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The effect of $AgNO_3$ on the bioenergetic processes and the ultrastructure of *Chlorella* and *Dunaliella* cells exposed to different saline conditions

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Abstract

The effect of $AgNO_3$, an inhibitor of the H^+ pump in the plasma membrane, on the bioenergetic processes and on the ultrastructure of the microalgae *Chlorella vulgaris* (salt sensitive) and *Dunaliella maritima* (salt resistant) was examined under varying salt concentrations. Differences between them were observed in changes of the cellular energy metabolism depending on their salt sensitivity and the inhibition of the H^+ pump activity.

A decrease was observed in the rates of heat production (about 45%), O_2 uptake (greater than 40–50% of the control) and particularly photosynthesis (more than 80%) in *Chlorella* cells under the simultaneous action of NaCl and $AgNO_3$. *Dunaliella* cells showed small to moderate rate increases for heat production (less than 7%), O_2 uptake (10–15%) and O_2 evolution (40%) in higher salt concentrations and under the action of $AgNO_3$.

The production of active oxygen species was studied as an early unspecific response of microalgal cells to possible unfavorable conditions, including salt stress. The amount of superoxide formed by the *Dunaliella* cells was higher than that by the *Chlorella* cells. However, Ag^+ ions increased the generation rate of superoxide radicals considerably in both *Chlorella* and *Dunaliella* cells.

The electron microscopy showed that changes of the algal ultrastructure of cells exposed to the action of Ag^+ ions were connected with the observed physiological changes and to a large extent with the alteration of the bioenergetic processes in them.

Keywords: *Chlorella vulgaris*; *Dunaliella maritima*; Heat production rate; Ion homeostasis; Plasma membrane; H^+-ATPases

1. Introduction

As a general feature, photosynthesizing organisms react with a cascade of defense responses on the action of various extreme factors. Their plasma membrane plays a crucial role in the perception, transduction and transport of environmental signals within the cells leading to a certain response [1–3]. The important enzymes which take part in the active transport are the H^+ pump or H^+-ATPases of the plasma membrane [1–4]. The plasma membrane cation translocation is characterized by the formation of phosphorylated intermediates [1–5]. High activities of the plasma membrane H^+ pump (in the following called "H^+ pump") in saline conditions are necessary for energization and stimulation of the plasma membrane resulting in an ion transfer from the cytoplasm to the internal medium [6,7]. The active transport of ions requires a considerable expenditure of metabolic energy, as it is connected with the work of numerous ATPases using the energy of ATP [2,8]. The activity of these enzymes can be inhibited by Ag^+ ions [9–11]. Therefore, we used Ag^+ ions to elucidate the changes of the energy yielding processes of plant cells at the inhibition of the H^+ pump in different saline conditions. Ag^+ ions do not penetrate into plant cells; they are adsorbed in the cell surface. Changes of permeability in the plasma membrane and in the efflux rate of ions from cells under Ag^+ action were already reported [9,10].

The unicellular green halotolerant microalga *Dunaliella maritima* and the salt sensitive microalga *Chlorella vulgaris* were used as suitable and controlled model systems. Both have been extensively used in biochemical and physiological investigations in many laboratories [11–15]. It was stated that *Chlorella* and *Dunaliella* may serve as useful plant models for clarifying mechanisms of tolerance to different stress conditions as their

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"photosynthetic and respiratory apparatus closely resembles that of higher plants" [13].

Alterations in the distribution of ions between the cytoplasm and the external environment under the influence of Ag^+ ions change the energy metabolism by depression of the H^+ pump rate [16,17]. The overall metabolic activity of algae in the presence of Ag^+ ions was determined by measuring their heat production. This is an integral indicator that reflects the changes in all catabolic and anabolic processes [18–21]. Photosynthesis and respiration rates were measured by a polarographic method [22]. In this paper, the production of the superoxide anion radical has been determined. It is known that the production of active oxygen species (AOS) is one of the early unspecific responses in plant cells to many stress situations including higher salt concentrations [23]. The enhanced generation of AOS may be an important primary defense response of plant cells to salt stress. Structural changes related to the physiological function of algae cells after exposure to Ag^+ ions have been observed employing electron microscopy.

