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Ionic Osmoregulation during Salt Adaptation of the Cyanobacterium *Synechococcus* 6311.[1]

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ABSTRACT

The mechanisms of salt adaptation were studied in the cyanobacterium *Synechococcus* 6311. Intracellular volumes and ion concentrations were measured before and after abrupt increases of external NaCl concentrations up to 0.6 molar NaCl. Equilibrium volumes, measured with a rapid and accurate electron spin resonance spin probe method, showed that at low NaCl concentrations the cells did not shrink as expected for an impermeable solute. However, when the NaCl concentration exceeded a critical value, volume losses occurred. These losses were not fully reversed by hypoosmotic treatment, suggesting membrane damage. The critical value of irreversible volume loss paralleled the increase in salinity during cell growth. Rapid mixing experiments showed that exposure of *Synechococcus* 6311 to non-damaging NaCl concentrations caused water extrusion from the cells; the volume decreases were time resolved to about 200 milliseconds. Subsequently, volumes increased rapidly as NaCl moved into the cells. Controls recovered their volumes within 15 seconds, while salt-adapted cells grown at 0.6 molar NaCl required 1 minute for volume equilibration. This decrease in the rate of cell volume recovery indicates that salt adaptation is accompanied by changes in cell membrane properties. Subsequent to these initial rapid volume changes, a more gradual sequence of ion movement and sugar accumulation was observed. Under conditions for photoautotrophic growth, significant Na^+ extrusion was observed 30 min after salt shock. Sucrose accumulation reached a maximum value after 16 hours and K^+ accumulation reached equilibrium after 40 hours. The final concentrations of K^+ and Na^+ and sucrose and glucose inside the 0.6 molar NaCl-grown cells indicate that the inorganic ions and organic 'compatible' solutes are the major osmotic species which account for the adaptation of *Synechococcus* 6311 to salt.

Osmoregulation in cyanobacteria grown at high external NaCl concentrations involves the accumulation of organic and inorganic solutes. The mechanisms of osmoregulation may vary in different species. For marine cyanobacteria, the accumulation of glucopyranosylglycerol in *Synechococcus* sp Nageli (4), and K^+ ions in *Aphanotheca halophytica* (14) have been reported. The role of low mol wt carbohydrates in the maintenance of osmotic balance has been shown for several fresh water cyanobacteria. When grown at high NaCl concentrations, accumulation of sucrose occurs in *Nostoc muscorum* (1), and in *Synechococcus* 6311 (3). Glucopyranosylglycerol accumulation occurs in *Synechocystis* 6308 (12). In *Synechococcus* 6311, the amount of accumulated sucrose increases with increasing external NaCl concentrations, but does not counterbalance the osmolar concentration of ions in the growth media, suggesting the involvement of other osmoregulants. It has been suggested that Na^+ ions have a specific ionic effect in the triggering of events that lead to the cellular adaptation to higher NaCl concentrations in the cyanobacterium *N. muscorum* (1, 2). The present study describes the permeability of *Synechococcus* 6311 to NaCl during NaCl-induced osmotic shock as measured by ESR[3] methods and shows the role of ionic regulation in the mechanism of osmoregulation.

MATERIALS AND METHODS

Culture and Growth Conditions. *Synechococcus* 6311 was obtained from E. Padan, Hebrew University of Jerusalem, Israel. The culture was grown in Kratz and Myers 'C' medium (8). Each culture originated from stock cells grown in Petri dishes with growth medium containing 1.5% agar. Two-week-old cultures were transferred to liquid medium supplemented with 10 mM $NaHCO_3$ and 1 mM Na_2CO_3, stirred magnetically, flushed with 1% CO_2 in air, and illuminated with cool white fluorescent light (I = 4 w/m^2 at 30°C).

