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$^{16}O_2/^{18}O_2$ analysis of oxygen exchange in *Dunaliella tertiolecta*. Evidence for the inhibition of mitochondrial respiration in the light

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Abstract. A mass spectrometric $^{16}O_2/^{18}O_2$-isotope technique was used to analyse the rates of gross O_2 evolution, net O_2 evolution and gross O_2 uptake in relation to photon fluence rate by *Dunaliella tertiolecta* adapted to 0.5, 1.0, 1.5, 2.0 and 2.5 M NaCl at 25 °C and pH 7.0.

At concentrations of dissolved inorganic carbon saturating for photosynthesis (200 μM) gross O_2 evolution and net O_2 evolution increased with increasing salinity as well as with photon fluence rate. Light compensation was also enhanced with increased salinities. Light saturation of net O_2 evolution was reached at about 1000 μmol $m^{-2} s^{-1}$ for all salt concentrations tested. Gross O_2 uptake in the light was increased in relation to the NaCl concentration but it was decreased with increasing photon fluence rate for almost all salinities, although an enhanced flow of light generated electrons was simultaneously observed. In addition, a comparison between gross O_2 uptake at 1000 μmol photons $m^{-2} s^{-1}$, dark respiration before illumination and immediately after darkening of each experiment showed that gross O_2 uptake in the light paralleled but was lower than mitochondrial O_2 consumption in the dark.

From these results it is suggested that O_2 uptake by *Dunaliella tertiolecta* in the light is mainly influenced by mitochondrial O_2 uptake. Therefore, it appears that the light dependent inhibition of gross O_2 uptake is caused by a reduction in mitochondrial O_2 consumption by light.

Abbreviations: DCMU - 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea, DHAP - dihydroxyacetonephosphate, DIC - dissolved inorganic carbon, DR_a - rate of dark respiration immediately after illumination, DR_b - rate of dark respiration before illumination, E_0 - rate of gross oxygen evolution in the light, NET - rate of net oxygen evolution in the light, PFR - photon fluence rate, RubP - rubulose-1,5-bisphosphate, SHAM - salicyl hydroxamic acid, U_0 - rate of gross oxygen uptake in the light

Introduction

Net O_2 evolution of photoautotrophic organisms in the light (NET) is composed of gross O_2 evolution (E_0) and gross O_2 uptake (U_0) (Canvin et al. 1980, Fock et al. 1981, Peltier and Thibault 1985b, Sültemeyer and Fock 1986). E_0 is only caused by the water splitting reaction of photosynthesis and, therefore, a measure for photosynthetically generated electrons. In contrast, U_0 may be composed of several processes including oxidation of RubP by RubP-carboxylase/oxygenase, O_2 reduction and mitochondrial O_2 uptake (Canvin et al. 1980, Peltier and Thibault 1985b, Sültemeyer et al. 1986, Brechignac and Furbank 1986). RubP oxidation, which contributes approximately 30–40% to total O_2 uptake in green algae (Fock et al. 1981, Peltier and Thibault 1985a, Sültemeyer and Fock 1986), is eliminated at high DIC concentrations in the medium so that at DIC saturation for photosynthesis only the other two reactions contribute to the remaining O_2 consumption in the light (Canvin et al. 1980, Peltier and Thibault 1985a, Sültemeyer and Fock 1986, Sültemeyer et al. 1986).

The contribution of mitochondrial respiration to O_2 uptake in the light is still an open and important question. Although not directly measured, it has been suggested that mitochondrial O_2 consumption is inhibited in the light in higher plants (Canvin et al. 1980) so that U_0 is mainly composed of O_2 reduction at DIC saturation (Sültemeyer et al. 1986). In contrast, Peltier and Thibault (1985b) in addition to the earlier work of Falkowski and Owens (1978) have recently reported no changes in mitochondrial O_2 uptake in the light by eucaryotic algae. In these experiments mitochondrial respiration appears to be the major contributor to U_0. However, the above mentioned authors used potent inhibitors such as KCN, DCMU and SHAM which create artifical systems and make an interpretation of results under in vivo conditions difficult.

