻¹ (Ben-Amotz & Avron, 1983b). When exposed to high light intensities, the rate of photosynthesis saturated at 2300 μE .m⁻².sec⁻¹ (3 μmol O₂ . mg⁻¹.chl⁻¹.min⁻¹). Cultures grown under high light intensity (2500 μE .m⁻².sec⁻¹) with 15.7 μg β-carotene . ml⁻¹, saturated at 9200 μE .m⁻².sec⁻¹ (10 μmol O₂ . mg⁻¹.chl⁻¹.min⁻¹). This demonstrated that the accumulation of high β-carotene has a protective function against injury by high intensity irradiation.
CHAPTER TWO:
MATERIALS AND METHODS
2. Materials and Methods

2.1. Materials:

2.1.1. Chemicals:
All chemicals were of analytical grade obtained from Sigma (U.S.A.), BDH (Australia) or from Univar-Ajax (Australia). Permablend, Triton X-100 were obtained from Packard (U.S.A.). The protein assay kit (BCA) was from Pierce (USA).

2.1.2. Biochemicals:
Glycerol kinase (EC 2.7.1.30) (from *Candida utilis*) and lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle) were purchased from Sigma (U.S.A.), and pyruvate kinase (from rabbit muscle) from Boehringer (Germany).

2.1.3. Radiochemicals.

<table>
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<th>Name</th>
<th>Company</th>
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<tr>
<td>$^{14}$C-NaHCO$_3$</td>
<td>Amersham</td>
</tr>
<tr>
<td>D-[1-1$^{14}$C]-mannitol</td>
<td>Amersham</td>
</tr>
<tr>
<td>$^{14}$C-Toluene</td>
<td>Amersham</td>
</tr>
<tr>
<td>$^{3}$H$_2$O</td>
<td>Packard</td>
</tr>
<tr>
<td>$^{22}$NaCl</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

2.1.4. Source of *Dunaliella*.

*Dunaliella tertiolecta* and *D. salina* Butcher were obtained from the University of Wollongong collection (R. McC. Lilley)
2.2. Methods:

2.2.1. Stock cultures and growth conditions.

Stock cultures were kept on 1.5% w/v agar slopes of the defined medium of Johnson et al. (1968) containing 0.17 M NaCl for *D. tertiolecta* and 1.5 M NaCl for *D. salina*. The slopes were placed in the light growth cabinet with 200 μE.m⁻².s⁻¹ photon flux density with a light/dark regime of 14/10 h at 27±2°C. The stock cultures were subcultured every 3-4 weeks.

A liquid culture (50ml in a 100ml Erlenmeyer flask) was seeded directly from an agar slope, and incubated in the light growth cabinet with 200 μE.m⁻².s⁻¹ photon flux density with a light/dark regime of 14/10 h at 27±2°C for one week with intermittent shaking by hand (3-5 times per a day). This served as a preinoculum. After one week 100 ml growth medium in a 250 ml Erlenmeyer flask was inoculated with 10 ml preinocula, and incubated for a further week under same conditions.

*D. tertiolecta* and *D. salina* were cultured in a defined medium (Ben-Amotz, Shish & Avron, 1989) containing 0.17 M or 0.6 M NaCl for *D. tertiolecta* and 1.5 M NaCl for *D. salina*. The experimental cultures (50 ml of algal suspension) were grown in 450 ml of growth medium in a 600 ml plastic tissue culture vessel at 27±2°C with continuous aeration by 5% CO₂ in air and stirred with a magnetic stirrer. The photon flux density was 280 μE.m⁻².s⁻¹ with a light/dark regime of 14/10 h. The cultures were harvested after five days.
Growth medium.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. KNO₃</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>2. MgSO₄</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>*3. CaCl₂</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>*4. KH₂PO₄</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>*5. FeCl₃+Na₂EDTA</td>
<td>2.0 μM + 5.0 μM</td>
</tr>
<tr>
<td>6. MnCl₂</td>
<td>7.0 μM</td>
</tr>
<tr>
<td>7. CuCl₂</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>8. ZnCl₂</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>9. CoCl₂</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>10. (NH₄)₆Mo₇O₂₄</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>11. NaHCO₃</td>
<td>10 mM</td>
</tr>
<tr>
<td>12. NaCl</td>
<td>depending on the molarity required</td>
</tr>
</tbody>
</table>

Adjust pH to 7.0 - 7.5 to 1000 ml distilled water.

*Reagents sterilised separately and added to media after sterilisation.

2.2.2. General techniques.

2.2.2.1. Chlorophyll determination

The chlorophyll (a+b) content of Dunaliella cells was determined in acetone extracts (Bruinsma, 1961; Walker, 1971). 100% acetone (1.0 ml) was added to 250 μl of algal suspension (in 3 replicates) and mixed well with a vortex mixer (Model K-550- GE) and centrifuged for 1 minute in a bench-top speed fixed centrifuge (Beckman, Microfuge, U.S.A) at 13,000 x g. The absorbance of the supernatant was measured by a spectrophotometer (Gliford, Stasar, U.S.A.) at 652 nm.

The chlorophyll content was calculated by the equation:

\[ \mu g \text{ chlorophyll (a+b) per ml} = E_{652} \times 27.78 \times 1.25 \times 4 \]
2.2.2. Cell density determination

A hemocytometer was used to determine *Dunaliella* cell counts. Assay buffer medium (900 μl) was added to 100 μl of algal suspension and a few iodine crystals was added to immobilise the alga. A small amount of cell suspension was transferred to both chambers of the Hemocytometer by Pasteur pipette and the cells counted under a light microscope in five squares in each two chambers.

The cell density was calculated by using the following equation:

\[
\text{Number of cells} \cdot \text{ml}^{-1} = \text{average cell count} \cdot 10^{-1} \text{ squares} \times \text{dilution factor} \times 10^4
\]

2.2.2.3. Photon flux density measurement

Photon flux density (photosynthetic photon flux fluence rate) was measured by spherical quantum sensor (Li-Cor, Li-193SB, SPQA 0331, USA) connected to a voltmeter. The photon flux density was then calculated as \(\mu\text{E.m}^{-2}.\text{s}^{-1}\) using the conversion factor supplied by manufacturer.

2.2.2.4. Osmotic pressure determination

Osmotic pressure was determined using a Wescor 5100C, USA vapour pressure osmometer. The osmometer was calibrated by 290 and 1000 mmol . kg\(^{-1}\) osmolality standard solutions. Samples (8 μl) were transferred on paper discs in the osmometer chamber. The osmolality of the sample was determined as mmol . kg\(^{-1}\).

2.2.3. O\(_2\) evolution.

2.2.3.1. Harvesting the experimental cultures for measuring O\(_2\) evolution

The cultures were harvested by centrifugation at 5000 rpm (2000 x g) for five minutes (Sorvall RC-5B Refrigerated Superspeed Centrifuge) following five days growth. The alga was resuspended in either phosphate buffer or Hepes buffer (Table 2.2). One ml of algal suspension was transferred to the reaction vessel of the O\(_2\) electrode.
Photosynthetic O$_2$ evolution or consumption rates were measured polargraphically (Delieu & Walker, 1972) before and after salt stress in light or dark conditions at 28 °C.

2.2.3.2. Application of salt stress and media for O$_2$ evolution measurement.

Salt stress was applied in a single step by adding appropriate volumes of NaCl concentrated solution according to the Table 2.1 and 2.2. The concentrated buffer solutions for salt stress or control were degassed in a vacuum desiccator.

Table 2.1: **Conditions for salt stress experiments.**

<table>
<thead>
<tr>
<th></th>
<th>volume of algal suspension</th>
<th>volume of concentrated NaCl buffer</th>
<th>[NaCl] in concentrated buffer</th>
<th>final volume</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D. tertiolecta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.17 M to 0.17 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>0.17 M</td>
<td>1.5 ml</td>
<td>0.17 M (control)</td>
</tr>
<tr>
<td>0.17 M to 1.0 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>2.66 M</td>
<td>1.5 ml</td>
<td>1.0 M</td>
</tr>
<tr>
<td>0.17 M to 0.28 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>0.5 M</td>
<td>1.5 ml</td>
<td>0.28 M</td>
</tr>
<tr>
<td>0.6 M to 0.6 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>0.6 M</td>
<td>1.5 ml</td>
<td>0.6 M (control)</td>
</tr>
<tr>
<td>0.6 M to 1.0 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>1.8 M</td>
<td>1.5 ml</td>
<td>1.0 M</td>
</tr>
<tr>
<td><strong>D. salina</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 M to 1.5 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>1.5 M</td>
<td>1.5 ml</td>
<td>1.5 M (control)</td>
</tr>
<tr>
<td>1.5 M to 2.5 M</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>4.5 M</td>
<td>1.5 ml</td>
<td>2.5 M</td>
</tr>
</tbody>
</table>
Table 2.2: **Media for O₂ measurement.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in phosphate buffer</th>
<th>Concentration in Hepes buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.25 mM</td>
<td>—</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.25 mM</td>
<td>—</td>
</tr>
<tr>
<td>Hepes</td>
<td>—</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.2 mM</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>5.0 mM</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>depending on molarity required</td>
<td>7.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

### 2.2.3.3. Measurement of O₂ evolution

Oxygen evolution was measured with two Clark-type O₂ electrodes in water-jacketed reaction vessels maintained at 28 °C. The O₂ electrode (Delieu & Walker, 1972) was connected to a measuring circuit and chart recorder (Rikadenki, Japan). Magnetic stirrers were used. A slide projector (Hanimex Rondette 1000) with a 150 Watt lamp was used for illumination, the white light beam (500 μE.m⁻².s⁻¹) was projected through a red filter and Infrared filter (Balzers) to obtain red light (250 μE.m⁻².s⁻¹). As required, either red or white light beam was projected through a spherical focusing lens (round bottomed flask filled with water) to obtain high photon flux density (red light = 770 μE.m⁻².s⁻¹ or white light = 2200 μE.m⁻².s⁻¹).

### 2.2.3.4. Photosynthetic ¹⁴C carbon dioxide fixation

Algal cultures were harvested (Section 2.2.3.1) and resuspended in buffer medium without bicarbonate. The measurements were performed in the O₂ electrode apparatus (Section 2.2.3.3). Algal suspension (1.0 or 2.0 ml) was transferred to the reaction
vessels. Photosynthetic $^{14}$C- fixation was initiated by adding 25 μl of NaH$^{14}$CO$_3$ containing 46.25 kBq to reaction vessels in the dark to a final concentration of 12.5 mM. At regular times, 10 μl samples were withdrawn (before and after salt stress) and immediately transferred to glass scintillation vials containing 0.5 ml 90% ethanol to stop photosynthesis. Then 0.5 ml of formic acid (3.0 M in ethanol) were added to each vial and evaporated to dryness in a fume cupboard on a hot plate at 80 °C to remove acid-labile $^{14}$CO$_2$. After cooling, 0.5 ml of 0.1 M HCl was added to dissolve fixed $^{14}$C. For counting, 5.0 ml scintillation cocktail was dispensed to the scintillation vials and the contents mixed.

### 2.2.3.5. Radioactivity measurement

The scintillation cocktail was prepared by dissolving 4.5 g of Permablend-III (Packard) in a mixture of toluene: triton X-100 (666:333 v/v) and stored in an amber coloured bottle.

Radioactivity was measured in a liquid scintillation spectrometer (LKB Wallac 1219 Rackbeta). Counting efficiency was determined with an internal standard ($^{14}$C-toluene, 985 dpm . μl$^{-1}$), and the results were corrected for counting efficiency. Generally the counting efficiency of the counter was 80 -90% with a background of 38-57 CPM. To determine the total $^{14}$C radioactivity in the reaction vessels, 10μl samples were withdrawn at the start and the end of experiments and mixed with 990 μl of 0.5 M Tris base. 20 μl of this mixture was transferred to glass vials containing 0.5 ml of 0.5 M Tris base and 5.0 ml of scintillation cocktail added before measuring in the scintillation counter.
2.2.4. Electroporation.

2.2.4.1. Electroporation apparatus.
The electroporator was constructed at the Science Faculty workshop of the University of Wollongong. The electroporator had a total capacitance of 2mF (3 x 200 μF, 1 x 500 μF, 1 x 900 μF) delivering pulses variable between 0 and 300V. Pulse duration was variable from 0 to 100ms. The chamber had two electrodes 4.3mm wide, 40mm tall, and 0.6mm thick pressed against the walls of the 1 cm³ spectrophotometer plastic cuvette (33mm tall, 10mm path length by 4.4mm wide) to give a current path length of 8.8mm. The electrodes were made initially of aluminium and later changed to copper, gold-plated to a thickness of 5.0 μm. The accuracy of voltage and pulse duration output were measured by a digital storage oscilloscope (Philips PM97).

2.2.4.2. Electroporation and post-electroporation media.
The composition of the electroporation medium was initially as in Table 2.3: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 6.0 mM CaCl₂, 5.0 mM NaHCO₃ (pH 8.1) with osmolality of 1189±10 mmol.kg⁻¹, equal to that of the growth medium which contained 600 mM NaCl (Section 2.2.1). Whenever the concentration of any components of this medium were changed (e.g. omission of CaCl₂, addition of the EDTA), mannitol concentration was adjusted to maintain the same osmotic pressure. The osmotic pressure was measured against standards of known osmolality (Section 2.2.2.4).
Table 2.3: Composition of electroporation media.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in electroporation medium</th>
<th>Concentration in post-electroporation medium (Hepes buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>150 mM</td>
<td>600 mM</td>
</tr>
<tr>
<td>Mannitol</td>
<td>730 mM</td>
<td>—</td>
</tr>
<tr>
<td>Tris-Cl</td>
<td>30 mM</td>
<td>—</td>
</tr>
<tr>
<td>Hepes</td>
<td>—</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>6.0 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>5.0 mM</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>pH</td>
<td>8.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Osmomolality</td>
<td>1189 ± 10 (mmol kg⁻¹)</td>
<td>1189 ± 10 (mmol kg⁻¹)</td>
</tr>
</tbody>
</table>

2.2.4.3. Electroporation procedure

Special precautions were used to minimise any mechanical damage to cells during harvesting (Section 4.3). The cells were centrifuged at the minimum possible RCF (relative centrifugal force) required to form a pellet and were resuspended as gently as possible.