Determination of Intracellular Volume. Cell volume was determined with ESR spectra (3, 9). Samples for ESR experiments contained (final volume, 40 μl) 1 mM TEMPONE, 20 mM $Na_3Fe(CN)_6$, 75 mM $Na_2MnEDTA$, and cells suspended at a final Chl concentration of 400 to 500 μg/ml in media described in the figure legends. The presence of ferricyanide ensured that if TEMPONE molecules were to become chemically reduced within cells, they would subsequently be reoxidized in the extracellular environment. The samples were placed into 100-μl capillaries, and spectra were recorded at room temperature in a Varian model E-109 E spectrometer. The measurements were conducted in the dark at 10 mw and a modulation amplitude of 0.4 G, with a time constant of 0.128 s and a scan time of 8 min.

Kinetic experiments were carried out by mixing the cell suspension with the different NaCl solutions using the rapid mixing system illustrated in Figure 1. This system consists of a T-junction capillary connected at one end to a vacuum line and containing electromagnetic valves, and, at the other end to tubes where the cell suspension and the test solutions were placed. Electromagnetic valves (4 and 5) were stabilized by a wooden support which was placed on the top of the ESR cavity. An electronic timer with a 20-ms delay was used between the opening

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[3] Abbreviations: ESR, electron spin resonance; TEMPONE, 2,2,6,6-tetramethyl-4-oxopiperidinooxy free radical.

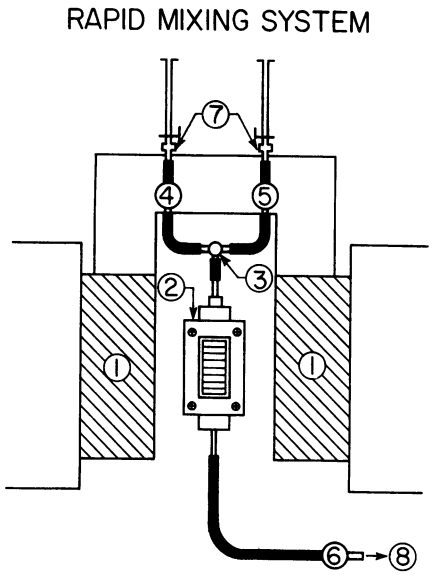

FIG. 1. Rapid mixing system for ESR studies. (1), Magnet coils; (2), ESR resonance cavity; (3), T-junction capillary; (4, 5, and 6), electromagnetic valves; (7), manual valves; (8), vacuum.

of the vacuum line and the opening of the other valves to avoid bubble formation within the ESR cavity. The driving force for mixing was atmospheric pressure relative to vacuum, and the mixing time was estimated to be about 100 ms. For kinetics measurements, the ESR spectrometer was locked on the low field peak and its line height change was recorded, with a time constant of 0.128 s.

Determination of Extracellular Volume. Cells were harvested by centrifugation (8000*g*, 5 min) and resuspended in growth medium at a final volume of 5 ml. Cell suspensions were labeled with [^{14}C]inulin (0.052 μCi/mg) (7) and after 5 min the cell suspensions were centrifuged at 10,000*g* for 15 min at 25°C. The supernatant was then discarded and the wall of the tube was dried with cotton swabs and any liquid adhering to the top of the pellet was removed. The pellet was digested with concentrated HNO_3. An aliquot of the cell digestion solution was dissolved in scintillation liquor and radioactivity was counted on a Packard Tri-Carb liquid scintillation spectrometer. The extracellular volume was calculated from the [^{14}C]inulin content.

Ion Determinations. An aliquot of the cell digestion described above was diluted 400 to 800 times with distilled H_2O, and Na^+ and K^+ content were measured with a Varian AA6 atomic absorption spectrophotometer. The ion concentration was then interpolated on a curve constructed from standard solutions with approximately the same ratio of ions as the unknowns.