The aim of the present investigation was to study the changes of bioenergetic processes and of the ultrastructure of *Chlorella* and *Dunaliella* cells at inhibition of the H^+ pump and to elucidate the energy cost for the adaptation of algae cells under these conditions.

2. Experimental

2.1. Cell culture

The unicellular algae *D. maritima* and *C. vulgaris* were the subjects of this investigation. *D. maritima* (from the collection of the Timiryazev Institute of Plant Physiology, Russian Academy of Sciences) was grown photoautotrophically in Tamiya medium (KNO_3; KH_2PO_4; $MgSO_4{\cdot}7H_2O$; $FeSO_4{\cdot}7H_2O$; $CaSO_4$ and H_3BO_3; $MnSO_4$; $ZnSO_4{\cdot}7H_2O$; MoO_3) with 500 mM NaCl, pH 6.8–7.2 and illuminated at 155 μmol photon m^{-2} s^{-1} with a light/dark photoperiod of 12/12. *Dunaliella* has the ability to survive and proliferate in extremely harsh environments. The culture was maintained in the logarithmic growth phase by daily dilution with fresh medium to maintain a cell density of about 1×10^6 cells/ml. *C. vulgaris* Beij. (a salt sensitive strain from the collection of the Botany Institute, Saint Petersburg, Russia) was grown in Tamiya medium, pH 6.8–7.2 [24] at 30 °C, and illuminated at 155 μmol photon m^{-2} s^{-1} with a light/dark photoperiod of 12/12. Cell suspensions were bubbled with 0.3% CO_2 in air. The cell density was maintained at (1–1.5) $\times 10^8$ cells/ml.

2.2. Incubation with stressors

Preliminary experiments showed that the appropriate concentration of $AgNO_3$ for *Dunaliella* cells is 10 μM. Smaller doses of the inhibitor did not change the rate of energy yielding processes compared to the control. But concentrations of $AgNO_3$ higher than 7 μM can lead to a total breakdown of *Chlorella* cells. Therefore, measurements were performed using the following experimental groups for *Chlorella*: (1) control (Tamiya medium), (2) cells incubated with 50 mM NaCl and (3) cells incubated with 50 mM NaCl and 7 μM $AgNO_3$. In the case of *Dunaliella*, they were: (1) control (Tamiya medium + 500 mM NaCl), (2) cells incubated with 1.5 M NaCl and (3) cells incubated with 1.5 M NaCl and 10 μM $AgNO_3$. The cells were preincubated with NaCl for 60 min before addition of the inhibitor of the H^+ pump ($AgNO_3$). The measurements were started 60 min after addition of the inhibitor.

Cell concentrations of 1–1.5 $\times 10^8$ cells/ml for *Chlorella* and 1×10^6 cells/ml for *Dunaliella* were used in the following experiments. All experiments were performed at 30 °C.

2.3. Analytical measurements

2.3.1. Polarographic determinations

Oxygen uptake and evolution rates were measured by a polarographic method using a Clark-type electrode. Each 3.2 ml sample was placed in a measuring vessel located in a water bath and equilibrated in the dark to 30 °C for 5 min. Then the oxygen uptake rate in the dark was measured for 5 min. After this, the cells were illuminated to record the rate of oxygen evolution for the next 5 min period [22].

2.3.2. Calorimetric measurements

The rate of heat production was measured with a heat conduction calorimeter, the LKB batch Bio Activity Monitor (BAM) whose direct calorimetric successor is the Thermal Activity Monitor (TAM) manufactured by Thermometric AB, Jarfalla, Sweden [20]. Suspensions of 1.5 ml were placed in unstirred 3 ml glass vessels that were hermetically sealed before thermal equilibration for 30 min. So, measuring the heat production rates started 30 min after adding NaCl to the *Chlorella* or *Dunaliella* cultures. The calorimetric signal was followed for 60 min.