The *D. tertiolecta* cells were harvested by centrifugation (Sorvall RC-5B Refrigerated Superspeed Centrifuge) at 161 x g for 6 minutes at 25 °C. The supernatant was discarded completely and the tube and surface of pellet rinsed briefly with electroporation medium. The pellet was then resuspended by slow swirling in electroporation medium which took about 10 to 15 minutes. The resuspended cells were concentrated by a factor of 10 from the growth medium. Then chlorophyll and cell number were determined (Section 2.2.2.1 & 2.2.2.2).

The resuspended pellet in the electroporation buffer medium was preincubated in darkness at 25 °C for 20 minutes, then placed on ice for 15 minutes. Electroporation
was carried out on 1.0 ml of algal suspension, which was transferred to the 1ml plastic cuvette on ice and then post-incubated for 5 minutes on ice. The algal suspension was subsequently removed by Pasteur pipette and used for glycerol or protein assay.

For determining the time-course of intracellular glycerol concentration and cell volume after electroporation, the electroporated and control suspension were transferred to a 25 ml glass conical flask, then diluted quickly with 6.0 ml of cold (2 °C) Hepes medium (Table 2.3). Under these cold conditions, 1.0 ml of sample was removed for glycerol assay and three 250 μl samples for chlorophyll assay. In some cases, samples of appropriate volume were removed for cell number counting or protein assay. The cell suspension was then brought to 25 °C by placing the flask in a 25 °C water bath by gently agitating for 30 seconds. The flask was then placed in a growth cabinet, illuminated at 200 μE.m⁻².s⁻¹ while the surface of the suspension was flushed continually with 5% CO₂ in air. At 30 minute intervals, further samples were taken for glycerol and chlorophyll assay.

2.2.5. Glycerol extraction and determination

2.2.5.1. Glycerol extraction

A sample of 1.0 ml of algal suspension was transferred to a weighed Eppendorf tube, and the volume (Vt) determined by weighing (Mettler AC 100 Electronic balance) and corrected by the previously determined specific gravity. The sample was centrifuged (Heraeus-Christ centrifuge) at 900 g for 5 minutes at 2 °C. An exact volume of about 700 μl of supernatant (Vs) was removed carefully by micro pipette without disturbing the pellet. The volume of the remaining supernatant and pellet fraction (Vps) was determined by weighing. To check the accuracy of weighing, the equivalence of the sum of Vs and Vps with Vt was used. Then immediately 62.5 μl of 70% perchloric acid was added to both fractions and mixed by a vortex mixer and centrifuged (Beckman Microfuge, USA) at 13,000 g for 1 minute. The extracts were neutralised
with a known volume of 5.0 M KOH, 1.0 M triethanolamine, centrifuged again for 1 minute. The supernatants of both fractions were removed and stored immediately at -80 °C. The glycerol content of extracts was determined enzymically (Section 2.2.5.2).

2.2.5.2. Glycerol determination.
Glycerol was determined from the oxidation of NADH to NAD by a coupled enzyme assay as followings (Eggstein & Kuhlmann, 1974).

The assay mixture contained in a final volume of 1.0 ml:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Required concentration in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine buffer (pH 7.5)</td>
<td>75 mM</td>
</tr>
<tr>
<td>MgSO4</td>
<td>10 mM</td>
</tr>
<tr>
<td>PEP</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>NADH</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>0.5 Units</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>2.0 Units</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>2.5 Units</td>
</tr>
</tbody>
</table>
For the glycerol assay, all reagents except glycerol kinase were added to a 1.5 ml quartz cuvette. A sample of known volume of glycerol extract was added to the reaction mixture and mixed. The cuvette was then placed in the spectrophotometer (Varian, Cary 210) at 25 °C, wavelength 340 nm and the absorbance followed with a chart recorder. Glycerol kinase was added to the cuvette and briefly but completely mixed. The decrease in absorbance was recorded and compared with a blank containing no glycerol. The amount of glycerol was taken as equivalent to the amount of NADH oxidised during pyruvate reduction, as measured by the decrease in absorbance at 340 nm (extinction coefficient 6.22).

### 2.2.5.3. Intracellular glycerol calculation

The glycerol concentration in the original supernatant ([Ge]) and the amount of glycerol in the remaining supernatant and pellet fraction (Gps) were calculated from the change in $E_{340}$. The intracellular (Gi) and extracellular (Ge) amounts of glycerol were calculated by assuming an intracellular volume of 125 µl per mg chlorophyll for *D. tertiolecta* (Marengo, Brown & Lilley, 1989) using the following equations:

- Intracellular volume $V_i$ (µl) = $V_t \times 125 \times $ sample chl concentration (mg . ml$^{-1}$) / 1000
- Extracellular volume $V_e$ (µl) = $V_t - V_i$
- Extracellular glycerol amount $G_e$ (nmol) = $[G_e] \times V_e / 1000$
- Intracellular glycerol amount $G_i$ (nmol) = $G_{ps} - (V_{ps} - V_i) \times [G_e] / 1000$
- Proportion of glycerol Extracellular = $\frac{G_e \times 100}{(G_e + G_i)}$

### 2.2.6. Soluble protein extraction and determination

A sample of 1.0 ml of cell suspension was diluted 4 times by distilled water and the cells broken by a Polytron 30% speed for 30 sec at 2 °C. The suspension was transferred to a Potter glass-teflon homogenizer and the plunger cycled at least 3 times. Microscopic inspection confirmed 100% cell breakage. This suspension was
centrifuged (Beckman Microfuge) at 13,000 g for 1 minute. The supernatant was removed and a known volume added to 0.5 ml of NaOH (100 mM), mixed and centrifuged again for 1 minute. The supernatant was removed and protein determined by the bicinchoninic acid procedure (Pierce Chemical Company). Bovine serum albumin was used as standard. A sample (0.5 ml) of protein extract was mixed with 0.5 ml of protein assay reagent and kept at 60 °C in a water bath for 60 min. After cooling, the absorbance was measured spectrophotometrically (Gilford, Stasar, USA) at 562 nm. In the case of soluble protein release determination after electroporation, protein was measured in the supernatant and pellet under the same conditions as for glycerol release determination. After centrifugation of 1.0 ml algal suspension, the supernatant was removed carefully and the pellet diluted with distilled water and then homogenised by Polytron and Potter homogeniser.

2.2.7. **Al^{3+} determination.**

Al^{3+} in the medium following electroporation using aluminium electrodes was determined by graphite furnace atomic absorption spectroscopy (Varian aa-SPECTRAA-20 GTA-96 Graphite Tube Atomizer) and calculated from a standard curve in the Department of Chemistry, University of Wollongong.

2.2.8. **Measurement of uptake of \textsuperscript{14}C-mannitol and \textsuperscript{22}Na.**

Centrifuged cell cultures were resuspended in electroporation medium containing additionally radiolabelled mannitol or Na\textsuperscript{+} and equilibrated for 15 min. For mannitol, 14.4 kBq . ml\textsuperscript{-1} of 1-\textsuperscript{14}C-mannitol (Amersham, England; Specific activity: 2.11 GBq.mmol\textsuperscript{-1}) was used. For Na\textsuperscript{+}, 6.90 kBq . ml\textsuperscript{-1} of \textsuperscript{22}Na (Amersham, England; original Specific activity: 35.26 MBq . ml\textsuperscript{-1}) was used. The actual activity of the \textsuperscript{22}NaCl solution was calculated from half-time and the date of standardisation.
One ml of electroporated or non-electroporated (control) cells were centrifuged (Heraeus-Christ centrifuge) at 900 x g for 5 minutes. The pellet was resuspended in 1.0 ml of Hepes medium. The cells were then centrifuged again and resuspended in this medium a further two times. Samples of resuspended pellet (100 µl) were transferred to scintillation vials containing 5.0 ml of scintillation cocktail and the radioactivity determined (Section 2.2.3.5). The amount of $^{14}$C-mannitol or $^{22}$Na uptake was expressed as nmol/mg chlorophyll.

2.2.9. Cell volume determination.

2.2.9.1. Solute exclusion method.

The Li-based procedure of Katz & Avron (1985) was used. One ml of electroporated or non-electroporated cell suspension was diluted with 6.0 ml of isotonic cold Hepes medium, containing also 50 mM LiCl and $1.10^7$ dpm . ml$^{-1}$ $^3$H$_2$O and mixed gently for 10 min. Cell number and chlorophyll content were measured. Samples (1.0 ml) of this suspension were centrifuged (Heraeus-Christ) at 900 x g for 5 min at 2 °C. The supernatant was completely and carefully removed by syringe without disturbing the pellet and retained (SUP-I). The algal pellet was diluted by 1.0 ml of cold water, mixed well and kept for 30 min, before centrifuging (Microfuge, Beckman) for 10 min. The supernatant was retained (SUP-II). For determining the time-course of cell volume, 1.0 ml samples were removed at specific times, cooled and assayed as described. SUP-I and SUP-II were diluted as required for Li and $^3$H$_2$O determination. Li was measured by flame photometry (Corning 410) and calculated from a standard curve. For $^3$H$_2$O radioactivity measurement, 10 µl of sample was transferred to scintillation vials and mixed with 5.0 ml of scintillation cocktail and radioactivity determined (Section 2.2.3.5). The cell volume was calculated by the following equation (Katz & Avron 1985):
$$^{3}\text{H}_{2}\text{O \text{SUP-II} (cpm.ml}^{-1}) \times \text{resuspended pellet (ml)} \times \left(1 - \frac{\text{Li SUP-II (mM)}}{\text{Li SUP-I (mM)}}\right)$$

Cell volume = \frac{\text{number of cells} \times ^{3}\text{H}_{2}\text{O SUP-I (cpm.ml}^{-1})}{(\mu m^3)}$

2.2.9.2. Cell volume determination by photography.

Cell volume was measured by microscopy of freshly-mounted cell suspensions using two procedures.

2.2.9.2.1. Still photography and measurement from projected slides.

A camera (Pentax K1000) was attached to a light microscope (Olympus BH) using differential interference optics. One drop of non-electroporated (control) or electroporated cells (immediately after electroporation while the cells were still cold) was put on the slide and covered by a cover slip. Random fields were photographed by 35 mm, 400 ASA Kodak film under the light microscope at 500x magnification. For the time-course of cell volume determination, 1.0 ml of the electroporated and control cell suspension was diluted with 6.0 ml of Hepes buffer (27 °C) (Table 2.3, Section 2.2.4.2) and kept in an illuminated growth cabinet at 27 °C. A drop of sample was transferred to a slide at specific times and photographed as described. An etched microscope ruler with 10 μm graduations was also photographed under the same conditions as a size scale. The still slides were projected onto a screen for measurement. The dimensions of all cells on each slide with both poles in focus were measured.

2.2.9.2.2. Video Recording

A video camera (National WV-CD) was mounted on a light microscope (Olympus CH-2) using darkfield. The video camera was connected to a video tape recorder (VCR Sharp VC-6V3X) (Fig. 2.2). A drop of cell suspension was placed on the slide,
covered by a cover slip and the cells photographed at a magnification of 400x on high resolution video tape (BASF). The recording of a number of random fields was completed within 1 min. The field focussing was checked by a monitor connected to the VCR. An etched microscope ruler with 10 μm graduations was also recorded under the same conditions as a size scale. Recordings were viewed with a monitor and individual motile cells were followed frame by frame using an Editing VCR (Panasonic AG-7500) which gives a very sharp fixed image. A frame was selected that displayed an image of the motile cell with both poles in focus. The selected cell images were digitised (software: Screen Play) and transferred to a computer (Apple Macintosh IIfx) connected to the Editing VCR. Cell dimensions were measured digitally (software: Adobe Photoshop).
Fig 2.1: Cell Volume Measurement by Video Microscopy
2.2.9.3. Measurement of cell dimensions:

For both methods, four measurements of each cell were made and the volume calculated by approximating the shape to two end-hemispheres and two frustums (Fig. 2.2). This approach was adopted because the cell diameters (especially a and b) were more sensitive to change and have a much larger influence on the calculated volume than cell length (d). The cell volume was calculated summing the volume of the two hemispheres and two frustums.

Volume, Hemisphere (1) = \( \frac{2}{3} \pi \left( \frac{a}{2} \right)^3 \)

Volume, Frustums (2) = \( \pi \times \frac{h}{3} \times \left\{ \left( \frac{a}{2} \right)^2 + \left( \frac{a}{2} \times \frac{b}{2} \right) + \left( \frac{b}{2} \right)^2 \right\} \)

Volume, Frustums (3) = \( \pi \times \frac{h}{3} \times \left\{ \left( \frac{b}{2} \right)^2 + \left( \frac{b}{2} \times \frac{c}{2} \right) + \left( \frac{c}{2} \right)^2 \right\} \)

Volume, Hemisphere (4) = \( \frac{2}{3} \pi \left( \frac{c}{2} \right)^3 \)

\[ h = \frac{d - \left( \frac{a}{2} \right) - \left( \frac{c}{2} \right)}{2} \]
CHAPTER THREE:
PHOTOSYNTHETIC O₂ EVOLUTION AND
RESPIRATORY CONSUMPTION IN D. tertiolecta AND
D. salina AFTER SALT STRESS
3.1. Introduction

The known effects of salt stress and dilution stress on photosynthetic O₂ evolution and respiratory O₂ consumption in *Dunaliella* have been described in Chapter One. There is some uncertainty regarding immediate effects of salt stress on photosynthesis. The burst of rapid O₂ evolution by *D. salina* after an increase in salinity, reported by Kaplan, Shreiber & Avron (1980) and Avron (personal communication to R. McC. Lilley) has not been observed by other researchers. However the other researchers used *D. tertiolecta* and widely varying conditions, thus it is difficult to determine whether the differences observed are due to experimental conditions or to physiological differences between species of *Dunaliella*.