To overcome these difficulties we used an in vivo system with an organism in which O_2 and another electron acceptor(s) compete for reducing power (thus reducing the rate of O_2 reduction). This is the case with species from the euryhaline genus *Dunaliella tertiolecta* which forms large amounts of glycerol from DHAP using light generated electrons (Craigie and McLachlan 1964, Kaplan et al. 1980, Ben-Amotz et al. 1982, Avron 1986). Therefore, one should expect a reduced rate of O_2 reduction especially under conditions of an enhanced demand for glycerol.

In this paper it will be demonstrated that U_0 by *D. tertiolecta* adapted to high NaCl concentrations is inhibited with increasing PFR and that this light dependent inhibition of U_0 is probably caused by a reduction in the rate of mitochondrial O_2 uptake.

Materials and methods

Growth and preparation of algae

Dunaliella tertiolecta was obtained from K.S. Thomson (University of Kaiserslautern) who originally received it from A.H. Latorella. The cells were maintained axenically in shake cultures at a PFR of 100 μmol $m^{-2} s^{-1}$ and pH = 7.0 with nutrient according to Latorella and Vadas (1973) except that NO_3^- was replaced by NH_4^+. The shake cultures were supplemented with 0.5, 1.0, 1.5, 2.0 or 2.5 m NaCl. These cultures were renewed each week by sterile transfer of 1 ml of the suspension to fresh medium with the required NaCl content.

At the start of each experiment, 1 ml of stock culture was transferred to 800 ml sterile culture medium with the corresponding NaCl content under sterile conditions in cylindrical glass vessels. The cultures were incubated in a temperature controlled water bath at 25 °C, pH = 7.0 and a PFR of 270 μmol $m^{-2} s^{-1}$ (400–700nm) for 5 days and bubbled with air. The advantage of this procedure was that all the cells were fully adapted to the salt concentration of the external medium indicated by the exponential phase of growth (data not shown). Under these conditions cell numbers per ml packed cell volume increased about two fold from 0.5 to 2.5 M NaCl indicating that cells adapted to higher salinities were smaller than those adapted to lower salt concentrations. The chlorophyll content per cell was almost constant (5.8–6.3 $\times$ 10^{-7} μg/cell) over the whole range of salinities tested (data not shown).

For the gas exchange measurements, cells were centrifuged at 5000 rpm for 5 min and then resuspended in 500 μl of fresh medium containing the appropriate NaCl concentrations and bubbled with CO_2-free air at 25 °C in the dark for up to 1 h. Microscopic examination of the cultures before each experiment ensured that the algae were free of bacteria.

Measurement of O_2 gas exchange

All O_2 gas exchange measurements were performed in a thermostated plexiglass chamber connected to an O_2 electrode and a mass spectrometer (GD 150/4, Finnigan, D-2800 Bremen) via a membram inlet system (Sültemeyer and Fock, 1986). Both systems were calibrated for the five different salt concentrations at 25 °C against air. The chamber was filled with 40 ml O_2 free medium (pH = 7.0) containing the appropriate NaCl concentration. Subsequently the chamber was sealed by a stopper. Then an $^{36}O_2$ gas bubble of 1 ml (^{18}O, 99.88 atom%) was injected into the chamber and the

medium was stirred until the total O_2 concentration ($^{36}O_2$ + $^{32}O_2$) reached about 250 μM (about 21% O_2). The bubble was removed and a aliquot of the concentrated algal suspension adapted to the corresponding NaCl concentration was injected into the chamber to make a final chlorophyll content of 1–3 μg/ml. Thereafter steady state dark respiration was obtained following the decrease in the O_2 electrode signal.

The light dependent experiments were carried out at DIC saturation for photosynthesis. Therefore, 200 μM DIC (Aizawa and Miyachi 1984) was introduced into the chamber in the dark. Light intensity was increased from darkness to 1300 μmol photons $m^{-2}s^{-1}$ by using neutral density filters (Balzers Limited, Liechtenstein; Sültemeyer et al. 1986). Each PFR was applied for 3 min. The rates of gross O_2 evolution and uptake were calculated according to (Radmer and Ollinger 1980) from the increase in the 32-signal (m/e = 32; $^{32}O_2$ concentration) and decrease in the 36-signal (m/e = 36; $^{36}O_2$ concentration), respectively. After the highest PFR the light was switch off and dark respiration was measured again for at least 5 min. Finally. an aliquot of cell suspension was removed from the cuvette and the chlorophyll content was determind after extraction in 100% methanol over night according to (McKinney 1941). The complete gas-exchange experiment from darkness before illumination over 8 light intensities to darkness after illumination took about 40 min. Four separate experiments were carried out and the data in Fig. 1-4 represent the mean values of the four individual experiments.

