Light and Calcium Interactions in Chlorella Inhibited by Sodium Chloride\textsuperscript{1,2}

PHROSENE E. CHIMIKLIS\textsuperscript{3} AND EDWARD P. KARLANDER
Department of Botany, University of Maryland, College Park, Maryland 20742

Received for publication June 5, 1972

ABSTRACT

Analysis of NaCl toxicity in Chlorella sorokiniana showed decreased growth rates, increased dry weight per cell, increased intracellular Na\textsuperscript{+} and Cl\textsuperscript{−}, and more total chlorophyll per cell, a decreased chlorophyll a to chlorophyll b ratio, increased rates of O\textsubscript{2} evolution, and decreased rates of CO\textsubscript{2} fixation when the extracellular concentration of NaCl was increased from zero to 0.3 M. Cultures did not grow at concentrations greater than 0.3 M NaCl unless 10 mM calcium salts were present. Inclusion of that concentration of Ca\textsuperscript{2+} extended the tolerance to 0.5 M NaCl before growth stopped. Increasing the light intensity from 1.2 to 9.4 mw/cm\textsuperscript{2} increased growth rates for cultures in 0.10 to 0.45 M NaCl. At 14 mw/cm\textsuperscript{2} added Ca\textsuperscript{2+} reduced growth rates of cultures in 0.3 M NaCl compared to controls without added Ca\textsuperscript{2+}. Maximal growth rates for cultures in NaCl media were achieved by 10 mM CaSO\textsubscript{4} and maintenance of the light intensity at 9.4 mw/cm\textsuperscript{2}. The maximal growth rate of the organism was 9.6 doublings/day achieved at 2.7 mw/cm\textsuperscript{2} for control cultures. In 0.3 M NaCl the growth rate was 4.3 doublings/day at 2.7 mw/cm\textsuperscript{2} and 8.2 doublings/day at 9.4 mw/cm\textsuperscript{2} with 10 mM CaSO\textsubscript{4} added.

Increasing light intensities from 2.7 to 9.4 to 14 mw/cm\textsuperscript{2} decreased intracellular Na\textsuperscript{+} in cells cultured in 0.3 M NaCl medium without added Ca\textsuperscript{2+} and increased Cl\textsuperscript{−} uptake in cells cultured in 0.3 M NaCl medium with and without added Ca\textsuperscript{2+}. For cells cultured in 0.3 M NaCl medium at 14 mw/cm\textsuperscript{2} intracellular Na\textsuperscript{+} was 0.68 meq/g dry weight with Ca\textsuperscript{2+} added and 0.81 meq/g dry weight without Ca\textsuperscript{2+} added. Addition of Ca\textsuperscript{2+} at 2.7 mw/cm\textsuperscript{2} reduced intracellular Na\textsuperscript{+} to similar values. It is postulated that energy requirements for active Na\textsuperscript{+} exclusion were reduced by addition of Ca\textsuperscript{2+} allowing more energy to be used for cell growth resulting in increased growth rates.

O\textsubscript{2} evolution and CO\textsubscript{2} fixation studies indicated that increased photosynthetic energy, probably actuated by a high proton gradient accompanying Cl\textsuperscript{−} influx and uncoupled from CO\textsubscript{2} fixation, was available for maintenance of cellular integrity and active control of intracellular ionic ratios. The O\textsubscript{2} evolving capacity was destroyed at 12 and 29 mw/cm\textsuperscript{2} for cells cultured in 0.3 M NaCl medium respectively with and without addition of Ca\textsuperscript{2+}. Control cultures continued producing O\textsubscript{2} at light intensities up to 115 mw/cm\textsuperscript{2}.

Physiological mechanisms of salt tolerance have been primarily concerned with ionic compositions and fluxes. Regulation of ionic composition in marine algae consists of Na\textsuperscript{+} exclusion with accumulation of K\textsuperscript{+} and Cl\textsuperscript{−} and generally requires cellular energy and the presence of Ca\textsuperscript{2+} (27). Studies of ionic fluxes in the red marine alga Gracilaria foliifera (12), in the fresh water algae Hydrodictyon africanum (23) and Chlorella pyrenoidosa (2), and on the internodal cells of Nitella transluens (18) have demonstrated light-dependent active transport for Na\textsuperscript{+} exclusion and K\textsuperscript{+} and Cl\textsuperscript{−} accumulation. Work on the red marine alga Porphyra perforata showed a requirement for Ca\textsuperscript{2+} in the medium for intracellular retention of K\textsuperscript{+} (7).