If conditions could be found where this initial burst of photosynthetic O₂ evolution could be consistently demonstrated, then the underlying cause might be expected to indicate some of the cellular processes involved in the response to salinity stress. Moreover, since a further aim of this research project is to find a way of eliciting glycerol synthesis without changing the osmotic pressure of the medium (Chapter 4), the burst in O₂ evolution could be a useful diagnostic aid.

For this reason, the initial part of this project was a comparative study of photosynthetic O₂ evolution and respiratory consumption in *D. tertiolecta* and *D. salina*.

**Aims:**

The primary aim of these experiments, accordingly, was to determine whether there is any fundamental difference in the response of *D. tertiolecta* and *D. salina* to osmotic stress in terms of photosynthetic O₂ evolution. In particular, it was important to establish whether an initial burst of photosynthetic O₂ evolution does occur in *D. tertiolecta* after an increase in salinity.
The secondary aims were to determine the effects of salt stress on mitochondrial respiration, and to investigate influence of buffer composition on these responses. Hepes and phosphate based buffers were compared.

### 3.2. Special procedures.

*D. tertiolecta* were grown in 0.17 M, and 0.6 M NaCl and *D. salina* in 1.5 M NaCl, and harvested after 5 days. Algal suspension (35 ml) was centrifuged and resuspended in 10 ml of Pi or Hepes buffer (Table 2.2, Section 2.2.3.2). Salt stress was applied in light or dark (in the sequences indicated below) by adding appropriate amounts of concentrated NaCl in Pi or Hepes buffer according (Table 2.1, Section 2.2.3.2).

- **Dark** → **Light** → **Salt stress** → **Dark**
- **Light** → **Dark** → **Salt stress** → **Light**

Oxygen evolution and consumption were measured as described (Section 2.2.3.3) in red light with intensity of 250 μE.m⁻².sec⁻¹ for *D. tertiolecta*. For *D. salina*, red or white light with intensity of 700 or 2200 μE.m⁻².sec⁻¹ were used respectively.

### 3.3. Results and discussion.

An example of an O₂ electrode recorder traces from two typical experiments are shown in Figs. 3.1 and 3.2. The rates of photosynthesis and respiration were determined from the slope of the linear sections of the record. No burst of O₂ evolution after salt addition was apparent for *D. tertiolecta* (Fig. 3.1) or for *D. salina* (Fig. 3.2). The significant inhibition of O₂ evolution and consumption following salt stress (from 0.17 M to 1.0 M NaCl) was visible in *D. tertiolecta* (Fig. 3.1).
3.3.1. Buffer effects.

For both *D. tertiolecta* and *D. salina*, no major difference between Hepes and phosphate buffers was apparent with regard to photosynthesis and respiration under any conditions investigated here (Figs. 3.3, 3.4, 3.5, 3.6, 3.7, Table 3.1 and Appendix I Tables 1 - 5). *Dunaliella* accumulates Pi to polyphosphate (Bental *et al*., 1991b), while Hepes is metabolically inert. The results show that the absence of Pi in Hepes buffer is without effect for the duration of the experiment.

3.3.2. Effect of salt stress on photosynthesis.

When illuminated *D. tertiolecta* were subjected to stress from 0.17 M to 1.0 M NaCl (salinity increase factor: 5.88) photosynthesis was inhibited for a few minutes and then recovered to a rate lower than before salt addition (Figs. 3.1, 3.3, Table 3.1 and Appendix I, Table 1). However, smaller salt stresses on illuminated *D. tertiolecta* (transfered from 0.17 M to 0.28 M or 0.6 M to 1.0 M, salinity stress factor: 1.66) increased net and total photosynthesis (Figs. 3.5a, 3.6a and Tables 3.1 and Appendix I, Tables 2 and 3). Thus, although high salt stress inhibited photosynthesis, salt stress of smaller magnitude caused it to increase. In *D. salina* salt stress from 1.5 M to 2.5 M (salinity increase factor: 1.66) had no substantial effect on photosynthesis (Figs. 3.7a, Table 3.1 and Appendix I, Tables 3 and 5). Hence there was a difference in the response of photosynthesis in *D. tertiolecta* and *D. salina* to salinity increases of the same relative magnitude. It is concluded that there may be an additional effect depending on the sodium chloride concentration in the medium in which the alga was adapted.

3.3.3. Effects of salt stress on mitochondrial respiration.

When *D. tertiolecta* adapted to dark were transferred from 0.17 M to 0.28 M, or 0.6 M to 1.0 M NaCl (salinity increase factor: 1.66) respiration decreased by about 10% and 3% respectively (Figs. 3.5b, 3.6b, Table 3.1 and Appendix I, Tables 2 and 3). This
effect was more marked at high salt stress from 0.17 M to 1.0 M (salinity increase factor: 5.88) (Figs. 3.1, 3.4, Table 3.1 and Appendix I, Table 1). However, a substantial increase was observed in *D. salina*, where salt stress (from 1.5 M to 2.5 M) increased respiration (Figs. 3.7b, Tables 3.1 and Appendix I, Tables 4 and 5) by a factor of 70%.

### 3.3.4. Lack of evidence for a burst in O₂ evolution after salt stress.

In *D. salina* there was no occurrence of high rates of O₂ evolution after salt stress in our experimental conditions (Figs. 3.2, 3.7 Table 3.1 and Appendix I, Tables 4 and 5). In *D. salina*, salt stress had only small effects on photosynthetic O₂ evolution. A burst of rapid O₂ evolution after salt stress in *D. salina* from 1.5 M to 2.5 M NaCl under similar conditions (M. Avron, personal communication to R. McC. Lilley) and from 1.5 to 2.1 M (Kaplan, Shreiber & Avron, 1980) has been reported. It is concluded that strain or species differences may be responsible for this difference in photosynthetic response.

### 3.3.5. Effect of photon flux density in *D. salina*.

Since the β-carotene-accumulating *D. salina* grows naturally in high photon flux densities, the effects of applying salt stress under high intensities of white light were determined.

There were no major differences between red light (intensity, 700 µE.m⁻².s⁻¹) and white light (intensity, 2200 µE.m⁻².s⁻¹) on photosynthesis in *D. salina* (Table 3.1 and Appendix I, Tables 4 and 5). This indicates that at the higher intensity white light was not a factor in causing the burst in O₂ evolution. No photoinhibition was observed of this higher photon flux density.
3.3.6. Effects of salt stress on carbon fixation.

The occurrence of a burst in O₂ evolution after a salt stress (Kaplan, Shreiber & Avron, 1980) might suggest that uncoupling of photoelectron transport from photophosphorylation can occur under these conditions. Such uncoupling should cause an inhibition of carbon fixation.

Carbon fixation was measured by ¹⁴C incorporation from ¹⁴CO₂ in to acid-stable compounds (Section 2.2.3.4 and 2.2.3.5). Oxygen evolution was measured simultaneously. The results (Fig. 3.8, and Table 3.2) show that substantial inhibition of carbon fixation did not occur after salt stress for both Hepes and phosphate buffer and that this salt stress had no significant effect (Table 3.2) given the size of the standard deviations.

This salt stress did cause a small increase in photosynthetic O₂ evolution (Table 3.2). The absence of the larger (24%) increase in photosynthetic O₂ evolution in this salt stress (comparison with results of similar salt stress in Table 3.1) may be due to O₂ loss during withdrawal of samples from the O₂ electrode for radioactivity determination.

3.3.7. Comparison of the salinity increase factor and final salt concentration.

Salt stress of equivalent magnitude (salinity increase factor 1.66) caused net photosynthesis to increase by about 25% (Table 3.1) for stresses from 0.17 M to 0.28 M and 0.6 M to 1.0 M NaCl. This suggests that the magnitude of a salt stress is more important than final salt concentration.
Fig. 3.1: Oxygen electrode recorder trace from two simultaneous experiments with *D. tertiolecta*. The rates of photosynthesis and respiration were calculated from the slope of the linear sections in light and dark respectively. (nb. Progress of experiments is from right to left)
Fig. 3.2: **Oxygen electrode recorder trace for D. salina.**

The rates of photosynthesis were calculated from the slope of the linear sections in light.

(nb. Progress of experiments is from right to left)
Fig. 3.3: **Time-course of O\textsubscript{2} exchange by* D. tertiolecta.

Effect of salt stress in light from 0.17 M to 1.0 M NaCl with phosphate or Hepes buffer.

Values are the mean ± S.D of three or four replicates.

Control: addition of an identical volume of medium containing 0.17 M NaCl.

Note: time (X-axis) is approximate. The vertical arrow indicates the addition of salt or equivalent volume of isotonic medium (control).
Fig. 3.4: Time-course of respiratory O₂ uptake by *D. tertiolecta*. Effect of salt stress in light from 0.17 M to 1.0 M NaCl with phosphate or Hepes buffer. Values are the mean ± S.D of three or four replicates. Control: addition of an identical volume of medium containing 0.17 M NaCl. Note: time (X-axis) is approximate. The vertical arrow indicates the addition of salt or equivalent volume of isotonic medium (control).
Fig. 3.5: Net photosynthetic $O_2$ evolution (a) or respiratory $O_2$ consumption (b) by *D. tertiolecta*. Effect of salt stress from 0.17 M to 0.28 M NaCl with phosphate or Hepes buffer. Values are the mean ± S.D of three or four replicates. Control: addition of an identical volume of medium containing 0.17 M NaCl.
Fig. 3.6: Net photosynthetic $O_2$ evolution (a) or respiratory $O_2$ consumption (b) by *D. tertiolecta*. Effect of salt stress from 0.6 M to 1.0 M NaCl with phosphate or Hepes buffer. Values are the mean ± S.D of three or four replicates. Control: addition of an identical volume of medium containing 0.6 M NaCl.
Fig. 3.7: Net photosynthetic $O_2$ evolution (a) or respiratory $O_2$ consumption (b) by *D. salina*. Effect of salt stress from 1.5 M to 2.5 M NaCl with phosphate or Hapes buffer (red light, 700 µE. m$^{-2}$.sec$^{-1}$). Values are the mean ± S.D of three or four replicates. Control: addition of an identical volume of medium containing 1.5 M NaCl.
Fig. 3.8: **Photosynthetic oxygen evolution and $^{14}$C fixation by *D. tertiolecta*.** Effect of salt stress from 0.6 M to 1.0 M NaCl with phosphate (a) or Heps (b) buffer. Data of one experiment..
Table 3.1: Photosynthetic O$_2$ evolution and O$_2$ uptake by *D. tertiolecta* or *D. salina* on control. Effect of three different salt stresses (with phosphate or Hepes buffer). "W": white light.

<table>
<thead>
<tr>
<th></th>
<th>D. tertiolecta</th>
<th>D. salina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>net photosynthesis</td>
<td>total photosynthesis</td>
</tr>
<tr>
<td>0.17 M to 1.0 M</td>
<td>-79</td>
<td>-72</td>
</tr>
<tr>
<td>0.17 M to 1.0 M</td>
<td>-61</td>
<td>-54</td>
</tr>
<tr>
<td>0.17 M to 0.28 M</td>
<td>+26</td>
<td>+7</td>
</tr>
<tr>
<td>0.17 M to 0.28 M</td>
<td>+24</td>
<td>+5</td>
</tr>
<tr>
<td>0.6 M to 1.0 M</td>
<td>+24</td>
<td>+17</td>
</tr>
<tr>
<td>0.6 M to 1.0 M</td>
<td>+25</td>
<td>+26</td>
</tr>
<tr>
<td>1.5 M to 2.5 M</td>
<td>-9</td>
<td>-8</td>
</tr>
<tr>
<td>1.5 M to 2.5 M</td>
<td>-2</td>
<td>+4</td>
</tr>
<tr>
<td>1.5 M to 2.5 M (W)</td>
<td>+9</td>
<td>+18</td>
</tr>
<tr>
<td>1.5 M to 2.5 M (W)</td>
<td>+11</td>
<td>+13</td>
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</table>
Table 3.2: Photosynthetic $^{14}$C fixation and $O_2$ evolution by *D. tertiolecta*. Effect of salt stress from 0.6M to 1.0M NaCl with phosphate or Hepes buffer. Values are the mean ± S.D of two or three replicates. Control: addition of an identical volume of medium containing 0.6M NaCl. The rate of $^{14}$C fixation was determined simultaneously with $O_2$ evolution measurement in an $O_2$ electrode.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Condition</th>
<th>Rate of $^{14}$C fixation (nmol $^{14}$C·min$^{-1}$·mg$^{-1}$ chl)</th>
<th>% change</th>
<th>% change on control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before stress</td>
<td>after stress</td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Control</td>
<td>632 ±261</td>
<td>614 ±286</td>
<td>-3</td>
</tr>
<tr>
<td>Pi</td>
<td>Stress</td>
<td>660 ±207</td>
<td>625 ±204</td>
<td>-5</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>500 ±197</td>
<td>466 ±193</td>
<td>-7</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>500 ±100</td>
<td>475 ±142</td>
<td>-5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Condition</th>
<th>Rate of $O_2$ evolution (nmol $O_2$·min$^{-1}$·mg$^{-1}$ chl)</th>
<th>% change</th>
<th>% change on control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before stress</td>
<td>after stress</td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Control</td>
<td>890 ±265</td>
<td>854 ±251</td>
<td>-4</td>
</tr>
<tr>
<td>Pi</td>
<td>Stress</td>
<td>810 ±290</td>
<td>850 ±185</td>
<td>+5</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>823 ±139</td>
<td>872 ±98</td>
<td>+6</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>883 ±198</td>
<td>1033±83</td>
<td>+17</td>
</tr>
</tbody>
</table>
CHAPTER FOUR:
MANIPULATION OF GLYCEROL CONTENT OF *DUNALIELLA*
BY ELECTROPORATION.
4.1. Introduction:
Glycerol is the major intracellular solute of *Dunaliella*, when growing in high salt concentration (Section 1.7.3). Adaptation of *Dunaliella* to changing osmotic pressure of the surrounding medium involves changes in cell volume and internal glycerol concentration. These changes are made by alterations to glycerol metabolism (Section 1.7, and Fig. 1.2). The mechanism by which the intracellular concentration of glycerol is regulated is unknown. Much research has been directed towards identifying the factor or factors involved in glycerol metabolism (Section 1.8). To a large degree this has been based on studying the effect of changing the salinity or some other solute to alter the osmotic pressure of the medium. This general approach has provided valuable information but is unable to distinguish between the direct effects of external salinity or osmotic pressure changes from indirect effects resulting from consequent physiological changes in the cells (e.g. changed cell volume). Investigation of the linkage between cell volume and glycerol synthesis while the effect of external osmotic pressure changes were eliminated, seemed necessary. Here an alternative approach is attempted to achieve this. This required the development of a technique to modify the glycerol content of the cells without changing the osmotic pressure of the medium. The electroporation technique was selected and developed to lower the glycerol content of cells by a small proportion with minimal cell damage. Electroporation induces the transient formation of pores in the plasma membrane, which allow the passage of a range of molecules (Saulis, Venslauskas & Naktinis, 1991), without disturbing the integrity of the intracellular organelles (Knight, 1981) (Section 1.9).