Results

Figure 1 shows the influence of PFR on NET at five different salinities. From Fig. 1A it is obvious that NET depends on both, PFR and osmotic stress. Light compensation increased with increasing NaCl concentration in the medium from 35 (0.5 M) to 98 μmol photons $m^{-2}s^{-1}$ (2.5 M). With increasing PFR and NaCl content in the external medium NET increased leading to a difference in NET of more than 300 μmol O_2 mg Chl^{-1} h^{-1} at light saturation between the lowest (0.5 M) and highest (2.5 M) salt concentration. Light saturated rates of NET were reached at a PFR of about 1000 μmol $m^{-2}s^{-1}$ for all salinities tested.

In Fig. 1B, the data on NET for weak PFR (up to 100 μmol $m^{-2}s^{-1}$) from Fig. 1A have been enlarged. The graph shows that the lower PFR protions of the O_2 responses are not linear. At 0.5 M the curve bends at 10 μmol $m^{-2}s^{-1}$, at 1.0 M there was almost no bend, while at 1.5 M, 2.0 M and 2.5 M the bend was more pronounced at 20 μmol $m^{-2}s^{-1}$.

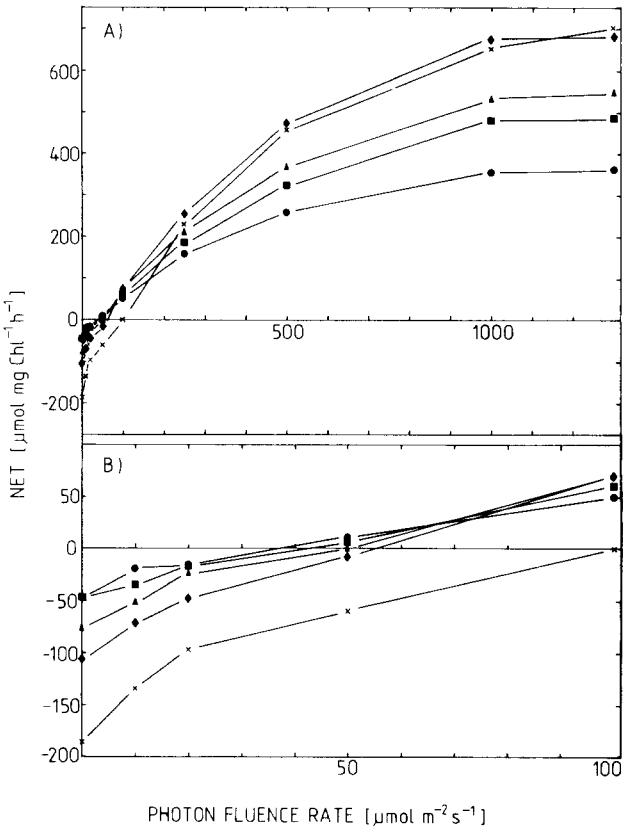

Fig. 1. The influence on photon fluence rate (PFR) on net O_2 exchange (NET) at 0.5 M (●), 1.0 M (■), 1.5 M (▲), 2.0 M (x) and 2.5 M (◆) NaCl. A) Includes the full range of PFR from 0 to 1300 μmol m^{-2} s^{-1} B) Expanded view at low PFR showing the changes in greater detail. Values are the means from four separate experiments with SE not greater than 10%. For conversion of rates from mg chlorophyll to packed cell volume units see 'Materials and methods'.

The rates of gross O_2 evolution (E_0) in relation to PFR at different salt concentrations are compared in Fig. 2. As was the case with NET (Fig. 1), the greater the salinity in the external medium the higher E_0 and consequently the rate of photosynthetic electron flow. At a PFR of 1300 μmol m^{-2} s^{-1} the difference in E_0 between 0.5 M and 2.5 M was more than 400 μmol O_2 mg Chl^{-1} h^{-1}. In contrast to the NET curves, no delay of E_0 was observed at the lower light intensities so that E_0 followed normal light saturation kinetics. In none of the experiments was E_0 completely saturated, even at a PFR of 1300 μmol m^{-2} s^{-1}.