2.3.3. Superoxide monitoring

The concentration of superoxide in the *Chlorella* and *Dunaliella* cultures was determined using the epinephrine method described in [25], incubating samples for 15 min with 1 mM epinephrine (pH 6.8). Epinephrine is converted to adrenochrome in the presence of oxygen radicals ($O_2^{\bullet -}$) and monitored photometrically at 480 nm (spectrophotometer SP-46; Lomo, St. Petersburg). The superoxide specifity of the epinephrine-adrenochrome system was confirmed by the significant inhibition of superoxide detection (up to 75%) in the presence of 250 units/ml of superoxide dismutase (SOD). Independent samples were taken at predetermined time intervals [25].

2.3.4. Electron microscopy

Ultrastructural changes of *Chlorella* and *Dunaliella* cells under the influence of salt compared with the controls were photographed by an electron microscope (JEM 100 CX; Joel Ltd., Japan). Samples were fixed in 2.5% (w/v) glutaraldehyde in phosphate buffer (pH 7.2) with a post-fixation in 1% (w/v) OsO_4 solution. They were embedded in Epon-812 resin after dehydration. Thin sections were obtained with a microtome Ultratome III (the defunct LKB AB, Bromma, Sweden) [26].

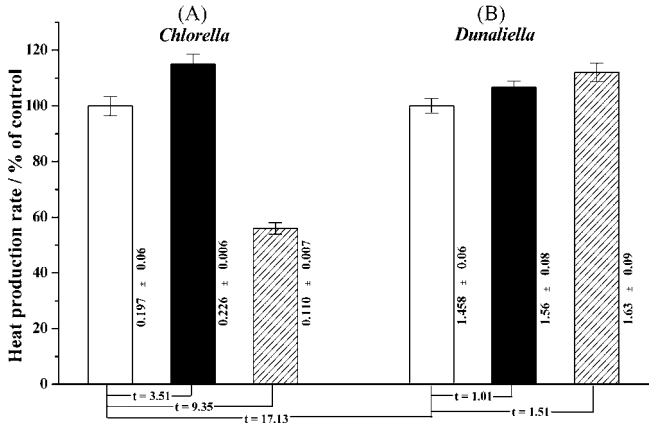

Fig. 1. Rate of *Chlorella* and *Dunaliella* cells under the action of NaCl and $AgNO_3$. (A) Control 100% (□); NaCl 50 mM (■); NaCl 50 mM + $AgNO_3$ 7 μM (▨) and (B) control 100% (□); NaCl 1.5 M (■); NaCl 1.5 M + $AgNO_3$ 10 μM (▨). (The absolute values are shown at the columns in $\mu W/10^6$ cells: (*t*) *t*-test value, calculated; at $P = 0.05$ and $n = 5$ (a) table value = 2.8.)

All experiments were repeated five times. The square error (S.E.) and *t*-test (at a significance level $P = 0.05$) were calculated using the program Microcal Origin™ V. 5.0.

3. Results

Fig. 1A shows that the rate of heat production of *Chlorella* cells treated with 50 mM NaCl slightly increased (10–15%, $t = 3.51$ and $P > 0.05$) compared to the control, but decreased to 50–55% after the simultaneous treatment with 50 mM NaCl and 7 μM $AgNO_3$. A similar increase was observed for *Dunaliella* cells under the action of 1.5 M NaCl alone and an even higher increase under the combined action of 1.5 M NaCl and 10 μM $AgNO_3$ (Fig. 1B).

Corresponding data for the O_2 uptake rate of *Chlorella* and *Dunaliella* cells under the influence of NaCl alone and together with $AgNO_3$ are presented in Fig. 2. The addition of $AgNO_3$ to a *Chlorella* suspension with NaCl resulted in a significant decrease to about 50% (Fig. 2A), while the addition of NaCl and $AgNO_3$ to *Dunaliella* cells increased the oxygen uptake (Fig. 2B). The net rate of oxygen evolution shows that the photosynthesis intensity of *Chlorella* cells is slightly reduced by 50 mM NaCl alone, but decreases by 80–90% under the combined action of NaCl and $AgNO_3$ (Fig. 3A). But the photosynthesis of *Dunaliella* cells increased drastically in 1.5 M NaCl medium (more than 80%) and by 40–50% under NaCl and $AgNO_3$ action (Fig. 3B).