Aims:
1. To find conditions for electroporation which allow a small but measurable release of glycerol with cell survival rates of near 100% at constant external osmotic pressure. The variable parameters investigated include: medium composition, voltage of the electric field, pulse duration, number of pulses.
2. To utilise electroporation as a tool to study the effects of such glycerol release on cell volume and glycerol synthesis in *Dunaliella*.

**4.2. Special procedures.**

To have the substantial intracellular glycerol concentration necessary for this investigation, *D. tertiolecta* was grown at an NaCl concentration of 600 mM NaCl. Goyal, Lilley & Brown (1986b) showed that when *D. tertiolecta* cells were grown in media containing 170 mM NaCl and subjected to increases in salinity, the rate of glycerol synthesis was at a maximum at final concentrations between 400 mM and 700 mM NaCl. After 5 days growth, 35 ml of algal suspension (5-6 mg chl . ml⁻¹) was centrifuged and resuspended in 3.5 ml of isotonic electroporation medium (Section 2.2.4.3). In preliminary experiments (Figs. 4.1, 4.2) solutes other than mannitol were used.

**4.3. Results**

**4.3.1. Selection of Electroporation medium, Centrifugation and Resuspending conditions**

The electroporation medium chosen initially contained 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 6.0 mM CaCl₂, 5.0 mM NaHCO₃ (pH 8.1), similar to that used for electroporation by Taylor & Larkin (1988) (described in Section 2.2.4.2). When algal suspensions were centrifuged and resuspended in this medium, the time taken to completely resuspend was about 10 minutes of vigorous agitation. When the glycerol release due to resuspending was determined, it was found to be more than 5% of total glycerol. A background of this magnitude, in non-electroporated cells, would make it difficult to accurately characterise release due to electroporation of 10 to 20% of total glycerol. Accordingly, other media of osmolality equal with that of growth medium were investigated (Fig. 4.1 and 4.2). The time required to completely
Fig. 4.1: Time taken to completely resuspended the algal pellet in the experimental medium, for five different isotonic media. The osmolality of each medium was 1180 ± 10 mmol . kg$^{-1}$ and the pH was 8±0.1. Values are the mean ± S.D of three replicates in A,B,C,E. and a single determination in D. Conditions: 35 ml of *D. tertiolecta* suspension adapted to 600 mM NaCl, centrifuged at 648 x g, 25 °C, for 5 minutes and resuspended in 3.5 ml of isotonic medium.

Media composition:
A: 600 mM NaCl, 0.5 mM Hepes-Cl, 0.5 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
B:170 mM NaCl, 490 mM Hepes, 190 mM Btp, 0.2 mM CaCl$_2$,
5.0 mM NaHCO$_3$.
C: 500 mM Mes, 500 mM Amp, 5.0 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
D: 1.1 M K$^+$-glycine, 5.0 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
E: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 5.0 mM CaCl$_2$,
5.0 mM NaHCO$_3$. 
Fig. 4.2: Proportion of glycerol released to the experimental medium. Comparison of four different isotonic media. The osmolality of each medium was $1180 \pm 10 \text{ mmol} \cdot \text{kg}^{-1}$ and the pH was $8 \pm 0.1$. Conditions: 35 ml of *D. tertiolecta* suspension adapted to 600 mM NaCl centrifuged at $648 \times g$, 25 °C, for 5 minutes and resuspended in 3.5 ml of isotonic medium. Values are mean $\pm$ Std. Error of 2 replicates for A, C, 6 for B, and 10 for D. 100% represents 19.5 μmol glycerol $\cdot \text{mg}^{-1}$ chl.

**Media composition:**
A: 600 mM NaCl, 0.5 mM Hepes-Cl, 0.5 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
B: 170 mM NaCl, 490 mM Hepes, 190 mM Btp, 0.2 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
C: 500 mM Mes, 500 mM Amp, 5.0 mM CaCl$_2$, 5.0 mM NaHCO$_3$.
D: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 5.0 mM CaCl$_2$, 5.0 mM NaHCO$_3$. 
resuspend the pellet, and the proportion of intracellular glycerol released were determined and compared for media containing mannitol and 600 mM NaCl.

The time taken to completely resuspend the cells from a pellet (Fig. 4.1) in electroporation medium containing 730 mM mannitol (E) (about 10 minutes) is double that for the Hepes medium containing 600 mM NaCl (A). This was similar to that of B and C media while the cell pellet resuspension in the D medium containing 1.1 M K⁺-glycine took more than an hour. Thus for further experiments, the medium containing 1.1 M K⁺-glycine was deleted. Resuspension was fastest in the 600 mM NaCl medium. When the proportion of glycerol released during resuspension in these media was measured (Fig. 4.2), 4% of the total glycerol was found in the 600 mM NaCl medium (A) and the medium containing mannitol (D). More than 6% of glycerol was released for medium B and C. Algal suspensions were centrifuged at four different relative centrifugal forces at 5 °C and 25 °C and resuspended in the medium containing mannitol (Fig. 4.3). At 25 °C there was little change in glycerol released from 40 to 161 x g (about 4%). At 648 x g, glycerol release increased to 5.5% of total glycerol. When cells were centrifuged at 5 °C, glycerol release due to resuspending was about more than 10% with no differences between the four different centrifugation forces. On this basis, for further experiments 161 x g at 25 °C was selected for harvesting algal suspensions. From the results (Figs. 4.1 and 4.2), the medium containing mannitol was selected as the most suitable low NaCl medium for subsequent experiments.

**4.3.2. Electroporation Conditions**

Preliminary experiments were done with aluminium electrodes (Taylor & Larkins 1988). It was found that electroporation in *D. tertiolecta* caused glycerol release but cell aggregation and loss of motility of the cells was also observed. When 6.0 mM EDTA was added before or after electroporation the cell aggregation was prevented.
Fig. 4.3: Proportion of glycerol released to the experimental medium. Effect of two different centrifugation temperatures at four different relative centrifugal forces.
Values are the mean ± Std. Error of two replicates. Conditions: 35 ml algal suspension centrifuged and pellet resuspended in the 3.5 ml of isotonic electroporation medium: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 5.0 mM CaCl₂, 5.0 mM NaHCO₃. 100% represents 20.4 μmol glycerol . mg⁻¹ chl.
Loomis-Husselbee *et al.* (1991) showed that during electroporation, Al\textsuperscript{3+} was released from aluminium electrodes. To check this, the amount of Al\textsuperscript{3+} released to the medium by electroporation was determined (Table 4.1). Measurable concentrations of Al\textsuperscript{3+} were released, for example 92.5 \(\mu\text{M}\) by two pulses at 300V and 100 ms duration.

Table 4.1. **Release of Al\textsuperscript{3+} from aluminium electrodes to the experimental medium by electroporation.**

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Pulse duration (ms)</th>
<th>number of pulses</th>
<th>Al\textsuperscript{3+} concentration in the medium ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No electroporation</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300</td>
<td>100</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td>300</td>
<td>100</td>
<td>2</td>
<td>92.5</td>
</tr>
<tr>
<td>300</td>
<td>50</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

To check the possibility of effects of Al\textsuperscript{3+} on glycerol metabolism of *D. tertiolecta* cells, salt stress was imposed in the presence of Al\textsuperscript{3+} (data not shown). These experiments showed that the rate and extent of glycerol synthesis after an increase in salinity from 0.6 M to 1.0 M, was decreased in the presence of 100 \(\mu\text{M}\) AlCl\textsubscript{3}. For this reason, the electrodes were changed from aluminium to copper, gold-plated to a thickness of 5 \(\mu\text{m}\).

Fig. 4.4 shows the effect on cells of electroporation or adding 100 \(\mu\text{M}\) AlCl\textsubscript{3}. When 100 \(\mu\text{M}\) EDTA was added to medium following electroporation, cell aggregation was mostly prevented. When electroporation was performed with copper, gold-plated electrodes no cell aggregations were observed. So for all subsequent work the copper, gold-plated electrodes were used.
Fig. 4.4: **Comparison of electroporation with aluminium and gold electrodes and AlCl₃.**

Conditions: 1 ml of algal suspension electroporated at five 300 V pulses, for 50 ms pulse duration at 2 °C. The following were added where indicated: 100 μM AlCl₃, 100 μM EDTA. Electroporation medium contained: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 6.0 mM CaCl₂, 5.0 mM NaHCO₃ (Electroporation electrodes and conditions as in Section 2.2.4.1)
4.3.3. Electroporation

The variable parameters for electroporation were pulse voltage, pulse duration, and the number of pulses with 4 sec. between each pulse. The time-course of voltage output of the electroporation apparatus when not connected to the electrodes was measured by a digital storage oscilloscope (Fig. 4.5). The time-course was also measured when connected to electrodes immersed in electroporation medium (Fig. 4.6).

The output of the equipment approximated a square wave and was close to the set voltage (Fig. 4.5). However, when connected to the immersed electrodes, for a set voltage of 250V, the initial potential was 226V, decaying approximately linearly to about 150V after 50 ms. For a set of 300V, it was 264, decaying to about 180V (Fig. 4.6). In the following, the set voltage rather than actual output are quoted.

Every electroporation experiment was accompanied by a control experiment in which the alga was subjected to identical treatment with the exception that electroporation was omitted. Control suspensions contained 3-6 % of total glycerol in the medium. Following electroporation, the cell suspensions were fractionated and glycerol determined enzymically (Section 2.2.5.2). An example of the calculation of the proportion of glycerol released is given in Table 4.2 (see also Section 2.2.5.3).

The proportion of intracellular glycerol released to the medium by a range of electroporation conditions is explored in Figs. 4.7, 4.8 and 4.9. When the set pulse voltage and duration increased, the amount of glycerol released increased (Figs. 4.7, 4.8). In all cases when the number of pulses increased, the amount of glycerol released increased, depending on the voltage and pulse duration. Increased set voltage (Fig. 4.7) and increased pulse duration (Fig. 4.8) both caused larger amounts of glycerol to exit the cells. When 6.0 mM CaCl₂ was omitted from the electroporation medium, a similar relationship was observed (Fig. 4.9). In the absence of added Ca²⁺,
Fig. 4.5: Time-course of the output potential for a set 300 V for 50 ms from the electroporator. The output was measured using a digital storage oscilloscope. Each vertical graduation represents 50 V. Each horizontal graduation represents 10 ms.
Fig 4.6: **Time-course of potential across the electrodes when submerged in 1 ml of electroporation medium.** Set voltage of 250 V (upper figure) or 300 V (lower figure) for 50 ms. Each vertical graduation represents 50 V. Each horizontal graduation represents 10 ms.
Table 4.2: **Example of determined volumes and glycerol content of control and electroporated *D. tertiolecta* suspensions following dilution with Hepes buffer.**

Electroporation was done with 2 pulses, 300 V, 50 ms duration in medium with CaCl₂ omitted. Alternative calculations based on two different estimations of cell volume on a chlorophyll basis.

<table>
<thead>
<tr>
<th>Intracellular volume on chlorophyll basis:</th>
<th>125 µl . mg⁻¹ chl</th>
<th>28 µl . mg⁻¹ chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Electroporated</td>
</tr>
<tr>
<td>sample chlorophyll concentration (mg . ml⁻¹)</td>
<td>0.084</td>
<td>0.081</td>
</tr>
<tr>
<td>sample volume (Vt, µl)</td>
<td>993</td>
<td>960</td>
</tr>
<tr>
<td>pellet fraction volume (Vps, µl)</td>
<td>286</td>
<td>250</td>
</tr>
<tr>
<td>intracellular volume (Vi, µl)</td>
<td>10.42</td>
<td>9.72</td>
</tr>
<tr>
<td>extracellular volume (Ve, µl)</td>
<td>983</td>
<td>950</td>
</tr>
<tr>
<td>remaining supernatant and pellet fraction glycerol content (Gps, nmol)</td>
<td>1,729</td>
<td>1,522</td>
</tr>
<tr>
<td>extracellular glycerol content (Ge, nmol)</td>
<td>79</td>
<td>189</td>
</tr>
<tr>
<td>extracellular glycerol concentration ([Ge], µM)</td>
<td>80</td>
<td>199</td>
</tr>
<tr>
<td>intracellular glycerol content (Gi, nmol)</td>
<td>1,707</td>
<td>1,474</td>
</tr>
<tr>
<td>proportion of glycerol extracellular Ge x 100 (%)</td>
<td>4.4</td>
<td>11.3</td>
</tr>
<tr>
<td>glycerol extracellular due to electroporation (%)</td>
<td>—</td>
<td>6.9</td>
</tr>
</tbody>
</table>
Fig. 4.7: Proportion of glycerol released from *D. tertiolecta* cells to the external medium by electroporation. Effect of pulse voltage and number of pulses.