Two considerations indicate that light-dependent ion fluxes in green plants are controlled by photosynthetic rather than respiratory energy. First, light generally has little effect on ion fluxes in nongreen tissue, and second, the action spectrum of ion fluxes in green tissue matches that of chlorophyll absorption (23). The activation of K\textsuperscript{+} and Cl\textsuperscript{−} fluxes in Nitella was separated respectively between photosynthetic systems II and I (18). Under conditions of either DCMU inhibition or far red light irradiation, where system I was functional, K\textsuperscript{+} absorption was not affected and Cl\textsuperscript{−} absorption was reduced compared to conditions where both photosystems were operative (2, 18, 24).

The selective participation of these systems was also reported in Chlorella pyrenoidosa (2) and Hydrodictyon africanum (24). System I was also implicated in Na\textsuperscript{+} influx (24).

Direct involvement of ATP from cyclic phosphorylation for light-dependent Na\textsuperscript{+} and K\textsuperscript{+} fluxes was established by using uncouplers of phosphorylation. Addition of carbonyleanide-\textit{m}-chlorophenylhydrazone (2, 17, 25, 28) and imidazol (18) to algae resulted in decreased fluxes of Na\textsuperscript{+} and K\textsuperscript{+}, while Cl\textsuperscript{−} uptake was either unaffected or only slightly inhibited. Under CO\textsubscript{2}-free air, N\textsubscript{2}, or O\textsubscript{2} there was no inhibition of Na\textsuperscript{+} and K\textsuperscript{+} fluxes in these algae (18, 24, 28). This suggests the absence of an O\textsubscript{2} or of a CO\textsubscript{2}-fixation requirement in the active flux mechanisms of Na\textsuperscript{+} and K\textsuperscript{+}.

Several approaches have been used to study the mechanism of energy participation in Cl\textsuperscript{−} uptake. Uncouplers of phosphorylation had little or no effect on Cl\textsuperscript{−} uptake (2, 9, 17, 18), suggesting either no ATP involvement or a small noncyclic demand for its transport. Several inhibitors of electron transport however, did reduce the Cl\textsuperscript{−} fluxes, indicating some involvement with electron flow within the energy apparatus and perhaps a requirement for both systems. Cl\textsuperscript{−} influx was inhibited by the absence of O\textsubscript{2} (17, 24). Raven (24) has suggested this requirement may indicate the participation of pseudocyclic electron transport with Cl\textsuperscript{−} uptake. Energy requirements would still involve system II excitation, but utilization would be at some point between electron transport from system II through system I to molecular O\textsubscript{2}.

Other studies of ion fluxes reported an inward movement...
of protons associated with efflux of Na\(^+\) and other cations of marine algae (3, 4). Proton uptake, associated with the illumination of isolated chloroplasts presumably into the intralamellar spaces, is thought to build an electrochemical gradient across the lamellar membrane and to be one component of the driving force for ATP formation (15, 19). The involvement of photosynthesis appears to bear two relationships to this phenomenon. It is suggested that proton influx in whole cells which, along with O\(_2\) evolution, is inhibited by DCMU (2) is also associated with ATP formation. In *Ulua* (4), there is a requirement for an influx of HCO\(_3^-\), accompanying the influx of protons which presumably supplies CO\(_2\) for dark reactions of photosynthesis.

An actual demand for increased photosynthetic energy may not be a valid interpretation of a mechanism for salt tolerance as the accumulation of carbohydrates observed in plants exhibiting salt toxicity may indicate that photosynthesis is not limiting growth (10). Growth inhibition may be related to the inability of these plants to utilize photosynthates.

Several investigators reported that salt toxicity resulted in decreased respiration, whereas others reported increased respiration in less salt-tolerant species (13, 14, 21). Further observations on metabolic changes related to salt toxicity are decreased activity of the glycolytic pathway with a concomitant increase in the pentose pathway and increase in NADPH + NADP: NADH + NAD levels (13, 14, 31).