Figs. 4 and 5 show the production of the superoxide anion as function of NaCl and $AgNO_3$ and of time. Samples were taken in 1 h intervals from independent cell cultures and treated with

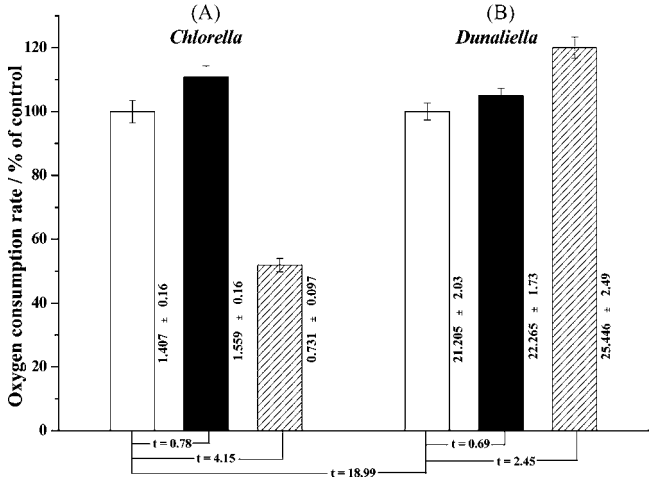

Fig. 2. Relative oxygen uptake rate of *Chlorella* and *Dunaliella* cells under the action of NaCl and $AgNO_3$. (A) Control 100% (□); NaCl 50 mM (■); NaCl 50 mM + $AgNO_3$ 7 μM (▨) and (B) control 100% (□); NaCl 1.5 M (■); NaCl 1.5 M + $AgNO_3$ 10 μM (▨). (The absolute values are shown at the columns in nmol $O_2/h/10^6$ cells; (*t*) *t*-test value, calculated; at $P = 0.05$ and $n = 5$ (a) table value = 2.8.)

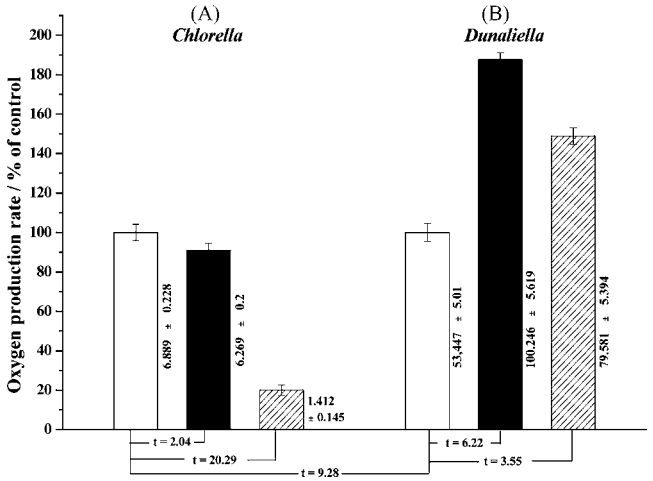

Fig. 3. Relative oxygen evolution rate of *Chlorella* and *Dunaliella* cells under the action of NaCl and $AgNO_3$. (A) Control 100% (□); NaCl 50 mM (■); NaCl 50 mM + $AgNO_3$ 7 μM (▨) and (B) control 100% (□); NaCl 1.5 M (■); NaCl 1.5 M + $AgNO_3$ 10 μM (▨). (The absolute values are shown at the columns in nmol $O_2/h/10^6$ cells; (*t*) *t*-test value, calculated; at $P = 0.05$ and $n = 5$ (a) table value = 2.8.)