Conditions: 1 ml of algal suspension electroporated at 200, 250 or 300 V with 20, 50 or 100 ms pulse duration at 2 °C. Electroporation medium: 150 mM NaCl, 730 mM mannitol, 30m M Tris-Cl, 6.0 mM CaCl₂.

100% represents 22.7 µmol glycerol·mg⁻¹ chl.
Fig. 4.8: Proportion of glycerol released from *D. tertiolecta* cells to the external medium by electroporation. Effect of pulse duration and number of pulses. Conditions: 1 ml of algal suspension electroporated at 200, 250 or 300 V with 20, 50 or 100 ms pulse duration at 2 °C. Electroporation medium: 150 mM NaCl, 730 mM mannitol, 30 mM Tris-Cl, 6.0 mM CaCl₂.

100% represents 22.7 μmol glycerol . mg⁻¹ chl.
Fig. 4.9: Proportion of glycerol released from *D. tertiolecta* cells to the external medium by electroporation. Effect of omission of 6.0 mM Ca^{2+}. Conditions: 1 ml algal suspension electroporated at 300 or 250 Volts and 50 ms pulse duration at 2 °C. Electroporation medium: 150 mM NaCl, 763 mM mannitol, 30 mM Tris-Cl or plus or minus 6.0 mM CaCl_{2}. 100% represents 22.7 μmol glycerol . mg^{-1} chl.
the amount of glycerol released was higher than when CaCl₂ present. For example, in
the electroporation medium containing 6.0 mM CaCl₂, 20% of glycerol was released
following five 300V pulses delivered. In the absence of CaCl₂ the same amount of
glycerol was released by three 300V pulses. There was also a linear relationship
between the number of 50 ms pulses (3-7 at 250V, 2-5 at 300V) and the amount of
glycerol released when CaCl₂ were omitted.

The release of a small proportion of intracellular glycerol could be due to: (a) total
rupture of some small proportion of the cells that are affected by electroporation with
the remaining cells unaffected, (b) release of a similar proportion of glycerol by all
cells, (c) some combination of (a) and (b). To investigate this, in parallel with the
release of glycerol, the amount of soluble protein released was also determined. When
intact cells were electroporated in the medium without CaCl₂ (Fig. 4.10), the
proportion of soluble protein released was much smaller than for glycerol. For
example, two pulses released 11% of glycerol, but only 1.4% of the soluble protein.

To investigate the effect of electroporation on metabolism in *D. tertiolecta* cells, the
effects of electroporation on photosynthesis and respiration were measured in medium
without 6.0 mM CaCl₂. As shown in Fig. 4.11, no substantial inhibition of the rate of
O₂ evolution in the light (net photosynthesis) or of O₂ consumption in the dark
(mitochondrial respiration) was observed following the delivery of up to four pulses.
However, the delivery of five 300V pulses (50 ms duration), which caused the release
of 50% of the glycerol (Fig. 4.9), resulted in major inhibition of both net
photosynthesis and respiration. Mannitol and Na⁺ uptake were determined following
the delivery of two 300V pulses for 50ms duration. To do this, ¹⁴C-mannitol and ²²Na
were included in the medium in separate experiments. Following three washes (which
reduced the ¹⁴C and ²²Na content of the controls to background level), the amount of
¹⁴C-mannitol remaining in electroporated cells was about 0.28 μmol . mg⁻¹ chl (Table
4.3). For ²²Na, the amount remaining was 0.03 μmol . mg⁻¹ chl. Under these
Fig. 4.10: Comparison of intracellular soluble protein and glycerol release from *D. tertiolecta* with increasing number of electroporation pulses at 300 V and 50 ms duration. The values represent % of the soluble protein and glycerol released from totally ruptured cells (Section 2.2.6). Electroporation medium: 150 mM NaCl, 763 mM mannitol, 30 mM Tris-Cl. 100% represents 20.5 µmol glycerol . mg⁻¹ chl or 15.7 mg protein . mg⁻¹ chl.
Fig. 4.11: Photosynthetic O\textsubscript{2} evolution and O\textsubscript{2} consumption by *D. tertiolecta* following electroporation. Effect of electroporation pulse number (300 V and 50 ms duration per pulse) on O\textsubscript{2} evolution in the light (positive bars) and consumption in the dark (negative bars).

Conditions: 1 ml algal suspension electroporated at 2 °C in electroporation medium (150 mM NaCl, 763 mM mannitol, 30 mM Tris-Cl), diluted with 6 ml of Hepes medium (Table 2.3, Section 2.2.4.2), before photosynthetic O\textsubscript{2} evolution or consumption were measured (Section 2.2.3.3).
conditions, the amount of glycerol lost to the medium due to electroporation was typically was 1.55 μmol . mg⁻¹ chl.

Table 4.3: **Comparison of $^{14}$C-mannitol and $^{22}$Na uptake with glycerol release following electroporation (uptake by control deducted).**

Values are mean±S.D of two experiments. Conditions: 1 ml algal suspension electroporated with 2 pulses at 300 Volts, 50ms pulse duration at 2 °C and then centrifuged and washed three times with Hepes medium. Electroporation medium: 150 mM NaCl, 763 mM mannitol, 30 mM Tris-Cl.

<table>
<thead>
<tr>
<th>Uptake or release due to electroporation (μmol . mg⁻¹ chl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-mannitol uptake</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>$^{22}$Na uptake</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Glycerol release</td>
<td>1.55 ± 0.13</td>
</tr>
</tbody>
</table>

**4.4. Discussion:**

A medium containing a low concentration of NaCl (150 mM) with the balance of osmotic pressure provided by mannitol (initially 730 mM) was developed. Electroporation in this medium released a small proportion of the glycerol content of *D. tertiolecta* cells. The NaCl concentration of 150 mM is optimal for electroporation of plant cells for DNA uptake (Taylor & Larkin, 1988). A medium containing 600 mM NaCl has a high conductivity which would cause the current flow to be around, rather than through the cells. Mannitol is a non-electrolyte and was chosen as a relatively inert sugar which is compatible with electroporation (Taylor & Larkin, 1988).

The use of mannitol as the main osmoticum was compared with Hepes-Cl, Hepes-BTP, Mes-Amp and K⁺-glycine. Each of these buffers was present in amounts that gave the correct osmolality and pH. In terms of allowing easy pellet resuspension (Fig. 4.1) and minimising glycerol release during centrifugation and resuspension (Fig.
4.2), the medium containing mannitol as osmoticum was clearly superior to the media containing buffers as osmotica.

There was a marked effect of temperature on the release of glycerol during centrifugation and resuspension. The cells seems to be more fragile at 5°C. For all subsequent studies, the cells were harvested at 25 °C and at a relative centrifugal force of 161 x g.

The calculation of the proportion of intracellular glycerol released during electroporation included the assumption that the intracellular volume of *D. tertiolecta* is 125 μl . mg⁻¹ chl (Marengo, Brown & Lilley, 1989). In fact, this value is not constant during the experiment. Following the release of intracellular glycerol during electroporation, a decrease in intracellular volume will be expected (this will discussed in the Chapter 6). This inaccuracy, however, is trivial. Altering the value for intracellular volume ± 20% changes the calculated value of Gi, Ge and the proportion of extracellular glycerol by less than 1%.

The value of 125 μl. mg⁻¹ chl was determined for intracellular volume by centrifugation in a haematocrit tube (Marengo, Brown & Lilley 1989). This is likely to be an overestimation (Section 1.5.2.2). This value affects the calculated intracellular volume (Vi) substantially but has very little effect on the calculated proportion of extracellular glycerol. If, for example in Table 4.2, a value of 25 μl . mg⁻¹ chl is taken (i.e., a value smaller by a factor of 5) the proportion of extracellular glycerol increases slightly to 4.5% for the control and 11.4 % for the electroporated cells, while the % of glycerol which is extracellular due to electroporation remains unchanged (6.9%).

From measurements of cell density (Section 2.2.2.2) and chlorophyll concentration (2.2.2.1) made simultaneously on suspensions of *D. tertiolecta* during this project, it is
possible to calculate an average value for the chlorophyll content per cell. This value is 6.1 pg chlorophyll per cell. From the measurements of cell volume by video microscopy (Section 2.2.9.2.2, and 2.2.9.3), a typical value for the average cell volume is 168 µm³ (Section 6.3 and Table 6.2). Combining these two figures gives an estimation of cell volume on a chlorophyll basis of 28 µl . mg⁻¹ chl.

When this estimate of 28 µl . mg⁻¹ chl for *D. tertiolecta* is adopted for the calculation of glycerol release, instead of the value from Marengo, Brown & Lilley (1989), it has little effect on the proportion of glycerol released (as discussed in the foregoing). However, if an attempt is made to calculate the intracellular concentration of glycerol from the data (e.g. Table 4.2), the accuracy of Vi becomes important.

Using the data in Table 4.2 for Vi and Gi, the intracellular concentration of glycerol can be calculated as 164 mM in control cells. This value seems low for *D. tertiolecta* growing in medium containing 600 mM NaCl and having an osmolality of 1189±10 mmol . kg⁻¹, since glycerol is regarded as the major solute in these cells (Section 1.7.3). If, on the other hand, the value of 28 µl . mg⁻¹ chl is applied to the data of Table 4.2, Vi is reduced from 10.4 µl to 2.33 µl. On the basis of this value, the intracellular glycerol concentration becomes 732 mM. This value might be expected on the basis that glycerol is the major solute. It is interesting to note that the volume of the chloroplast stroma in a higher plant (spinach) has been estimated at the comparable value of 25 µl . mg⁻¹ chl (Heldt & Sauer, 1971).

Loomis-Husselbee et al. (1991) showed that release of Al³⁺ during electroporation of mouse lymphoma cells stimulated conversion of inositol 1,3,4,5-tetrakisphosphate into inositol 1,4,5-trisphosphate (Ins P₃) (see also Section 1.8.9).

Our results also showed release of Al³⁺ of similar magnitude. In our initial experiments, electroporation of *D. tertiolecta* was accompanied by aggregation of the
cells and loss of their motility. Moreover, we found that Al\textsuperscript{3+} affected the glycerol synthesis of *D. tertiolecta* cells following salt stress. After we changed the electrodes to gold-plated copper, no cell aggregation and no loss of motility were observed after electroporation. The use of inert gold-plated electrodes is advisable for any study where effects on metabolism or on cell physiology should be minimised.

The semi-linear relationship between the number of pulses and proportion of intracellular glycerol released in absence of added CaCl\textsubscript{2} (Fig. 4.9) indicates one of the following alternatives: (a) Each sequential pulse caused the formation of new pores in a fresh area of the plasmalemma because the cells are motile and their rotation changes the orientation of each cell to successive applied fields. Hence, the number of open pores increase with each pulse. (b) The pore resealing time is less than the time delay between each pulse (approximately 4 sec). Hence, the number of open pores is constant but very short-lived. To distinguish between these two possibilities, it would be necessary to know the average time that pores remain open after a single electroporation pulse. We have no information on this for *D. tertiolecta*. With other types of the cell, estimates of the pore resealing time vary widely from milli-seconds to hours (Saulis, Venslauskas & Naktinis, 1991, also see Section 1.9).

Our experiments also do not give any information about the size of the pores formed in the plasmalemma of *D. tertiolecta*. However, the results do show that the pore size is large enough to permit the release of glycerol.

The amount of solute crossing the cell membrane might be expected to depend on both the relative size of the solute molecule and pore diameter and on the concentration gradient across the membrane. For mannitol, the concentration gradient was about 763 mM assuming there was initially no mannitol in the cells. For glycerol, the intracellular glycerol content was about 733 mM (see above), hence the concentration gradient for both solutes was of the same magnitude. On the basis of an intracellular
volume of 28 \mu l . mg^{-1} chl, the intracellular mannitol concentration (after electroporation) was 10 mM. Considering this, it seems likely that the much smaller uptake of mannitol may be the result of size discrimination, i.e. that the size of the pores formed by electroporation is not much larger than the mannitol molecule and that mannitol influx was restricted. Otherwise, the equilibration of mannitol with the internal medium of the cells would be expected.

In plant protoplast membranes, CaCl$_2$ at 4-6 mM, but not MgCl$_2$, causes a phase transition. Ca$^{2+}$ is generally included in media for electroporation of plant cells for DNA uptake (Taylor & Larkins, 1988). As shown in Fig. 4.9, the release of glycerol is reduced by the presence of 6.0 mM CaCl$_2$ in the electroporation medium. In living cells the level of free Ca$^{2+}$ ion in the cytosol is commonly kept below 0.2 \mu M. Any small increase in the level of cytosolic Ca$^{2+}$ has major effects on metabolism (Darnel, Lodish & Baltimore, 1990) (Section 1.8.9). To prevent the possibility of uptake of Ca$^{2+}$ to \textit{D. tertiolecta} cells during electroporation, CaCl$_2$ was omitted from the medium. In Chapter 5, glycerol synthesis following electroporation will be studied in both the presence or absence of CaCl$_2$.

The very low release of protein by electroporation, compared to glycerol (Fig. 4.10), indicates that the glycerol appearing in the medium following electroporation is not due to the total disruption of a small proportion of cells. Of that protein released, some may originate from the extracellular matrix, since Oliveira, Bisalputra & Antia (1980) reported that \textit{D. tertiolecta} releases glycoprotein to the medium constantly.