The complex interactions between PFR and the NaCl content in the external medium of U_0 are shown in Fig. 3. An increase in the salinity,

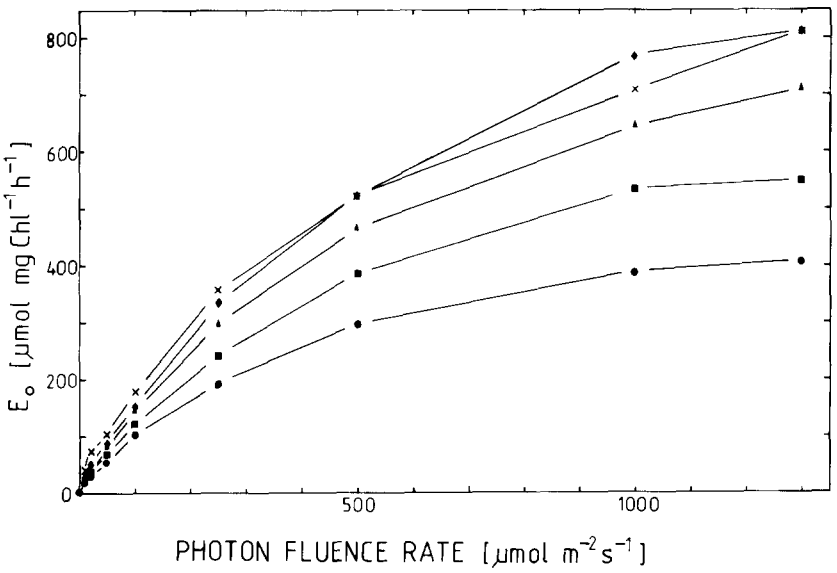

Fig. 2. The influence of photon fluence rate (PFR) on gross O_2 evolution (E_0) at 0.5 M (●), 1.0 M (■), 1.5 M (▲), 2.0 M (×) and 2.5 M (◆) NaCl. Values are the means from four separate experiments with SE not greater than 10%. For further details see legend of Fig. 1.

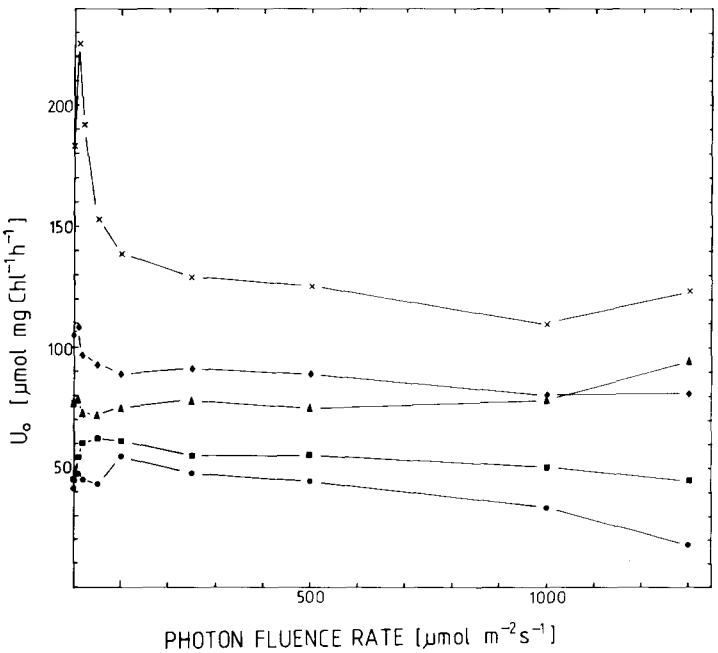

Fig. 3. The influence of photon fluence rate (PFR) on gross O_2 uptake (U_0) at 0.5 M (●), 1.0 M (■), 1.5 M (▲), 2.0 M (×) and 2.5 M (◆) NaCl. Values are the means from four separate experiments with SE not greater than 10%. For further details see legend of Fig. 1.