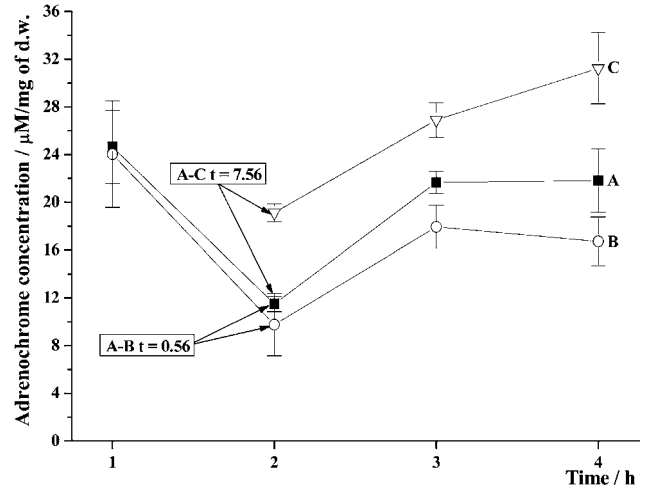

Fig. 4. Concentration of superoxide (according to determination of adenochrome concentration) in *Chlorella* suspensions under the action of NaCl and $AgNO_3$. ■ *Chlorella*; ○ *Chlorella* + 50 mM NaCl; ▽ *Chlorella* + 50 mM NaCl + 7 μM $AgNO_3$; ((*t*) *t*-test value, calculated; at $P = 0.05$ and $n = 5$ (a) table value = 2.8.)

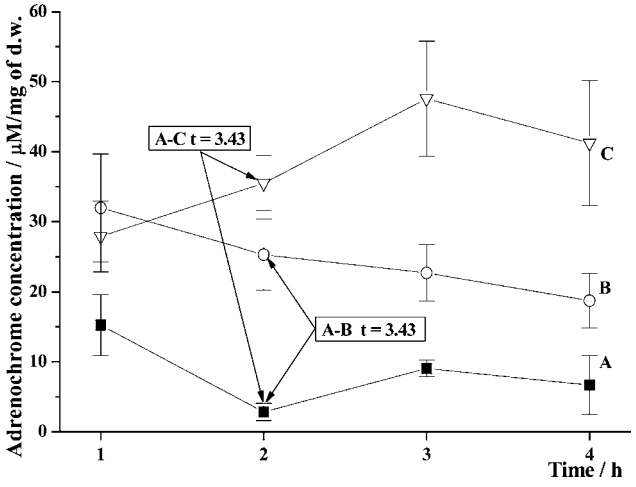

Fig. 5. Concentration of superoxide (according to determination of adenochrome concentration) in *Dunaliella* cells under the action of NaCl and $AgNO_3$. ■ *Dunaliella*; ◯ *Dunaliella* + 1.5 M NaCl; ▽ *Dunaliella* + 1.5 M NaCl + 10 μM $AgNO_3$; ((*t*) *t*-test value, calculated; at $P = 0.05$ and $n = 5$ (a) table value = 2.8.)

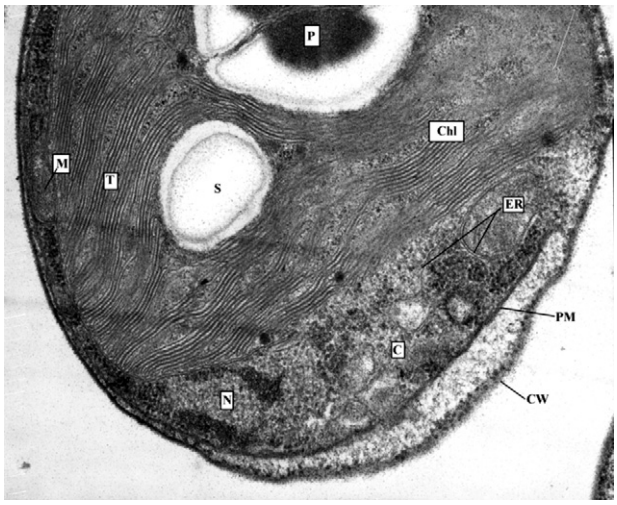

Fig. 7. *Chlorella* cells under the NaCl (50 mM) and $AgNO_3$ (7 μM) action. CW: cell wall; PM: plasma membrane; N: nucleus; C: cytoplasm; Chl: chloroplast; T: thylakoid; P: pyrenoid; S: starch; ER: endoplasmic reticulum.

epinephrine as described above. The amount of superoxide anion produced by *Chlorella* cells under the action of 50 mM NaCl was little decreased by about 15% (Fig. 4; 2 h) while superoxide generation by *Dunaliella* cells increased considerably (8.5 times at 2 h) under the action of 1.5 M NaCl (Fig. 5). But the addition of $AgNO_3$ to the medium significantly increased the superoxide generation by *Chlorella* cells as did $AgNO_3$ with *Dunaliella* cells (Figs. 4 and 5; 2 h).