The purpose of this investigation was to study the adaptive mechanisms in *Chlorella sorokiniana* Shihira and Krauss to high levels of NaCl. This high temperature, fresh water species of *Chlorella* was found to be more tolerant to various dilutions of sea water than other *Chlorellas* examined (11). Various investigators have reported both increased and unchanged photosynthetic activity in response to NaCl, even though there was a toxic effect on growth (21). Part of this research investigated the extent to which increasing light intensities could be utilized by this alga to overcome NaCl toxicity. In some plants the combination of NaCl and high intensities have been injurious to plant growth. For example the application of NaCl to *Phaseolus vulgaris* resulted in stimulated growth at lower intensities, whereas at higher intensities growth was suppressed (22). Also the observation that marine phytoplankton exhibit optimal growth at relatively low light intensities may be further evidence of inhibition from a combination of NaCl and high light intensities (6).

**MATERIALS AND METHODS**

**Growth Conditions.** Stock cultures of *C. sorokiniana* Shihira and Krauss (29) were grown in inorganic medium consisting of the following nutrients in g per liter: KNO\(_3\), 1; MgSO\(_4\)·7H\(_2\)O, 0.25; KH\(_2\)PO\(_4\), 1; KHPO\(_4\), 0.25; MoO\(_3\), 0.0015; EDTA Na\(_2\)Fe, 0.0385; EDTA Na\(_2\)Mn, 0.0071; EDTA Na\(_2\)Co, 0.0077; EDTA Na\(_2\)Zn, 0.0093; EDTA NaCu, 0.0077; EDTA Na\(_4\)Sn, 0.0067. Other media used consisted of CaCl\(_2\), Ca(NO\(_3\))\(_2\), or CaSO\(_4\), all at 10 mm and various concentrations of NaCl incorporated into the inorganic medium. Sterilization of NaCl media with and without Ca salts was accomplished by passing it through a presterilized Millipore filter apparatus containing a Millipore filter with a pore diameter of 0.45 \(\mu\)m. Inorganic medium was sterilized by autoclaving at 138 km/min and 121 C for 20 min.

 Cultures were grown in test tubes measuring 23 × 250 mm placed in a lucite water bath maintained at 38 ± 1 C. The test tubes were closed with cotton plugs through which cotton-plugged glass bubbling tubes were placed to provide the culture with a 1% CO\(_2\)-in-air mixture and to keep the cells in suspension.

 Experimental cultures were grown at four light intensities: 1.22, 2.70, 9.40, and 14.0 mw/cm\(^2\) as measured with a YSI Kettering Model 65 radiometer placed inside the water bath. Light was provided by two banks of cool-white and power-groove, General Electric lamps F48 T-10-CW and F48 PG 17-CW. The higher intensity of 14.1 mg/cm\(^2\) was attained by using one air fan to cool each bank. Light intensities were reduced by placing wire screens and cheesecloth between the lamps and water bath.

 Inoculation into NaCl media from cultures growing in either inorganic medium or NaCl medium produced no observable differences in final growth rates or concentration of NaCl tolerated, so both methods were utilized. Constant growth rates were reached approximately 4 days after serial transfers into NaCl medium. Cultures were then considered adapted and used in further investigations. Experimental cultures were maintained in exponential growth by serially transferring to fresh medium when the absorbance reached 0.6 for cells grown in inorganic medium, and 0.4 for cells grown in NaCl media. Absorbance was measured at 550 nm in 23 mm diameter tubes in a Coleman junior spectrophotometer. Experimental cultures were inoculated initially to at least 0.1 absorbance for those grown in NaCl medium and not less than 0.05 absorbance for those grown in inorganic medium. Growth rates were based on absorbance measurements.