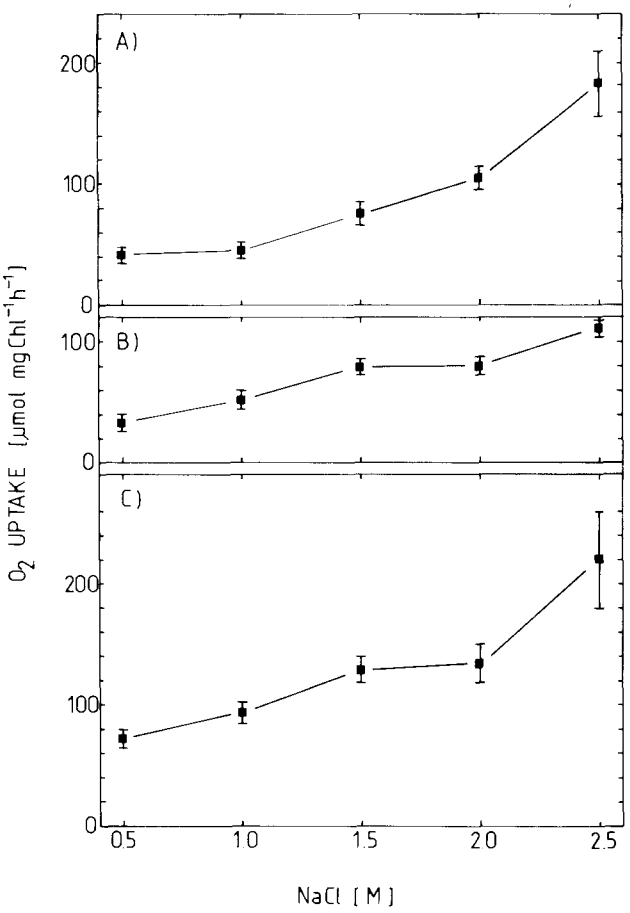

Fig. 4. Comparison of A) steady state O_2 uptake before illumination (DR_b), B) O_2 uptake in the light (U_0) at a PFR of 1000 µmol $m^{-2}s^{-1}$ and C) O_2 uptake immediately after darkening (DR_a). Bars indicate +/− SE, n = 4. For futher details see legend of Fig. 1.

generally resulted in an increase in U_0. For all salinities tested weak light (10–20 µmol photons $m^{-2}s^{-1}$) stimulated U_0 resulting in a maximal U_0 value. Further increase of PFR, however, had only a small effect on U_0 at low salt concentrations (e.g. at 1.0 and 1.5 M) or caused a inhibition of U_0 at high NaCl content (e.g. 2.0 and 2.5 M NaCl).

In Fig. 4 the rates of dark respiration and O_2 uptake in the light were compared. With increasing salinity steady state dark respiration (DR_b) increased. At 2.5 M NaCl, DR_b was almost five times the rate at 0.5 M NaCl (Fig. 4A). The rates of gross O_2 uptake at light saturation (1000 µmol $m^{-2}s^{-1}$) are shown in Figure 4B. An important observation was that U_0 paralleled steady state dark respiration and that U_0 was significantly lower than DR_b, especially at higher salinities. That this is also true at other

Table 1. Comparison of the ratios of U_0/DR_b, U_0/E_0 and DR_b/E_0 at different salt concentrations.

	Salt concentration				
Ratios	0.50	1.00	1.50	2.00	2.50
U_0/DR_b[a]	0.80[b]	1.10	1.01	0.74	0.59
U_0/E_0	0.09	0.09	0.12	0.10	0.16
DR_b/E_0	0.10	0.08	0.12	0.14	0.26

[a] U_0 and E_0 were obtained at light saturation (1000 μmol $m^{-2} s^{-1}$).
[b] Standard error for all values was not greater than 0.03 (n = 4).

PFR can be seen from Fig. 3. These results become even more obvious by comparison of the U_0/DR_b ratios (Table 1) where these ratios reached unity at 1.0 and 1.5 M NaCl but decreased to at least 0.59 at 2.5 M NaCl. Upon darkening, the rate of dark respiration measured immediately after illumination increased to the DR_a value which was always higher than DR_b and U_0 for all salinities tested but again mirrored O_2 consumption in the light (Fig. 4C).