There was no inhibition of net photosynthesis and respiration (Fig. 4.11) by electroporation up to four pulses where 35% of intracellular glycerol was lost from the cells (Fig. 4.7). This indicates that electroporation (in medium without CaCl$_2$) at fewer than four pulses (300 V, 50ms) should have little effect on metabolism. Other combinations of voltage, pulse number and duration causing the release of smaller
proportions of glycerol might also be expected not to affect metabolism greatly. When five 300 V pulses of 50 ms were delivered, major inhibition of photosynthesis and respiration with loss of more than 50% of the intracellular glycerol occurred (Fig. 4.7), suggesting significant disturbance to the cells.

The amount of mannitol (the major component of the electroporation medium) taken up by electroporation was less than 20% of glycerol release (Table. 4.3). Mannitol is a compatible solute (Davison & Reed 1985) and the entrance of such amounts of mannitol may not affect significantly the metabolism of the cells. Once taken up, the mannitol could contribute to intercellular osmolality for adjustment of the intracellular osmotic pressure. When similar techniques were used for $^{22}\text{Na}$ uptake, the amount of $^{22}\text{Na}$ take-up was very low. Although the concentration of Na$^+$ in the medium was low (150 mM) compared with mannitol, it seems that this technique is unsuitable to determine the actual uptake of Na$^+$. Cellular Na$^+$ may be exchanged with the medium more rapidly than the washing procedure. On the other hand, in *Dunaliella* photosynthesis is strongly inhibited by the presence of intracellular Na$^+$ and Cl$^-$ (Gilmour, Hipkins & Boney, 1984a; Krist, 1989). If significant amounts of Na$^+$ and Cl$^-$ entered the cells during electroporation (at two pulses of 300V for 50ms duration) inhibition of photosynthesis would be expected. The lack of inhibition of photosynthesis (Fig. 4.11) after the first few minutes (the time needed for transfer electroporated cell suspensions to the $O_2$ electrode) following electroporation suggests that there was no massive influx of Na$^+$ and Cl$^-$ to *D. tertiolecta* cells.

### 4.5. Conclusions

In conclusion, the results show that under specific conditions, electroporation can be used for lowering the intracellular glycerol by a small proportion without significant structural or metabolic disturbance to the cells. Moreover, the intracellular glycerol content was lowered while the osmotic pressure of the external medium remained essentially constant. The extracellular volume ($V_e$) is greater than the intracellular
volume or about 400 based on the lower value) (Table 4.2). Hence any solute lost from the cell will have a trivial effect on the external osmotic pressure. Thus, electroporation can be used as a tool for study of the linkage between external osmotic pressure and glycerol synthesis.
CHAPTER FIVE:
THE TIME-COURSE OF GLYCEROL CONTENT AFTER ELECTROPORATION:
EVIDENCE FOR NET SYNTHESIS OF GLYCEROL AT CONSTANT OSMOTIC PRESSURE.
5.1. Introduction

The response of *Dunaliella* cells to changing osmotic pressure of the medium has been described in Section 1.7 and Fig. 1.2. In previous work, glycerol synthesis has been extensively studied following changes to salinity (or osmotic pressure) of the medium (Chapter 4). This work has given much information about the regulation of glycerol synthesis, but always involved changing the external osmotic pressure. The electroporation technique has been selected and optimised for artificially lowering the glycerol content of *Dunaliella* cells (Section 4.5). Here, the linkage between glycerol content and the regulation of glycerol synthesis was studied at constant osmotic pressure. The time course of glycerol synthesis (at constant osmotic pressure of the medium) following loss of glycerol by electroporation has been determined to answer the following question: Does the regulatory mechanism that sets the glycerol content of the *Dunaliella* cells sense external osmotic pressure *per se*?

5.2. Special procedures.

*D. tertiolecta* cells were grown at 600 mM NaCl. After five days 35 ml algal suspension were harvested by centrifugation at 161 x g at 25°C for 6 minutes (Section 4.3.1 or 2.2.4.3) and resuspended in the 3.5 ml isotonic electroporation medium (2.2.4.2) with or without added 6.0 mM CaCl₂, or with 50 μM EDTA. The resuspended algae (1.0 ml) were electroporated (Section 2.2.4.3) and the time-course of the glycerol content determined (Section 2.2.5.2) and compared with that of non-electroporated controls.

In some cases Student's t-test (independent) was used for comparing the total glycerol in the culture or the intracellular glycerol content of electroporated cells and control cells at zero-time and at subsequent 30 minute intervals. In other cases, the rate of change of glycerol content was compared between electroporated and control cells. The fitted linear slopes of the time-course were compared using JMP software on a Macintosh computer.
This statistical comparison was done for both intracellular and total glycerol content, based on the equation (see also Appendix II):

\[
t = \frac{\text{control slope} - \text{electroporated slope}}{\sqrt{\text{(Std.Error, control slope)}^2 + \text{(Std.Error, electroporated slope)}^2}}
\]

5.3. Results

The conditions selected for electroporation were two or three 300V pulses for 50ms duration with Ca\(^{2+}\) omitted from electroporation medium (Section 4.3.1). But, for study of possible effects of Ca\(^{2+}\), a set of experiments was performed in medium containing 6.0 mM CaCl\(_2\), delivering 5 to 9 pulses of 250V or 300V for 50 ms duration.

5.3.1. Electroporation in medium containing 6.0 mM CaCl\(_2\)

When 5 or 6 pulses of 250V for 50 ms were applied, the amount of glycerol inside the cells decreased, while the total amount of glycerol in the cell suspensions was unchanged (Fig. 5.1). Following electroporation, during the subsequent incubation, intracellular glycerol did not return to the control level and total glycerol remained similar to the control. When the number of pulses increased to seven (Fig. 5.2), variable results were obtained. However, after performing many repeat experiments, it became clear that the results fell into three categories. Category (a) shows similar results to those of when 5 or 6 pulses applied (Fig. 5.2a). Category (b) shows an increase of total glycerol following electroporation above the control while intracellular glycerol content did not recover. In category (c), the intracellular glycerol content recovered during the 120 minutes time-course, accompanied by increase in the total amount of glycerol for electroporated cells. It should be noted that most of the results were category (a), while results (b) and (c) were from one experiment each and they were not repeatable. When a large amount of glycerol (70%) was lost to the
Fig. 5.1: **Time-course of glycerol content in control and electroporated cell suspensions.**
Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). (a) single experiment. (b) mean ± Std. Error of three replicates. Conditions: electroporation at 2 °C with 5 pulses (a) or 6 pulses (b), 250 V and 50 ms pulse duration. Electroporation medium included 6.0 mM CaCl₂. 100% represents 24.0(a) and 26.0 (b) μ mol glycerol . mg⁻¹ chl.
Fig. 5.2: **Time-course of glycerol content in control and electroporated cell suspensions.**

Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). Comparison of three categories of results. (a) mean ± Std. Error of five replicates. (b and c) single experiments.

Conditions: electroporation at 2 °C with 7 pulses, 250 V and 50 ms pulse duration. Electroporation medium included 6.0 mM CaCl₂.

100% represents 20.3(a), 23.2(b), 28.6(c) μ mol glycerol . mg⁻¹ chl.
medium by the application of nine pulses (Fig. 5.3) the total glycerol content during incubation remained lower than the control, showing that glycerol synthesis was inhibited. When 5 pulses of 300V for 50ms duration were applied, two categories of results were obtained (Fig. 5.4). The types of response in Fig. 5.4a and b were similar to Fig. 5.3a and c (where 7 pluses of 250V-50ms were delivered) respectively.

5.3.2. Electroporation in media without added CaCl$_2$.

For this series of experiments, Ca$^{2+}$ was deleted from the medium and two or three pulses of 300V for 50ms were delivered. With two pulses, the results show (Fig. 5.5a) that the amount of glycerol inside the cells decreased significantly after electroporation compared to the control ($p<0.05$, independent t-test). However the total amount of glycerol in the suspension was unchanged immediately after electroporation. During the subsequent incubation, the amount of intracellular glycerol returned to a level not significantly different to the control and similar to that before electroporation. The amount of total glycerol also increased. When 50 μM EDTA was added to the electroporation medium to bind any trace of Ca$^{2+}$ which might be present as an impurity, a basically similar result was obtained (Fig. 5.5b). In this case, the intracellular glycerol also decreased significantly ($p<0.05$, independent t-test) but recovered to the control level after 30 minutes. For these two sets of experiments, the slope of total glycerol content with time in the control and electroporated cells was compared statistically (Section 5.2). This was also done for intracellular glycerol. The results (Table 5.1) show that the rate of total glycerol increase (Fig 5.5a) was significantly ($p<0.05$) higher than the control. The rate of increase of intracellular glycerol was also significantly ($p<0.05$) higher than the control. When 50 μM EDTA was included (Fig. 5.5b), the rate of total ($p<0.01$) and intracellular ($p<0.05$) glycerol increase were again higher than in the control. After three pulses were applied (Fig. 5.6), the rate of total glycerol increase was significantly ($p<0.05$, Table 5.1) higher
Fig. 5.3: Time-course of glycerol content in control and electroporated cell suspensions.
Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). Values of single experiment.
Conditions: electroporation at 2 °C with 9 pulses, 250 V and 50 ms pulse duration. Electroporation medium included 6.0 mM CaCl₂
Fig. 5.4: **Time-course of glycerol content in control and electroporated cell suspensions.**
Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). Comparison of two categories of results. (a) mean ± Std. Error of three replicates. (b) single experiment.
Conditions: electroporation at 2 °C with 5 pulses, 300V and 50ms pulse duration. Electroporation medium included 6.0 mM CaCl₂.
100% represents 19.2 (a), 22.5 (b) µmol glycerol . mg⁻¹ chl.
Fig. 5.5: **Time-course of glycerol content in control and electroporated cell suspensions.**

Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). Effect of addition of EDTA. No Ca²⁺ was added to the electroporation medium (a & b). EDTA (50 μM) was added to (b).

(a) mean ± Std. Error of four replicates. (b) mean ± Std. Error of three replicates.

Conditions: electroporation at 2 °C with 2 pulses, 300 V and 50ms pulse duration.

100% represents 21.2 (a), 22.2(b) μmol glycerol . mg⁻¹ chl.
Fig. 5.6: Time-course of glycerol content in control and electroporated cell suspensions.
Post-electroporation incubation in Hepes medium at 25 °C (Section 2.2.4.3). Effect of addition of 50 μM EDTA. No Ca^{2+} was added to the electroporation medium. Values are mean ± Std. Error of two replicates.
Conditions: electroporation at 2 °C with 3 pulses, 300 V and 50 ms pulse duration.
100% represents 23.7 μmol glycerol . mg^{-1} chl.
Table 5.1: **Statistical comparison of the time-courses for total and intracellular glycerol content of electroporated cells with the control (not electroporated) using Student's t-test (two-sided test).** The average rate of glycerol synthesis was obtained from the slope of the linear best fit to the data. t values obtained were tested for significance by comparison with value from statistical table.

<table>
<thead>
<tr>
<th>Condition</th>
<th>comparison of slope of glycerol synthesis</th>
<th>t value obtained</th>
<th>table value</th>
<th>***</th>
<th>df</th>
<th>difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>300V-50ms 2pulse</td>
<td>total</td>
<td>2.23</td>
<td>&gt; 2.06</td>
<td>24</td>
<td>significant *</td>
<td></td>
</tr>
<tr>
<td>300V-50ms 2pulse</td>
<td>intracellular</td>
<td>2.29</td>
<td>&gt; 2.06</td>
<td>24</td>
<td>significant *</td>
<td></td>
</tr>
<tr>
<td>300V-50ms 2pulse (plus EDTA)</td>
<td>total</td>
<td>3.62</td>
<td>&gt; 2.84</td>
<td>20</td>
<td>significant **</td>
<td></td>
</tr>
<tr>
<td>300V-50ms 2pulse (plus EDTA)</td>
<td>intracellular</td>
<td>2.82</td>
<td>&gt; 2.08</td>
<td>20</td>
<td>significant *</td>
<td></td>
</tr>
<tr>
<td>300V-50ms-3pulse</td>
<td>total</td>
<td>3.34</td>
<td>&gt; 2.92</td>
<td>16</td>
<td>significant **</td>
<td></td>
</tr>
<tr>
<td>300V-50ms-3pulse</td>
<td>intracellular</td>
<td>0.56</td>
<td>&lt; 2.12</td>
<td>16</td>
<td>not significant</td>
<td></td>
</tr>
</tbody>
</table>

* represents difference is significant at p<0.05
** represents difference is significant at p<0.01
*** df represents degree of freedom.
than the control while intracellular glycerol content did not return to the control level during the 120 minute incubation.

5.4. Discussion

In a number of experiments (Figs. 5.2 - 5.6), a rate of increase of glycerol content was observed which was significantly higher in electroporated cells compared with controls. This clearly represents the occurrence of net glycerol synthesis in the electroporated cells. The central role of Ca\textsuperscript{2+} in triggering many cellular responses has been described in Section 1.8.9. When Ca\textsuperscript{2+} was included in the medium in these experiments, in just a few cases (which were not reproducible), the intracellular glycerol recovered (Figs. 5.2c, 5.5b) following electroporation. In one case (Fig. 5.2b), an increase in total glycerol at a rate higher than the control was observed, while the intracellular glycerol remained unchanged. This suggests that the pores formed by electroporation did not reseal completely or that some other permanent damage were done to cell integrity. In this case, the cells synthesised more glycerol but it was lost to the medium. Generally, however, with Ca\textsuperscript{2+} included in the medium, the glycerol content in electroporated and control cells was the same. The occasional but non-repeatable occurrence of net glycerol synthesis in the presence 6.0 mM CaCl\textsubscript{2} suggests that Ca\textsuperscript{2+} in the electroporation medium is not inhibitory to net glycerol synthesis. However, the variability in these results does not allow a confident conclusion.