 **Analysis of Na\(^+\) and Cl\(^-\).** Samples of *Chlorella* cells were prepared for Na\(^+\) and Cl\(^-\) analysis by filtering 15-ml portions of a culture using a Millipore Filter of 0.45 \(\mu\)m-pore diameter. The cells retained on the filter were rinsed twice with 15 ml of distilled water. Comparisons of Na\(^+\) concentrations and number of washings showed this 30-sec procedure removed extracellular and not intracellular ions. Na\(^+\) analyses were performed by digesting cells and filter with fuming nitric acid and 72% perchloric acid according to the procedure of David (5). Two blank samples were included in this procedure. The filter, with cells retained from 15 ml of culture, was placed in 15 ml of nitric acid and allowed to digest for 1 hr. Then 2 ml of perchloric acid were added, and the solution was mixed and slowly heated on a hot plate. The samples were allowed to evaporate to about 2 ml and then made to 25 ml with distilled water. Na\(^+\) concentrations of both cell and medium samples were determined using a Na\(^+\) hollow cathode lamp in a Model 303 Perkin-Elmer atomic absorption spectrophotometer. A standard curve of absorbance against concentration was prepared from known concentrations. Concentrations of experimental samples were determined from this curve.

 Intracellular Cl\(^-\) was determined from samples prepared in the same manner as those for Na\(^+\) analysis. Cells and filter were placed in a 10-ml beaker and allowed to digest in a solution of 0.1 N HNO\(_3\) in acetic acid. Samples were then titrated with a Buchler-Cutlove chlorideidometer, and concentrations were determined from known values of standard solutions.

 Dry weights were measured using 25-ml samples centrifuged on an international centrifuge at 3500g for 15 min. Two washings were used in each determination. Cell numbers were obtained by averaging three separate totals on a 1 mm field of an American Optical Company bright line S-5145 hemacytometer.

 **Rates of Oxygen Exchange.** Rates of oxygen evolution were obtained from samples taken from the exponential phase of growth of cultures grown at 2.7 mw/cm\(^2\). Samples were placed in a 1-ml chamber maintained at 38 ± 0.004 C by a Model FS Haake constant temperature circulator. Light was provided by a 500-W Sylvania CZA projection lamp. Infrared and ultraviolet wavelengths were removed by passing the light through a glass water bath and a 5% CuSO\(_4\) solution. Variations in light intensity were obtained by placing neutral
density and wire screen filters between the cell chamber and the CaSO₄ solution. Cells were exposed to alternate periods of light and dark. Light exposures were not greater than 4 min and dark periods varied from 4 to 10 min. Rates of O₂ evolution and uptake were monitored by an oxygen electrode positioned within the cell chamber. The Clark type electrode was calibrated in the appropriate solution for each experiment. The signal from the electrode was amplified and recorded by a VOM 5 Bausch and Lomb recorder. Concentrations of O₂ were determined by comparing experimental values with those of known concentrations in distilled water. Rates of photosynthetic O₂ evolution were calculated from those observed in the light with corrections for respiration using respiration rates observed in the dark immediately following the light period.

Rates of CO₂ Fixation. Relative rates of CO₂ fixation were determined for cultures growing in inorganic medium and in 0.3 M NaCl medium. Triplicate samples of these cultures grown at 2.7 mw/cm² were incubated at 38 ± 1°C with 3°C-NaHCO₃ in lucite culture baths for 10 min at the four light intensities used for growth rate determinations (1.2, 2.7, 9.4, and 14.0 mw/cm²). The cells were collected on a 0.45 μm pore-diameter Millipore filter and washed twice with distilled water using 15 ml each time. Relative rates at each light intensity were determined from the average of two 10-min counts for each sample using a Tracer Lab SC - 19A utility scaler with an end window (2.0 mg/cm²) Geiger tube in an SC-10A sample holder. Photosynthetic rates of CO₂ fixation were corrected for background and respiration.

Chlorophyll. Samples (25 ml) of cell suspensions were taken directly from cultures in their exponential phase of growth and centrifuged on an International centrifuge at 3500g for 15 min. The pellet was then resuspended in a pigment extracting solvent (26) of methanol-dimethylsulfoxide 4:1 v/v and placed in a dark cold room for 20 min. This solution was then centrifuged for 10 min and the supernatant containing the pigments decanted into a separatory funnel containing 25 ml of diethyl ether. The remaining pellet was again resuspended in the extracting solvent, centrifuged, and decanted. The total chlorophyll was washed into ether with approximately 1 liter of about 2% saline solution. Following washing the chlorophyll-ether solution was dried over anhydrous Na₂SO₄ for 20 min. The dried chlorophyll solution was then made to 25 ml volume with anhydrous ether. Absorbance of the chlorophyll extract was determined on a Model 14 Cary recording spectrophotometer. Absorbance values at 662 nm and 644 nm were obtained and substituted in the formula of Smith and Benitez (30).