For all salinites and at saturating PFR the U_0/E_0 ratios were very small and never exceeded 0.20 (Table 1). The observed increase in this ratio with increasing salt concentration is accompanied by an increase in the DR_b/E_0 ratio.

Discussion

Net oxygen exchange (NET)

The observed increase in NET with increasing salt concentrations in the medium (Fig. 1) is in accordance with earlier results (Ben-Amotz and Avron 1972, Frank and Wegmann 1974) but contradicts those reported by Gilmour et al. (1984) and Wegmann (1977). The main reason for these discrepancies probably is that the latter authors used non-adapted cells for their measurements of salt dependent photosynthetic activity thus recording stress characteristics. As pointed out earlier (Frank and Wegmann 1974, Latorella and Vadas 1973), adaptation of *D. tertiolecta* to another salt concentration may take several days. Therefore, we used the pre-culture procedure (see Materials and Methods) to ensure complete adaptation of the cells to all salinities.

The rise in the compensation point in relation to salt concentration is probably due to an increase in the rate of mitochondrial O_2 uptake at higher salinities (Fig. 4A). Reduction in the slope of the net O_2 response curves after 20 μmol photons $m^{-2} s^{-1}$ (Fig. 1b) has also been reported from other labora-

tories (Falkowski and Owens 1978). The differences in the absolute rates between Falkowski's and Owens' data and those reported here are probably due to the lower temperature (15 °C) during their experiments. The general trends are the same. Because E_0 does not show any delay or transient from dark to light for any NaCl concentration (Fig. 2), it is suggested that the small increases in NET at low light intensities (Fig. 1) are mainly caused by an enhanced O_2 consumption observed at these low PFR (Fig. 3).

Falkowski and Owens (1978) also recorded an hysteresis effect during net O_2 measurements by *D. tertiolecta*. They considered that it was the result of the altered spectrum of the light source, when power was reduced. We suggest that the hysteresis observed was the effect of increased respiration following a period of irradiation, because the higher DR_a (Fig. 4C) continued for 10–20 minutes after darkening (data not shown) until it declined to the steady state levels of dark respiration (DR_b) within one hour.

Gross oxygen evolution (E_0)

Although growth rates are suppressed at high salinities (data not shown), E_0, as a measure for the rate of photosynthetic electron flow, is increased in relation to the salt concentration when expressed in $\mu mol\, O_2\, mg\, Chl^{-1}\, h^{-1}$ but also in $\mu mol\, O_2\, h^{-1}$ (ml packed cell volume)$^{-1}$ (Fig. 2). This could be explained as follows. Firstly, the demand for glycerol by cells grown at higher salinities may be enhanced (Frank and Wegmann 1974, Borowitzka et al. 1977, Ben-Amotz et al. 1982). Glycerol is accumulated within the cells in an amount directly proportional to the external salt concentration (Avron 1986). Moreover, using ^{14}C-tracer techniques it has been demonstrated that glycerol is an early photosynthetic product (Craigie and McLachlan 1964, Wegmann 1971, Frank and Wegmann 1974) and that it is synthezised by reduction of DHAP (Ben-Amotz et al. 1982). The required reduction equivalents are generated from the water-splitting reactions of photosynthesis (Kaplan et al. 1980). Together, these results indicate that light generated electrons are needed to produce DHAP (via CO_2 fixation) and then to reduce DHAP to glycerol.

For the second possibility of enhanced E_0 the metabolic pathway of glycerol biosynthesis and degradation may be considered (Ben-Amotz et al. 1982). Within fully salt-adapted cells the glycerol pool will be in a steady state concentration, where the synthesis of glycerol is as fast as its breakdown. During steady state only ATP is consumed by dihydroxyacetone kinase which is thought to act on the degradation side of the glycerol pathway (Lerner et al. 1980, Ben-Amotz et al. 1982). Consequently, following these two explanations, with an increased demand for glycerol (e.g. increased salinity), photosynthesis supplies both reducing aquivalents and ATP for the

glycerol pathway and therefore, E_0 should increase with increasing salt concentration in the external medium (Fig. 2).