The results of the series of experiments where CaCl\textsubscript{2} was omitted from the electroporation medium show clearly that in *D. tertiolecta*, following loss of a small proportion of intracellular glycerol, net synthesis of glycerol can be triggered at constant osmotic pressure. When 50 µM EDTA was added to the medium to ensure that no trace of free Ca\textsuperscript{2+} was present, similar results were obtained. This confirms that intracellular glycerol synthesis following electroporation is not triggered by Ca\textsuperscript{2+} uptake from medium. Another possible way that electroporation might affect the cytosolic Ca\textsuperscript{2+} concentration is by disturbance of the endoplasmic reticulum or other
intracellular vesicles that store Ca$^{2+}$. The evidence that the internal membranes and organelles of *D. tertiolecta* are not affected by electroporation with two or three pulses was presented in Section 4.4. The general evidence that the plasma membrane of cells can be electroporated without disturbing the membranes of intracellular organelles and their functions has reviewed by Knight (1981). The intracellular volume is between 100 and 400 times smaller than extracellular volume (Table 4.2). For example, when 0.112 µmol of intracellular glycerol is released by electroporation, this adds (0.112 µmol) to the medium with initial osmolality of 1189 mmol kg$^{-1}$, increasing the osmolality less than 0.01%. Hence the released glycerol has a trivial effect on the external osmolality (and so osmotic pressure and water activity). The increase in total glycerol content of the cells subsequent to electroporation in cases where the intracellular glycerol did not recover fully (Fig. 5.6), may be the result of the plasma membrane being more leaky to glycerol.

In summary, following electroporation of *D. tertiolecta* cells under appropriate conditions, a small proportion of intracellular glycerol was lost to the medium. During subsequent incubation in the Hepes medium, the cells produced new glycerol to regain the original level. The entire process occurred at constant medium osmotic pressure.

It may be concluded that the regulatory mechanism responsible for setting the intracellular glycerol content does not sense external osmotic pressure *per se.*
CHAPTER SIX:
THE TIME-COURSE OF CELL VOLUME CHANGES DURING ELECTROPORATION AND SUBSEQUENT INCUBATION AT CONSTANT OSMOTIC PRESSURE
6.1. Introduction
The response of the cell volume of *Dunaliella* to changes in external salinity and osmotic pressure has been described in Section 1.8.1 and Fig 1.3. Following the release of glycerol by electroporation under the conditions established in Chapter 5, water efflux and shrinkage of the cells is expected. The water efflux is in response to the lowered osmotic pressure, and hence raised water potential inside the cell due to this loss of solute.

**Aim:**
The aim of this section is the determination of cell volume of *D. tertiolecta* cells following loss of glycerol by electroporation and during the subsequent time-course during which the intracellular glycerol content recovered (Chapter 5). As previously, this work was performed with media of constant osmotic pressure, equal to that of the growth medium.

6.2. Special procedures.
Cell suspensions (1.0 ml) were electroporated in the electroporation medium (6.0 mM CaCl₂ omitted) with 2 pulses of 300V, 50 ms duration. The cell volume was measured at zero time, 30 and 90 minutes after electroporation (Section 2.2.4.3, 2.2.9.2.2). All procedures were the same for glycerol determination (Section 5.3.2) except that at zero time, before dilution of cells by Hepes medium, the cell volume was measured directly after of electroporation. As before, every electroporation experiment was accompanied by a control in which the alga was subjected to identical manipulations with the exception of electroporation.

6.3. Results
Preliminary measurements of cell volume were done using the Li⁺ exclusion method of Katz & Avron (1985) (see Section 2.2.9.1). It was found, however, that the 1.0 ml
volume of algal suspension available after electroporation (in comparison to 16 ml recommended by these authors) was not enough for an accurate measurement.

Cell volume changes following loss of glycerol by electroporation were measured on motile cells, initially by still photography and then by video microscopy (Fig. 6.1). Preliminary results (not shown) using still photography showed that immediately after electroporation the average cell volume decreased from 215 μm³ to 197 μm³, an 8% decrease compared with the control. When the video microscopy technique was used, immediately after electroporation (Figs. 6.2, 6.3 and Table 6.1), the average cell volume decreased (from 168 μm³ to 131 μm³) by 23% compared with the control. The distribution of size in the control cells was approximately symmetrical, while that in the electroporated cells was skewed towards the smaller volume. A statistical comparison of the distribution of control and electroporated cell volumes showed that, immediately after electroporation, the cell volume decreased significantly (Table 6.1) (p<0.001, Mann-Whitney U test). After incubation for 30 minutes, the average electroporated cell volume returned to the control level. The distributions of cell volume for control and electroporated cells were not significantly different at 30 and 90 minutes following electroporation. Following electroporation, the cell density did not increase and did not change significantly during the further 90 minutes incubation for either the control or electroporated cells (Table 6.2).

Table 6.2: Cell density for D. tertiolecta in control (non-electroporated) and electroporated cells immediately after electroporation (zero time) and after a further 90 minutes incubation in Hepes medium.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Electroporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>zero-time</td>
<td>1.29</td>
<td>1.18</td>
</tr>
<tr>
<td>90 minutes</td>
<td>1.34</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Fig. 6.1: Digitized image of a *Dunaliella* cell superimposed on the size scale. (Small graduations represent 10 μm).
Fig. 6.2: Digitized images of \textit{D. tertiolecta} cells. Comparison of control and electroporated cells immediately after electroporation.
Fig. 6.3: **Distribution of cell volume in control and electroporated *D. tertiolecta* cells measured by video microscopy.**

The x-axis represents the size categories (20 μm³ per category) and the y-axis the percentage of total cells counted in each range. Number of cells measured (control and electroporated respectively): (a) 114 & 91, (b) 60 & 39, (c) 59 & 87. Conditions: 1 ml algal suspension electroporated with two pulses of 300 V, 50 ms pulse duration at 2 °C. (a) Cell volume at zero time (measured immediately after electroporation) (b) at 30 minutes (c) at 90 minutes. Electroporation medium without added Ca²⁺ (see Section 6.2). Cells incubated cells in Hepes medium post-electroporation at 25 °C (Table 2.3, Section 2.2.4.2).
Table 6.1: Cell dimensions and calculated volumes (Mean ± S.D) by video microscopy.
Comparison of control (non-electroporated) and electroporated *D. tertiolecta* cells during the time-course at zero, 30, and 90 minutes. The values in parenthesis are the number of cell measured.
(see Section 2.2.9.3).

<table>
<thead>
<tr>
<th></th>
<th>Cell dimensions (µm)</th>
<th>Cell volume (µm³)</th>
<th>Z-value obtained by Mann-Whitney U test. Non-parametric equivalent of a 2-sample (control and electroporated) t-test.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td><strong>Zero-Time</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (114)</td>
<td>5.8±0.6</td>
<td>5.5±0.6</td>
<td>3.4±0.6</td>
</tr>
<tr>
<td>Electroporated (91)</td>
<td>4.8±0.7</td>
<td>4.9±0.7</td>
<td>2.5±0.7</td>
</tr>
<tr>
<td><strong>30-minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (60)</td>
<td>5.4±0.6</td>
<td>5.3±0.6</td>
<td>3.3±1.1</td>
</tr>
<tr>
<td>Electroporated (39)</td>
<td>5.3±0.7</td>
<td>5.3±0.7</td>
<td>3.7±1.1</td>
</tr>
<tr>
<td><strong>90-minute</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (59)</td>
<td>5.5±0.5</td>
<td>5.5±0.7</td>
<td>3.5±0.9</td>
</tr>
<tr>
<td>Electroporated (87)</td>
<td>5.5±0.6</td>
<td>5.4±0.7</td>
<td>3.4±0.8</td>
</tr>
</tbody>
</table>

* Difference in the distribution of volume of control and electroporated cells is significant at the \(p<0.001\) level.
6.4. Discussion

The cell volume of Dunaliella can be measured by using solute exclusion methods such as the Li\(^{+}\)-based procedure of Katz & Avron (1985) (Sections 2.2.9.1, 1.5.2.2 and Fig. 1.1). Using such methods gives an average of cell size rather than distribution of volumes. The average value obtained may be more accurate due to the large number of cells included in solute exclusion methods. However, the possibility of effects of Li\(^{+}\) on the cells (e.g. entry during pore formation by electroporation) with unknown outcomes for the accuracy of the volume calculation was another reason for choosing direct photographic methods.

Still photography was investigated first to measure the cell volume size of the *D. tertiolecta* cells (Section 2.2.9.2.1). The control cell volume measured (215 \(\mu m^3\)) was 20% larger than when video microscopy was used (168 \(\mu m^3\)). However, with still photography one cannot distinguish between motile cells and non-motile cells. A proportion of cells attach by their flagella to the glass of the cover slip or slide. When *Dunaliella* cells are immobilised, they change their shape and swell slowly (Lilley, unpublished). Hence it was decided to make the volume measurement only on cells which were motile. For this purpose, the video microscopy technique was chosen because of its ability to identify and measure the motile cells (Section 2.2.9.2.2). In addition video microscopy has the capacity to chase an individual mobile cell, frame by frame, until both poles are in focus. This gives an image which in the axis of the cell (i.e. dimension d in Section 2.2.9.3) is perpendicular to the viewing axis. This procedure will be most accurate for microscopes with a shallow of depth of focus: measuring cells which do not have perpendicular orientation will result in an underestimation of cell volume.

The average cell volume of *D. tertiolecta* obtained here by video microscopy was 169 \(\mu m^3\). This is close to the 179 \(\mu m^3\) value of *D.tertiolecta* cells (adapted to 410 mM NaCl) obtained from video images by Ehernfeld & Cousin (1982). They also reported
different cell sizes of 274 and 283 μm$^3$ for *D.tertiolecta* cells adapted to media containing 0.02 M and 1.64 M NaCl respectively.

The accurate determination of cell dimensions is essential because the radius is cubed in the volume calculation. Any small error in linear measurement brings a big error in cell size estimation (Blackwell & Gilmour, 1989). Our procedure makes three determinations of cell diameter in different places (Section 2.2.9.3). Many authors (e.g. Ehrenfeld & Cousin, 1982; Zimiri, Wax & Ginzburg, 1984; Ginzburg & Ginzburg, 1985a) have determined the dimensions of *Dunaliella* cells by assumption that the cell is ellipsoid. For this they has measured the half-length and half-width of the cells. We believe our procedures approximate more closely the shape of *Dunaliella*, which can vary between almost spherical and pear-shaped depending on the degree of swelling or shrinkage. After electroporation, the shrinkage is due to a change in diameters, rather than the pole-to-pole length (Table 6.1). In addition, the measurement of cell diameters in digitised images of the cells using the computer is more accurate and faster than by a manual measurement. The disadvantage of microscopic measurement is that for more reliable results large numbers of cells (at least 50 to 100 cells) must be measured; this is a time-consuming process.

Our results clearly showed that immediately after the electroporation, there was a significant decrease of cell volume (23% compared within the control), which recovered after incubation for 30 minutes. This shrinkage and recovery agreed with our expectations. We expected that following loss of glycerol by electroporation, the cell would shrink. After the intracellular glycerol content had recovered during post-electroporation incubation in Hepes medium, the cell volume was also expected to recover.

Asexual reproduction in *Dunaliella* produces two smaller daughter cells from each parental cell (Section 1.1). The absence of cell density changes after electroporation
and during subsequent incubation shows that the decrease in cell volume after electroporation was not due to cell division.

Katz & Avron (1985) determined the cell volume of *Dunaliella* over a range of salinities and found it to be essentially constant. They concluded "that *Dunaliella* cells attempt to maintain a constant osmotic volume independent of the osmotic pressure to which they are exposed". For *Dunaliella*, during osmotic adjustments immediately following a change in external osmotic pressure, volume changes are relatively large and turgor pressure changes extremely small but not zero (Brown, 1990). Here under the conditions we used for electroporation, the osmotic pressure of the medium was constant, while the cell volume decreased by about 23%. The turgor pressure changes would be extremely small. Hence, we believe that the most likely mechanism by which glycerol metabolism participates in turgor-volume control in *Dunaliella* is by a regulatory signal from a cell volume-linked parameter to one or more enzymes of glycerol metabolism. The identity of this signal and its receptor remain to be established.
CHAPTER SEVEN:
GENERAL CONCLUSIONS
7. General conclusions.

7.1. O₂ evolution.

No major difference was found between Pi and Hepes buffer when measuring photosynthetic O₂ evolution, carbon fixation and respiration (in the dark) and following salt stress in *D. tertiolecta* and *D. salina*. This shows that during the duration of our experiments, the presence of Pi in the buffer did not have any metabolic consequences that translated to effects on photosynthesis or respiration. Increasing salinity of the medium by a factor of 5.88 in *D. tertiolecta* caused inhibition of both photosynthesis and dark respiration. An increase in salinity by a factor of 1.66 caused a small increase in photosynthesis and no major effect in respiration in *D. tertiolecta*. Increasing salinity by the same factor caused no major effect on photosynthesis but a substantial increase in respiration in *D. salina*. While the relative magnitudes of salt stress of these two species was the same, the salinity in which the cells were grown was different. It is not possible to conclude whether the difference in response of photosynthesis and respiration is species specific or is due to the concentration of salt in the growth medium having additional effects.

The absence of the reported burst in O₂ evolution in *D. salina* after salt stress (Kaplan, Shreiber & Avron, 1980 and Avron, M, personal communication to R. McC. Lilley) in our work (Section 3.3.4) may be due to differences within the species. No inhibition of O₂ evolution was observed in high photon flux density with *D. salina*, indicating that photon flux density is not a factor in causing the reported burst of O₂ evolution. The similar response in net photosynthesis in *D. tertiolecta* following an increase in salinity by a factor of 1.66 for stresses from 0.17 M to 0.28 M and from 0.6 M to 1.0 M NaCl suggests that the magnitude of a salt stress is more important than the initial or final concentrations.
7.2. Glycerol synthesis and cell volume recovery following electroporation.