Electron microscopy was used to look for structural alterations that could be connected with the observed physiological changes in the two organisms. The basic structure of the *Chlorella* cells is shown in Fig. 6. The typical components like cell wall, nucleus, large chloroplasts, relatively small mitochondria and highly characteristic pyrenoids with starch grains are visible. It can be seen that overall structure changes occur in *Chlorella* cells under the action of NaCl and $AgNO_3$ (Fig. 7). For instance, cells contain thylakoid membranes which have spread from the ordinary compact arrangement of lamella in slightly expanded mitochondria. More starch granules appear, and a part of the cells show an exfoliation of the cell wall from the plasma membrane. Basic structures of the *Dunaliella* cells are presented in Fig. 8 in which cells possess a plasma membrane, but are devoid of a recognizable cell wall. The thylakoid membranes of the chloroplasts run in parallel to each other, pyrenoids are bounded by starch particles. The nucleus is usually found

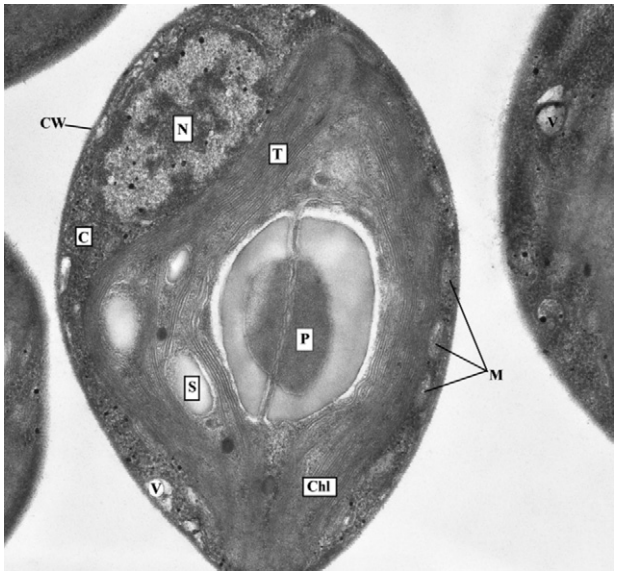

Fig. 6. Electron microscopic photography of a *Chlorella* cell (control). Cells of *Chlorella* are oval or round of, approximately, 3.5 μm × 2.5 μm. The thylakoid membranes of the chloroplast run parallel to each other. There are small starch grains and osmophilic granules. CW: cell wall; N: nucleus; C: cytoplasm; Chl: chloroplast; T: thylakoid; P: pyrenoid; S: starch; M: mitochondrion; V: vacuole.