The internal Na^+ and K^+ concentrations were calculated using the formula:

$$I_i = (I_t \cdot V_t - I_e \cdot V_e)/V_i$$

where:
- I_i = internal Ion concentration in mM.
- I_t = total Ion concentration in μmol/ml, as determined by atomic absorption.
- V_t = final volume of the digested pellet in ml.
- I_e = Ion concentration in the growth medium in μmol/ml.
- V_e = extracellular volume in ml, as determined by [^{14}C]inulin.
- V_i = intracellular volume in ml, as determined by ESR.

In a typical experiment, 200 ml of cells were centrifuged at 10,000*g* for 15 min to a final Chl concentration of 2.5 to 3.0 mg/ml. This pellet represents an extracellular volume (V_e) = 350 to 380 μl, and an internal volume (V_i) = 100 to 120 μl.

RESULTS

Effect of NaCl-Induced Stress on Cell Volume. The change in cell volume as measured by ESR spectra of control and salt-adapted cells incubated for 30 min in the light at 30°C, with increasing NaCl concentrations, is shown in Figure 2. For con-

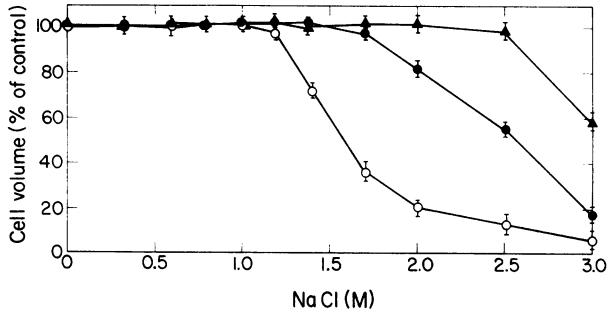

FIG. 2. Changes in cell volume of *Synechococcus* 6311 grown in the presence of NaCl. Cells were harvested after 90 h of growth, incubated with different NaCl concentrations at 30°C in the light, for 30 min. Values are mean ± SD (n = 6). (O), Control cells; (●), 0.3 M NaCl-grown cells; (▲), 0.6 M NaCl-grown cells.

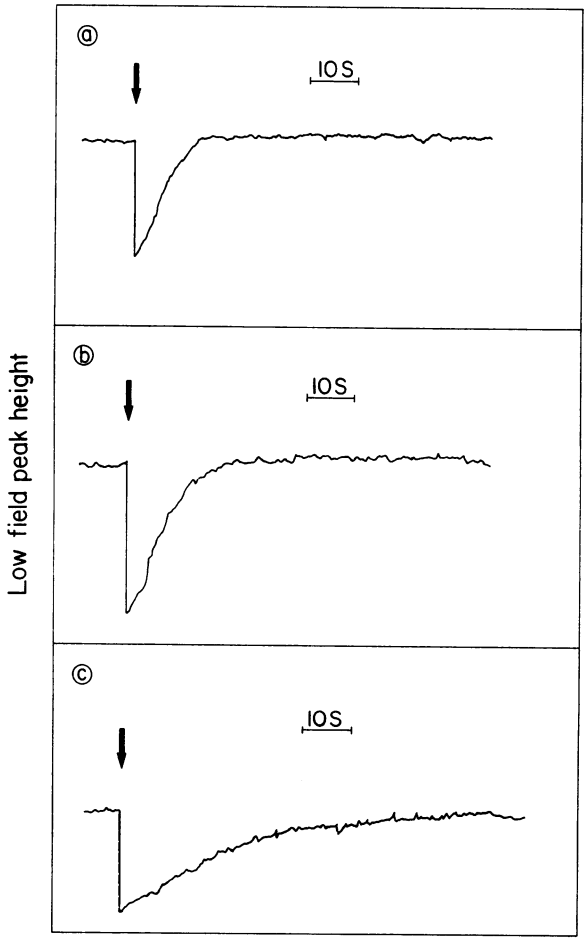

FIG. 3. Kinetics of the osmotic response to 1.0 M NaCl of *Synechococcus* 6311 cells grown in NaCl. The cells were harvested after 90 h of growth, and resuspended in fresh growth media at a Chl concentration of 2.5 mg/ml. Arrows indicate initiation of rapid mixing with NaCl. a, Control cells; b, 0.3 M NaCl-grown cells; c, 0.6 M NaCl-grown cells.