RESULTS AND DISCUSSION

Growth. Salt tolerance in C. sorokiniana was initially measured by observing the highest concentrations of NaCl in which the alga would grow at a light intensity of 2.7 mw/cm². At this light intensity and without NaCl added to the inorganic medium, the alga maintained its maximal growth rate at 9.6 doublings per day (Fig. 1A). Growth rates of cultures adapted to three concentrations of NaCl (0.1 M, 0.2 M, 0.3 M) in inorganic medium decreased as the concentration of NaCl increased. The growth rate in 0.3 M NaCl medium was 3.4 doublings per day. Cultures in 0.35 M NaCl medium did not grow and were bleached after 3 days.

Although Ca²⁺ was present in the inorganic medium as a micronutrient at 1 μg/ml its addition at 400 μg/ml (the same concentration of Ca²⁺ as found in oceanic sea water) increased the NaCl tolerance of C. sorokiniana. Growth rates of cultures adapted to 10 mM CaCl₂, NaNO₃, or CaSO₄ in 0.3 M NaCl medium showed stimulated growth in varying degrees relative to cultures growing without the addition of Ca salts (Fig. 1B). The conditions of maximal antagonism to NaCl toxicity were 9.4 mw/cm² light intensity and the addition of 10 mM CaSO₄. Inclusion of 10 mM CaSO₄ in inorganic medium also extended the concentration of NaCl tolerated by cultures of C. sorokiniana (Fig. 1C). Growth rates were examined at concentrations of 0.35 M, 0.45 M, and 0.5 M NaCl media. Cultures did not grow in 0.55 M NaCl medium. In 0.5 M NaCl medium cells grew very slowly, 0.5 doublings per day and only below 2.7 mw/cm² of light. This phenomenon, of increasing salt tolerance by addition of Ca salts, has been reported for the higher plant Phaseolus vulgaris (17).

The effects of increasing light intensity on salt toxicity in C. sorokiniana were also investigated. Growth rates of cells in several concentrations of NaCl without 10 mM CaSO₄ were examined at four light intensities (Fig. 1A). With no CaSO₄ added to the medium increases in light intensity from 1.2 to 2.7 to 9.4 mw/cm² resulted in increased growth rates. An increase to 14 mw/cm² produced no further increase in growth rates at any concentration of NaCl examined. Without addition of Ca²⁺ to the medium growth rates were the same at 14 mw/cm² as they were at 9.4 mw/cm². In 0.1 M NaCl medium the growth rate of the organism was 9.6 doublings per day—the same as cultures in control conditions where no NaCl was added. However, this salt culture was different from the control, because exponential growth could not be maintained at absorbance values greater than 0.5 while the control cultures grew exponentially to an absorbance of at least 1.0. The growth rate for cultures adapted to 0.30 M NaCl medium was 6.3 doublings per day at 9.4 and 14 mw/cm².

Increases in light intensity from 1.2 to 2.7 to 9.4 mw/cm² for cells cultured in NaCl medium to which CaSO₄ was added also resulted in increased growth rates (except for cultures in 0.5 M NaCl) (Fig. 1, B and C). At 9.4 mw/cm² cultures in 0.3 M NaCl medium maintained growth rates of 8.2 doublings per day, and those in 0.45 M NaCl medium maintained rates of 4.7 doublings per day. At 14 mw/cm² growth rates of cultures with 10 mM CaSO₄ decreased below those observed at 9.4 mw/cm².