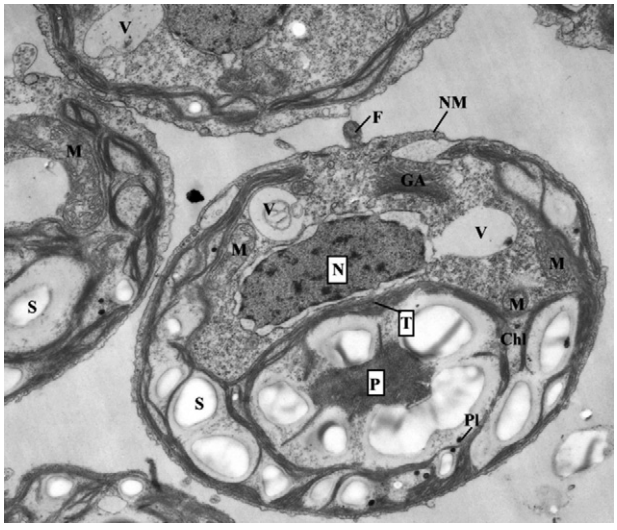

Fig. 8. Electron microscopic photography of *D. maritima* (control). *Dunaliella* cells are ellipsoid, approximately, 9 μm × 7 μm. NM: naked membrane (plasma membrane)—no cell wall; N: nucleus; GA: Golgi apparatus; F: flagellum; Chl: chloroplast; T: thylakoid; P: pyrenoid; S: starch; Pl: plastoglobule; V: contractile vacuole; M: mitochondria.

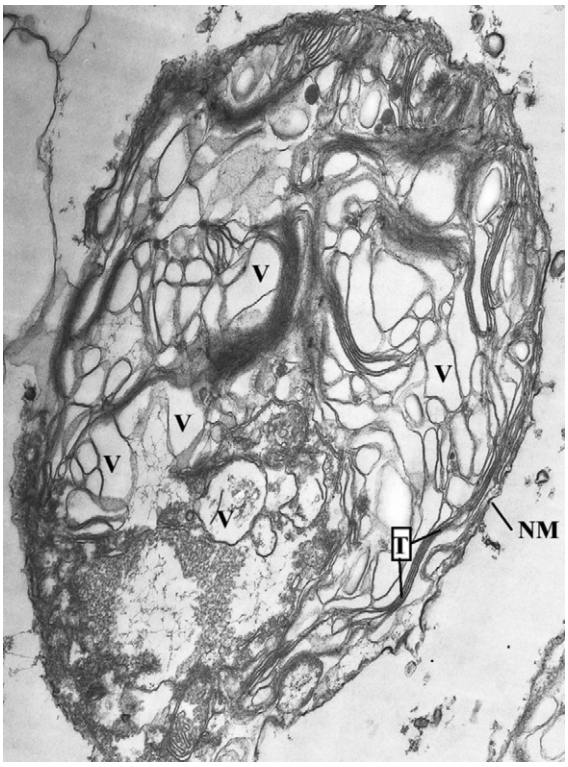

Fig. 9. *Dunaliella* cells under the NaCl (1.5 M) and $AgNO_3$ (10 μM) action. NM: naked membrane (plasma membrane)—no cell wall; T: thylakoid; V: contractile vacuole.

at the center of the cytoplasm. Cells contain a Golgi apparatus, mitochondria and one or more contractive vacuoles. Fig. 9 demonstrates the severe structural changes under the influence of $AgNO_3$ with many contractile vacuoles.

4. Discussion

The adaptation of algae to salt stress depends to a great extent on supporting the ion homeostasis in cells [12,28]. Alterations in the distribution of ions between the cytoplasm and the external environment may cause changes in the energy metabolism of the cells because ion homeostasis is an active, energy-requiring process. The active transport of ions is carried out by numerous ATPases, using the energy of ATP [16,17,27]. One of the important plant cell enzymes taking part in the active ion transport is the H^+ pump in the plasma membrane [1,4,5]. What is the contribution of the plasma membrane electron transport system to the overall Na^+ export capacity in salt sensitive and salt resistant algae cells?

As *Chlorella* and *Dunaliella* are photoautotrophic organisms with a photosynthetic apparatus closely resembling that of higher plants, they are typical plant cells [5,15]. However, the energetic potential of *Dunaliella* cells is to a great extent higher than that of *Chlorella* cells. The respiration rate of *Dunaliella* cells in the control was 16 times greater than in *Chlorella* cells (21.20 nmol O_2/h/10^6 cells and 1.40 nmol O_2/h/10^6 cells, respectively). The photosynthesis rate was in a ratio 7:1 (53.45 nmol O_2/h/10^6 cells and 6.88 nmol O_2/h/10^6 cells, respectively). But one has to keep in mind that their volumes differ at a rate 20:1. *Chlorella* and *Dunaliella* exhibit different changes of their energy yielding processes at the action of $AgNO_3$ under salt stress. A decrease in heat production and oxygen uptake rates to 40–50% was observed in *Chlorella* cells under the influence of $AgNO_3$ (Figs. 1 and 2A). The photosynthesis intensity in *Chlorella* cells under these conditions was reduced by 80–90% of the control (Fig. 3A). So, *Chlorella* cells are deprived of two major sources of ATP from photosynthesis and respiration in these conditions under $AgNO_3$ action, inhibiting Na^+ transporting ATPases in the plasma membrane. Under these conditions, an interruption of the ion homeostasis occurred. The increasing amounts of Na^+ ions in the cytoplasm have a toxic effect on the electron transport during photosynthesis and respiration. Five hundred millimolar NaCl were found to be the critical concentration for the adaptive processes of *Chlorella* cells.