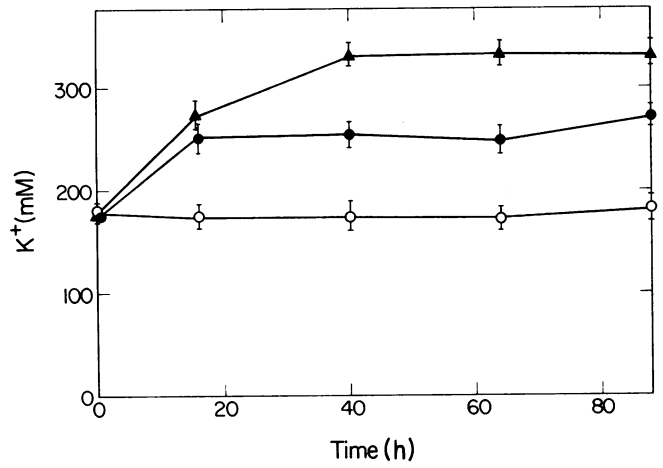

FIG. 4. Comparison of the internal K^+ concentrations of *Synechococcus* 6311 cells grown in NaCl. Cells were grown in control growth medium and resuspended in new growth media containing 0.3 and 0.6 M NaCl. Two hundred ml of cell suspension were harvested by centrifugation, resuspended in growth media at Chl concentration of 0.7 to 1.1 mg/ml. K^+ in control and NaCl-containing growth media = 19 ± 0.9 mM; values are mean ± SD (n = 18). (○), Control; (●), 0.3 M NaCl-grown cells; (▲), 0.6 M NaCl-grown cells.

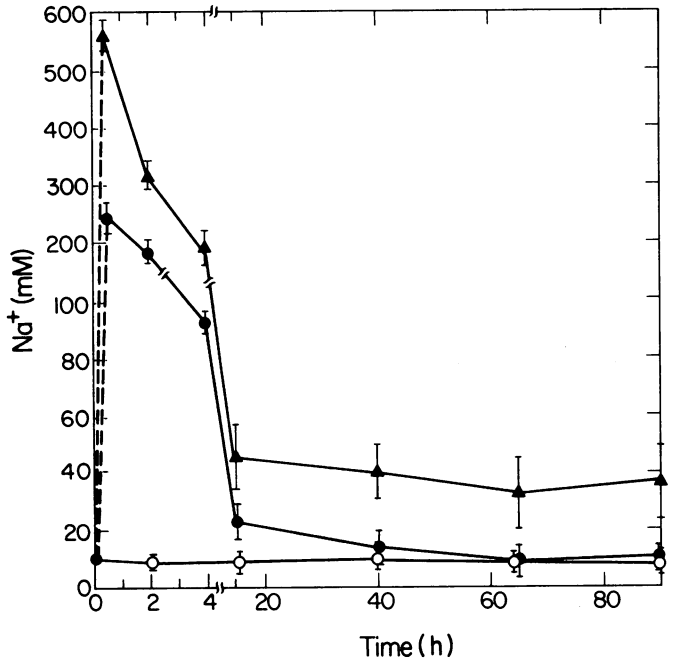

FIG. 5. Comparison of the internal Na^+ concentration of *Synechococcus* 6311 grown in NaCl. Cells were grown in control growth medium and resuspended in new growth media containing 0.3 and 0.6 M NaCl. Two hundred ml of cell suspension were harvested by centrifugation, resuspended in growth media at Chl concentration of 0.7 to 1.1 mg/ml. Na^+ in growth medium = 14 ± 0.8 mM; values are mean ± SD (n = 18). (○), Control cells; (●), 0.3 M NaCl-grown cells; (▲), 0.6 M NaCl-grown cells.

centrations up to 1.0 M NaCl, no change in volume was observed; at higher NaCl concentrations, different plasmolysis curves were obtained. A 50% volume reduction required 1.5 M NaCl for control, 2.5 M NaCl for cells grown in 0.3 M NaCl, and 3.0 M NaCl for cells grown in 0.6 M NaCl. This volume reduction was not reversible. When cells that underwent a volume reduction higher than 50% were resuspended in lower NaCl concentrations, they did not recover their initial volume (results not shown).