Oxygen uptake by photorespiration

Several mechanisms including oxygenation of RubP, O_2 reduction and mitochondrial O_2 consumption may contribute to U_0 (Canvin et al. 1980, Peltier and Thibault 1985b, Sültemeyer et al. 1986). However, the RubP oxygenase reaction was completely inhibited, since all experiments were carried out under DIC saturation (200 mM DIC; Fock et al. 1981, Aizawa and Miyachi 1984, Sültemeyer and Fock 1986). Therefore, the observed U_0 (Fig. 3) is mainly composed of mitochondrial O_2 uptake and O_2 reduction.

Oxygen uptake by O_2 reduction (Mehler reaction)

For all salinities tested U_0 remained either unchanged (1.0 and 1.5 M) or was suppressed below DR_b (0.5, 2.0 and 2.5 M) by light (Fig. 3). Under the same conditions, E_0 and, therefore, the flow of electrons simultaneously increased (Fig. 2). The light dependent inhibition of U_0 was in disagreement with the findings reported from higher plants (Canvin et al. 1980) and eucaryotic algae (Fock et al. 1981, Brechignac and Furbank 1986, Sültemeyer et al. 1986), where U_0 was stimulated by light and several fold higher than the rate of steady state dark respiration. In the case of *Chlamydomonas reinhardtii* Sültemeyer et al. (1986) reported an U_0/E_0-ratio which was 3–4 times higher than in *Dunaliella* at light saturation (Table 1). It was argued that this high ratio reflects an enhanced rate of O_2 reduction (e.g. Mehler reaction) which could provide extra ATP (pseudocyclic phosphorylation) for the CO_2/HCO_3^- concentrating mechanism. *Dunaliella*, however, uses DHAP formed in the light as an alternative electron acceptor to synthesize glycerol (Frank and Wegmann 1974, Borowitzka et al. 1977, Ben-Amotz et al. 1982). This could lead to a competition between O_2 and DHAP for light generated electrons. In addition, from the glycerol pathway (Ben-Amotz et al. 1982) it follows that glycerol synthesis via CO_2 fixation needs 3 $NADPH_2$ and 4 ATP. If we assume an average value of 1.33 ATP/2e in the non-cyclic photosynthetic electron flow (Gimmler 1977) then no extra energy is required for glycerol production. Therefore, we suggest that photosynthetically generated electrons are mainly channeled to DHAP and not to O_2. This may lead to a small contribution of O_2 reduction to U_0, especially at high salt concentrations. Consequently, it appears that the observed U_0 is mainly composed of mitochondrial O_2 consumption.

Oxygen uptake by mitochondrial respiration

The increase in steady state dark respiration (DR_b; Fig. 4A) in relation to

the adaptation to higher salinities has been reported earlier (Frank and Wegmann 1974, Gilmour et al. 1984). It probably reflects the formation of glycerol which also occurs in the dark (Borowitzka et al. 1977, Gilmour et al. 1982) and the energy required to ensure a low Na^+ concentration within the cells (Ben-Amotz and Avron 1972, Latorella and Vadas 1973, Avron 1986, Pick et al. 1986). Measurements of dark respiration immediately after illumination (DR_a) exhibited an almost two fold acceleration of O_2 consumption compared with DR_b (Fig. 4A, C) but declined to the basal rate of steady state dark respiration within an hour (data not shown). These results support the view that dark respiration depends on carbohydrate provided by photosynthesis and that steady state dark respiration is substrate limited (Azcon-Bieto et al. 1983, Furbank and Rebeille 1986).

The view of a persistent mitochondrial O_2 uptake in the light under in vivo conditions is further supported by the similar trends (but different rates) between U_0 and DR_b at several salt concentrations (Fig. 4). Consequently, the reduction of U_0 (Fig. 3) is probably caused by a partial suppression of mitochondrial O_2 consumption in the light. The light dependent inhibition of U_0 reached values between 50–70% of steady state dark respiration at 2.0 and 2.5 M NaCl, respectively. Thus reports on a continuing rate of mitochondrial O_2 uptake in the light by eucaryotic algae treated with potent inhibitors of the photosynthetic and respiratory chain (Falkowski and Owens 1978, Peltier and Thibault 1985b, Brechignac and Furbank 1986) which may change the ATP/ADP ratio (Jacobus et al. 1981) have been confirmed and extended to an in vivo system.

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