When electroporation was chosen for artificially lowering the glycerol content of *D. tertiolecta*, the variable parameters were investigated to find the best conditions for lowering glycerol content while maintaining cell survival rates near 100%. It was essential to use cells with a high intracellular glycerol content and this was achieved by growing the cells in 600 mM NaCl. The electroporation medium selected was isotonic to the growth medium. The electroporation medium contained a low amount of NaCl, (150 mM) with the balance of osmolality provided by mannitol chosen as a relatively inert non-electrolyte. The electroporation was applied initially with aluminium electrodes. The results clearly showed a release of Al$^{3+}$ during electroporation with these electrodes. Added Al$^{3+}$ caused cell aggregation and effects on glycerol metabolism. When the Al$^{3+}$ electrodes were replaced by gold-plated copper electrodes these effects were not observed. Accordingly, for all subsequent work the gold-plated electrodes were used.

A direct relationship between the number of pulses, pulse duration and voltage and the magnitude of glycerol release was shown. It was also shown that a small proportion of glycerol can be removed by electroporation under these constant osmotic pressure conditions with no observed effects on the metabolism of the cell. This was demonstrated by lack of effects on photosynthesis, respiration and cell motility following electroporation. Since the total intracellular cell volume of the pellet was very small (typically about 2.0 μl) compared to the external volume (about 1000 μl), the release of glycerol to the medium had an insignificant effect on the osmolality (less than 0.01 % increase) and hence on the osmotic pressure of the medium.

The release of soluble protein (1.4%) was low compared to the release of glycerol (11%) following electroporation. This indicated that glycerol release to the medium was due to the efflux through pores formed by electroporation and not due to rupture
of a small proportion of the cells. These experiments gave limited information regarding the size of the pores. The low uptake of mannitol (20% of glycerol release) under optimum electroporation conditions indicated a pore size small enough to partially exclude the six-carbon mannitol molecule. If the size and resealing time of pores was long enough, equilibration of intracellular glycerol and extracellular mannitol across the cell membrane would be expected. We were unable to make a valid measurement of Na⁺ uptake by cells after electroporation. However, the absence of inhibition of photosynthesis and respiration following electroporation indicates that the intracellular concentration of Na⁺ was unlikely to be high since, in Dunaliella, photosynthesis has been shown to be inhibited by the presence of intracellular Na⁺ and Cl⁻. For further study, a more thorough determination of the influx of Na⁺ and Cl⁻ during electroporation would be desirable.

Under optimised conditions, the amount of intracellular glycerol lost during electroporation of D. tertiolecta cells was shown to be fully replaced. This was demonstrated in the absence of Ca²⁺ in the medium and in the presence of a chelator. This shows that glycerol synthesis is triggered by a mechanism not dependent on Ca²⁺ influx from the medium.

The significant shrinkage of the cells following electroporation in Ca²⁺-free medium and the recovery of cell volume after 30 minutes indicates that, following loss of glycerol from the cells, the internal osmolality and hence osmotic pressure decreased. Consequently, the cells lost water within seconds after electroporation and shrank until the osmotic pressure inside the cells equalled that of the medium. The synthesis of new glycerol added to the internal osmolality and this drove the restoration of cell volume (see the general model below).
Since the osmotic pressure of the medium before and after electroporation was constant, the work in this thesis demonstrates that, in *Dunaliella*, the intracellular glycerol content is not regulated by external osmotic pressure *per se* but by some other factor or factors.

As pointed out in the introduction (section 1.8), no metabolite or inorganic ion has been positively identified as being involved in the mechanism for control of glycerol metabolism in *Dunaliella*. The findings in this thesis are consistent with the proposal of Cram (1976) that in wall-less cells the primary signal for adaptive responses might be a function of cell volume (section 1.8.1). Although the involvement of turgor pressure changes (Brown 1990) can not be rigorously excluded, it seems unlikely that the extremely low turgor pressure of *Dunaliella* could have a role in the signalling mechanism.

It is concluded that the relevant variable factor during electroporation of *Dunaliella* at constant external osmotic pressure is cell volume. Many of the components of the inositol-phospholipid signalling pathway are present in *Dunaliella*, and changes in the concentrations of some components have been shown to occur on osmotic stress (section 1.8.9). Hence it seems possible that the decrease in cell volume following electroporation could act as the primary signal for changing the rate of hydrolysis of plasma membrane-localised PIP$_2$. In turn, a secondary messenger (eg. release of internal Ca$^{2+}$ to the cytosol or generation of DAG) might increase the activity of glycerol phosphate dehydrogenase. Activation of this chloroplast-located enzyme increases intracellular glycerol concentration (section 1.7.1) resulting in the restoration of cell volume.
These conclusions are summarised in the following general model:

For further confirmation of these findings, the measurement of additional parameters after electroporation would be desirable. The following would be of particular relevance: (a) Determination of influx of Na\(^+\) and Cl\(^-\) (b) Determination of effects on ATP and other internal metabolites, especially components of the inositol-phospholipid signalling pathway (c) Measuring the cell volume under a wider range of electroporation conditions by video microscopy or other suitable techniques.
APPENDIX I
Table 1: **Photosynthetic O$_2$ evolution and O$_2$ uptake by D. tertiolecta.** Effect of salt stress from 0.17 M to 1.0 M NaCl with phosphate and Hepes buffer. Values are the mean ± S.D of three or four replicates.

Control: addition an of identical volume of medium containing 0.17 M NaCl

<table>
<thead>
<tr>
<th>Buffer</th>
<th>condition</th>
<th>Rate of O$_2$ evolution (nmol O$_2$. min$^{-1}$. mg$^{-1}$ chl)</th>
<th>% change</th>
<th>% change on control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before</td>
<td>after</td>
<td></td>
</tr>
<tr>
<td><strong>Net photosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Control</td>
<td>1012 ± 144</td>
<td>1066 ± 230</td>
<td>5</td>
</tr>
<tr>
<td>Pi</td>
<td>Stress</td>
<td>1149 ± 221</td>
<td>302 ± 162</td>
<td>-74</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>914 ± 176</td>
<td>865 ± 22</td>
<td>-5</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>890 ± 180</td>
<td>306 ± 118</td>
<td>-66</td>
</tr>
<tr>
<td><strong>Total photosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Control</td>
<td>1348 ± 266</td>
<td>1374 ± 288</td>
<td>2</td>
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<tr>
<td>Pi</td>
<td>Stress</td>
<td>1482 ± 218</td>
<td>438 ± 265</td>
<td>-70</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>1696 ± 241</td>
<td>1251 ± 200</td>
<td>-26</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>1600 ± 253</td>
<td>484 ± 173</td>
<td>-70</td>
</tr>
<tr>
<td><strong>Respiration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi</td>
<td>Control</td>
<td>-438 ± 111</td>
<td>-410 ± 128</td>
<td>-6</td>
</tr>
<tr>
<td>Pi</td>
<td>Stress</td>
<td>-411 ± 146</td>
<td>-211 ± 69</td>
<td>-48</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>-382 ± 150</td>
<td>-353 ± 138</td>
<td>-8</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>-329 ± 186</td>
<td>-178 ± 63</td>
<td>-46</td>
</tr>
</tbody>
</table>
Table 2: **Photosynthetic O₂ evolution and O₂ uptake by D. tertiolecta.** Effect of salt stress from 0.17 M to 0.28 M NaCl with phosphate and Hepes buffer. Values are the mean ± S.D of three or four replicates.

Control: addition an of identical volume of medium containing 0.17 M NaCl

<table>
<thead>
<tr>
<th>Buffer</th>
<th>condition</th>
<th>Rate of O₂ evolution (nmol O₂ . min⁻¹ . mg⁻¹ chl)</th>
<th>% change on control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before</td>
<td>after</td>
</tr>
</tbody>
</table>

**Net photosynthesis**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>condition</th>
<th>before</th>
<th>after</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>Control</td>
<td>874 ± 112</td>
<td>901 ± 158</td>
<td>3</td>
</tr>
<tr>
<td>Pi</td>
<td>Stress</td>
<td>990 ± 277</td>
<td>1274 ± 227</td>
<td>29</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>911 ± 141</td>
<td>977 ± 104</td>
<td>7</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>1127 ± 213</td>
<td>1480 ± 225</td>
<td>31</td>
</tr>
</tbody>
</table>

**Total photosynthesis**

<table>
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<th>Buffer</th>
<th>condition</th>
<th>before</th>
<th>after</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>Control</td>
<td>1188 ± 224</td>
<td>1276 ± 205</td>
<td>7</td>
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<tr>
<td>Pi</td>
<td>Stress</td>
<td>1374 ± 248</td>
<td>1566 ± 274</td>
<td>14</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>1235 ± 91</td>
<td>1351 ± 82</td>
<td>9</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>1753 ± 253</td>
<td>1996 ± 258</td>
<td>4</td>
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</tbody>
</table>

**Respiration**

<table>
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<th>after</th>
<th>% change</th>
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</thead>
<tbody>
<tr>
<td>Pi</td>
<td>Control</td>
<td>-389 ± 145</td>
<td>-374 ± 148</td>
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<td>Pi</td>
<td>Stress</td>
<td>-329 ± 144</td>
<td>-291 ± 115</td>
<td>-11</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>-359 ± 131</td>
<td>-346 ± 113</td>
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<tr>
<td>Hepes</td>
<td>Stress</td>
<td>-404 ± 152</td>
<td>-330 ± 131</td>
<td>-18</td>
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</table>
Table 3: **Photosynthetic O$_2$ evolution and O$_2$ uptake by D. tertiolecta.** Effect of salt stress from 0.6 M to 1.0 M NaCl with phosphate and Hepes buffer. Values are the mean ± S.D of three or four replicates.

Control: addition an of identical volume of medium containing 0.6 M NaCl

<table>
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<th>before (nmol O$_2$. min$^{-1}$. mg$^{-1}$ chl)</th>
<th>after (nmol O$_2$. min$^{-1}$. mg$^{-1}$ chl)</th>
<th>% change</th>
<th>% change on control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>Control</td>
<td>838 ± 83</td>
<td>921 ± 124</td>
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<td>+24</td>
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<tr>
<td>Pi</td>
<td>Stress</td>
<td>987 ± 236</td>
<td>1349 ± 245</td>
<td>37</td>
<td>+24</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>1303 ± 28</td>
<td>1501 ± 10</td>
<td>15</td>
<td>+25</td>
</tr>
<tr>
<td>Hepes</td>
<td>Stress</td>
<td>1084 ± 103</td>
<td>1441 ± 105</td>
<td>40</td>
<td>+25</td>
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</table>

**Net photosynthesis**

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<th>% change on control</th>
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<tbody>
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<td>Control</td>
<td>1059 ± 97</td>
<td>1159 ± 72</td>
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<td>+17</td>
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<tr>
<td>Pi</td>
<td>Stress</td>
<td>1235 ± 291</td>
<td>1599 ± 210</td>
<td>29</td>
<td>+17</td>
</tr>
<tr>
<td>Hepes</td>
<td>Control</td>
<td>1688 ± 76</td>
<td>1772 ± 45</td>
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<tr>
<td>Hepes</td>
<td>Stress</td>
<td>1308 ± 185</td>
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**Total photosynthesis**

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<th>% change</th>
<th>% change on control</th>
</tr>
</thead>
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<td>Control</td>
<td>-252 ± 44</td>
<td>-251 ± 43</td>
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<tr>
<td>Pi</td>
<td>Stress</td>
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<td>-249 ± 70</td>
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<td>+3</td>
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<tr>
<td>Hepes</td>
<td>Control</td>
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<td>-287 ± 42</td>
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<tr>
<td>Hepes</td>
<td>Stress</td>
<td>-226 ± 41</td>
<td>-238 ± 45</td>
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<td>-3</td>
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</table>
Table 4: **Photosynthetic O$_2$ evolution and O$_2$ uptake by D. salina.** Effect of salt stress from 1.5 M to 2.5 M NaCl with phosphate and Hepes buffer in red light (intensity, 700 μE·m$^{-2}$·sec$^{-1}$). Values are the mean ± S.D of three or four replicates. Control: addition an of identical volume of medium containing 1.5 M NaCl

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<th>% change on control</th>
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<td>(nmol O$_2$·min$^{-1}$·mg$^{-1}$ chl)</td>
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<td></td>
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<td>Control</td>
<td>3217 ± 43</td>
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<td>Pi</td>
<td>Stress</td>
<td>3520 ± 455</td>
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<td>Hepes</td>
<td>Control</td>
<td>2895 ± 287</td>
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<td>Stress</td>
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<td>Control</td>
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<td>Heps</td>
<td>Control</td>
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<td>Heps</td>
<td>Stress</td>
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<td>Respiration</td>
<td>Heps</td>
<td>Stress</td>
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Table 5: **Photosynthetic O$_2$ evolution and O$_2$ uptake by D. salina.** Effect of salt stress from 1.5 M to 2.5 M NaCl with phosphate and Hepes buffer in red light (intensity, 2200 μE.m$^{-2}$.sec$^{-1}$). Values are the mean ± S.D of two replicates. Control: addition of an identical volume of medium containing 1.5 M NaCl.

<table>
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<th>Rate of O$_2$ evolution (nmol O$_2$. min$^{-1}$. mg$^{-1}$ chl)</th>
<th>% change</th>
<th>% change on control</th>
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<td>Control</td>
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<td>Pi</td>
<td>Stress</td>
<td>3083 ± 250</td>
<td>3198 ± 271</td>
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<td>Hepes</td>
<td>Control</td>
<td>3824 ± 300</td>
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<td>Hepes</td>
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<td>3777 ± 300</td>
<td>17</td>
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<td>Hepes</td>
<td>Control</td>
<td>3988 ± 300</td>
<td>4157 ± 380</td>
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<td>3731 ± 200</td>
<td>4376 ± 407</td>
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<td>Pi</td>
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<td>-578 ± 39</td>
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<tr>
<td>Hepes</td>
<td>Control</td>
<td>-445 ± 41</td>
<td>-444 ± 30</td>
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<tr>
<td>Hepes</td>
<td>Stress</td>
<td>-372 ± 10</td>
<td>-558 ± 25</td>
<td>50</td>
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</table>
APPENDIX II
References