The observed increase in growth rates for cultures in NaCl media as light intensity was increased above light saturation of growth for cultures in inorganic medium suggested the utilization of increased amounts of light energy for maintaining cellular integrity. Growth of cultures in NaCl media stimulated by increased light energy was enhanced by the addition of CaSO₄. As observed in the 0.3 M culture at 9.4 mw/cm² the addition of CaSO₄ increased the growth rate from 6.3 to 8.2 doublings per day but in the absence of CaSO₄, the growth rate at 9.4 and 14.0 mw/cm² was 6.3 doublings per day. The greatest stimulation of growth observed at concentrations of 0.3 M to 0.45 M NaCl was achieved by the addition of 10 mM CaSO₄, at a light intensity of 9.4 mw/cm². At 14 mw/cm² growth was inhibited with 10 mM CaSO₄ in the medium.

Changes in light intensity produced changes in the dry weight per cell for cultures growing in inorganic medium and in 0.30 M NaCl medium with and without 10 mM CaSO₄, (Fig. 2). Dry weight per cell increased for cultures growing in NaCl with and without 10 mM CaSO₄, compared to cultures growing without NaCl. This increase for cultures in NaCl medium was observed at all four light intensities and was greatest at 1.2 mw/cm² for cultures in NaCl without CaSO₄. The smallest dry weight per cell for each of the three culture media occurred at 2.7 mw/cm²—the intensity which was growth saturating for cultures in inorganic medium.

Intracellular Na⁺ and Cl⁻. Both Na⁺ and Cl⁻ increased in
Fig. 1. Growth rates of *Chlorella sorokiniana* grown at 39 °C in white light of 1.2, 2.7, 9.4, and 14 mw/cm² in inorganic medium and in NaCl medium with and without 10 mM CaSO₄.
intracellular concentration as the extracellular concentration of NaCl was increased (Fig. 3). This observed accumulation of ions indicated the presence of a highly permeable membrane in *C. sorokiniana* analogous to those found in marine algae (3). A high degree of permeability permits accumulation of ions in response to increased external osmotic pressure and prevents a plasmolytic reaction. Reduced growth rates observed with cultures in NaCl medium could have been due either to ion toxicity or diversion of energy normally used for growth into the control of ionic ratios, as in marine algae or some combination of these mechanisms.

Analyses of intracellular Na\(^+\) at four light intensities with and without the addition of 10 mM CaSO\(_4\), to cultures in 0.3 mM NaCl medium are presented in Figure 4A. For cultures growing in 0.3 mM NaCl medium the concentrations of intracellular Na\(^+\) decreased as light intensity was increased. At 1.2, 2.7, and 9.4 mw/cm\(^2\) the addition of Ca\(^{++}\) to 0.3 mM NaCl medium reduced the intracellular Na\(^+\) concentration of the cells. At 2.7 mw/cm\(^2\) addition of Ca\(^{++}\) reduced the intracellular concentration of Na\(^+\) to the lowest level observed. A further increase in light intensity produced no further decrease in intracellular Na\(^+\).

Decreased intracellular Na\(^+\) in response to increased light intensities, observed in the absence of added Ca\(^{++}\), suggested the functioning of an energy-dependent pump for Na\(^+\) exclusion. With the addition of Ca\(^{++}\) the light energy required for Na\(^+\) exclusion was much lower. Ca\(^{++}\) may have acted either to decrease passive permeability to Na\(^+\) or to enhance the utility of some ionic exclusion process. Decreased passive permeability would reduce the Na\(^+\) accumulation. Less energy would be required for active efflux leaving more energy available for cell growth. This was substantiated by the increased growth rates observed at all but the highest light intensity when Ca\(^{++}\) was added to salt cultures.

Concentrations of intracellular Cl\(^-\) appeared to be related to several factors. At the lower light intensities of 1.2 and 2.7 mw/cm\(^2\), where photosynthetic energy was low, the intracellular Cl\(^-\) appeared to be related to intracellular Na\(^+\) concentrations (Fig. 4B). This may represent a nonactive accumulation occurring through passive mechanisms for intracellular neutrality. For cultures at light intensities of 9.4 and 14 mw/cm\(^2\) active Cl\(^-\) influx probably occurred (Fig. 4B).