$AgNO_3$ at high salt concentration did not suppress the bioenergetic processes in *Dunaliella* cells. Increase of heat production (about 10%), O_2 uptake (10–15%) and O_2 evolution rates (up to 40%) were observed (Figs. 1–3B). The increase of bioenergetic processes at action of $AgNO_3$ suggests that alternative Na^+ export mechanisms may operate in parallel with the H^+-ATPases of the plasma membrane in *Dunaliella* [5,11,15]. Two transporting systems have been discussed, viz. the Na^+, H^+-antiporter energized by the H^+-ATPase and an Na^+-ATPase in the plasma membrane of the *Dunaliella* cells [12,13]. It is supposed that Na^+ rather than H^+ may be the predominant cation in the energization of transport processes in *Dunaliella* plasma membranes. Some authors stated [15] that this hypothesis is intriguing since most non-mammalian eukaryotes including plants utilize H^+-ATPases as their main energy generators for transport. The unique situation in *Dunaliella* and probably in some other microalgae may reflect an adaptation to hypersaline solutions.

It is known that the formation of superoxide by *Chlorella* and *Dunaliella* cells in saline conditions (Figs. 4 and 5) is a trigger in the defence reaction [17,25]. Probably, the source of the superoxide is NAD(P)H oxidase for which the cells have to produce electron donors. This process requires cellular energy that causes a change in enthalpy. The reduction of superoxide anion generation by *Chlorella* cells under salt action may be associated with the deficiency of cell energy.

But the data obtained with $AgNO_3$ show that both, the salt tolerant and the salt sensitive algae had a considerable increase of their superoxide production (Figs. 4 and 5). Probably, this phenomenon is not directly connected with an $AgNO_3$ action on energy yielding processes under salt stress. There are data that Ag^+ ions link to the SH groups in the membrane and induce an inhibition of the antioxidant systems of those algae [29,30].

The electron microscopy showed structural changes in chloroplasts and mitochondria of *Chlorella* cells and an exfoliation of the cell wall from the plasma membrane under the influence of $AgNO_3$ (Fig. 7). The activity of tonoplast ATPases of *Dunaliella* cells is increased under the action of $AgNO_3$. This hypothesis is supported by the formation of a great number of contractile vacuoles (*Dunaliella* cells) seen in electronmicrographs (Fig. 9).

5. Conclusion

The work examined the possibility that the Na^+ export in *Dunaliella* and *Chlorella* cells was linked to an electron transport system of the plasma membrane under saline conditions. The activation of this electron transport was associated with the increase of energy costs for maintaining the ion homeostasis in saline conditions.

It is shown, that an interruption of the ion homeostasis occurs under the action of $AgNO_3$ as a result of the depression of energy yielding processes of *Chlorella* cells. Actually, it becomes a critical situation for the adaptive processes of *Chlorella* cells already at low concentration of NaCl (50 mM) and $AgNO_3$ action.

The unique abilities of *Dunaliella* to maintain high rates of photosynthesis and respiration for the generation of extra ATP as a driving force for the ion transport across the plasma membrane were observed under the inhibition of the H^+ pump. These results correspond to the operation of Na^+-ATPases as well as H^+-ATPases of the plasma membrane and different tonoplast ATPases. A high energetic potential allows the cells to maintain the ion homoeostasis which promotes a quick adaptation of them to the hypersaline solution.

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