Light activation of Cl\(^-\) uptake is believed to occur by way of photosystem II. MacRobbie (18) has suggested that the energy comes from electron transport systems. The reducing potentials generated through photosynthesis migrate to several components some of which (quinones) require protons for the attainment of this potential (15, 19). If a high proton influx occurs, with the accompaniment of membranes highly permeable to Cl\(^-\) for electroneutrality, reductive potentials may migrate more rapidly through the electron transport chain of photosynthesis. The occurrence of more rapid electron transport might increase the efficiency of photosynthesis and provide more energy for growth. In this manner, high intracellular Cl\(^-\) would be accumulated as observed at 14.0 mw/cm\(^2\) for cultures in NaCl medium with and without added Ca\(^{++}\). The increased intracellular Cl\(^-\) found in cultures in NaCl medium with no Ca\(^{++}\) added when the light intensity was increased from 2.7 to 9.4 mw/cm\(^2\), would represent an active accumulation accompanying proton uptake. The reduced intracellular Na\(^+\) in these cultures would provide less intracellular cation site competition and proton uptake would occur.

**Photosynthetic and Respiratory Capacities.** Measurements of O\(_2\) evolution were based on dry weight, chlorophyll, and cell number for control cultures and for cultures in NaCl medium with and without added Ca\(^{++}\). Two phenomena were apparent for cultures in NaCl medium. At the same incident light intensities the rates of O\(_2\) evolution were greater while CO\(_2\) fixation rates were less than for controls (Figs. 5 and 6). This increased O\(_2\) evolution may indicate an increased efficiency for the whole photosynthetic apparatus or for just system II. The latter, uncoupling of photosystem II from photosystem I, may be the case in these whole cells; since it has been shown that NaCl does inhibit photoreduction of NADP, but does not inhibit photoreduction of cytochrome c or ferricyanide in an *in vitro* mixture (20). If more energy were transformed through the light reactions of photosynthesis for cultures in NaCl medium, it was uncoupled from CO\(_2\) fixation, since the photosynthetic quotient of salt cultures was
Fig. 4. Intracellular Na⁺ and Cl⁻ concentrations in *Chlorella sorokiniana* grown at 39°C in white light of 1.2, 2.7, 9.4, and 14 mw/cm² in 0.30 M NaCl medium with and without 10 mM CaSO₄.

considerably greater than nonsalt cultures (Figs. 5 and 6). Rates of O₂ evolution may have indicated an actual increase in quantum efficiency for cultures in NaCl medium. Increased energy not used for CO₂ fixation was probably utilized in the maintenance of Na⁺ efflux and Cl⁻ influx. The ability of cells to use more energy when grown in 0.3 M NaCl was demonstrated by the stimulated growth rates obtained by using light intensities greater than required to saturate growth of cells in inorganic medium.

The O₂-evolving mechanisms of cells cultured in NaCl medium was also more sensitive to higher light intensities than the controls. Cultures in 0.3 M NaCl medium did not continue producing O₂ at intensities greater than 29 mw/cm²; and cultures in 0.3 M NaCl medium with 10 mM CaSO₄ stopped at 12 mw/cm². Exposure of these cultures to higher light intensities caused O₂ production to drop to low levels and to eventually stop. The existence of competition between Cl⁻ and HCO₃⁻ influx as electroneutrality of entry is maintained may have limited usable CO₂. Further limitations on CO₂ availability may have resulted from NaCl inhibition of carbonic anhydrase, since 10 mM NaCl is known to inhibit this enzyme by 50% (8). With insufficient CO₂ available the increased reductive potential created by a large proton influx in cells cultured in NaCl medium would result in a blockage of the usual dark electron pathways for photoactivated reducing potentials.

Cells cultured in NaCl medium with Ca²⁺ added ceased producing O₂ at lower intensities than cells cultured in NaCl medium without Ca²⁺. Addition of Ca²⁺ to cells cultured in NaCl medium may have limited further the CO₂ available for photosynthetic fixation by decreasing membrane permeability. Uncoupled photoreductive potentials for cultures with less available oxidant would then exist at lower intensities as did the observed photodestruction of cells cultured in NaCl medium with added Ca²⁺ (Fig. 5). Decreased growth rate for cells cultured in NaCl medium with Ca²⁺ added as light intensity increased was increased from 9.4 to 14 mw/cm² may also be interpreted as a similar photodestructive phenomenon.

The increased dry weight per cell for cultures in NaCl medium may have been due to the accumulation of carbohydrates which were not metabolized as suggested by the lower rates of respiration (Table I). The reduced growth rates observed in response to NaCl may result from a combination of insufficient energy for activation of ion pumps and cellular functions and reduced capacity to utilize photosynthates to manufacture materials of the next generation.

Chlorophyll. Investigations of the photosynthetic mechanisms were extended by determining chlorophyll concentrations (Fig. 7). Total chlorophyll on a per cell basis was greater for cultures in NaCl medium than in controls and greatest for cultures in NaCl medium with no Ca²⁺ added (Fig. 7A). Ratios of chlorophyll a to chlorophyll b were lower for cultures in NaCl medium than in controls at the four light intensities examined (Fig. 7B). Concentrations of chlorophylls a and b (except for b at 1.2 mw/cm²) were greater for cultures in NaCl medium than in controls, but the increase of chlorophyll b was greater than of chlorophyll a (Fig. 7C and D).

Predominantly more chlorophyll b is found in system II than system I of the photosynthetic apparatus (1). At 2.7 mw/cm² the above increase in chlorophyll b, which was larger than the increase in chlorophyll a, in response to salt, correlates with the increased rate of O₂ evolution for cultures in NaCl medium and may represent an increase in the capacity or amount of system II. Chlorophyll b for cultures in NaCl medium without Ca²⁺ added increased more than for cultures in NaCl medium with added Ca²⁺. At 2.7 mw/cm²
In -J o E N9, u w 0 w =0 C, I w 0

C, I w 0

0 2 4 6 8 10
LIGHT INTENSITY (10

115 mW/cm2)

FIG. 5. Rates of oxygen evolution at several light intensities for Chlorella sorokiniana grown at 39 C in white light of 2.7 mw/cm2 in inorganic medium and in 0.30 m NaCl medium with and without 10 mM CaSO4. Arrow of +Ca to the vertical line for the culture grown in 0.30 m NaCl medium with 10 mM CaSO4 indicates the light intensity above which oxygen evolution became unstable and rapidly declined. The various symbols distinguish replicates.

the increase in chlorophyll a, however, was the same in both cultures. Therefore, the observed identical increases in O2 evolution for cultures in NaCl medium with and without added CaSO4 could be related to identical increases of chlorophyll a, since chlorophyll a is found in both system I and system II of photosynthesis. Both photosynthetic systems are reportedly functional for active maintenance of favorable intracellular Na+ and Cl- ratios and are presumed to be operational in C. sorokiniana. Proceeding from cultures in inorganic medium to cultures in NaCl medium with added Ca2+ to cultures in NaCl medium without added Ca2+ the amount of chlorophyll b increased and so did energy requirements for active Na+ efflux. The increased amounts of chlorophyll b may have been used to enlarge system I. Therefore, increases in total chlorophyll and proportionately greater increases in chlorophyll b for cultures in NaCl medium are postulated to be cellular adaptations to meet increasing demands of NaCl toxicity.

FIG. 6. Relative rates of CO2 fixation for Chlorella sorokiniana grown at 39 C in white light of 2.7 mw/cm2 in inorganic medium and in 0.30 m NaCl medium. One hundred is the rate of CO2 fixation for 10 min at 14 mw/cm2 for cells in inorganic medium on a per cell, per chlorophyll, and per dry weight basis. The control with no NaCl is the top unlabelled curve with halved circles.

Table I. Dark respiration rates of Chlorella sorokiniana

The cells were grown in 2.7 mw/cm2 white light in inorganic medium and in 0.30 m NaCl medium with and without 10 mM CaSO4.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Respiratory Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles O2/hr/mg dw</td>
</tr>
<tr>
<td>Inorganic medium</td>
<td>3.74</td>
</tr>
<tr>
<td>0.30 m NaCl medium</td>
<td>1.78</td>
</tr>
<tr>
<td>0.30 m NaCl medium with 10 mM CaSO4</td>
<td>1.59</td>
</tr>
</tbody>
</table>
Fig. 7. Chlorophyll content and ratios in *Chlorella sorokiniana* grown at 39°C in white light of 1.2, 2.7, 9.4, and 14 mw/cm² in inorganic medium and in 0.3 M NaCl medium with and without 10 M NaCl.

**LITERATURE CITED**