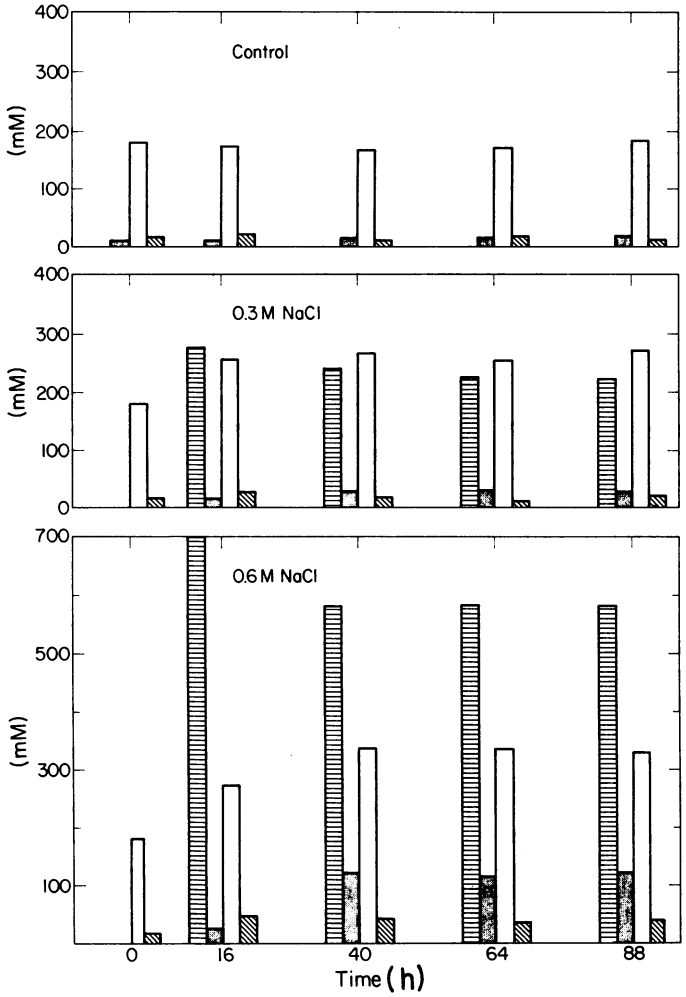

FIG. 6. Comparison of the internal solute concentrations of *Synechococcus* 6311 grown in the presence of NaCl. Na^+ and K^+ values from Figures 4 and 5. Sucrose and glucose values were assumed to be the same as previously determined (3) under identical growth conditions. (▤), Sucrose; (▨), glucose; (□), K^+; (▧), Na^+.

A comparison of the kinetics of the osmotic response to NaCl of control and salt-adapted cells is shown in Figure 3. When transferred to 1.0 M NaCl, control and 0.3 M NaCl-grown cells showed a rapid decrease in volume, reached a minimum within 300 ms, and recovered their initial volume within 15 s (Fig. 3, a and b). Cells grown in 0.6 M NaCl showed full volume recovery within 1 min when transferred to 1.0 M NaCl (Fig. 3c), and 1.5 and 2.0 M NaCl (results not shown). An estimate for the rate of full recovery was made by measuring the time required for 50% of volume recovery ($t_{1/2}$). The $t_{1/2}$ for control cells and cells grown in the presence of 0.3 M NaCl is 5 s, while the $t_{1/2}$ for cells grown in the presence of 0.6 M NaCl is 25 s.

Intracellular K^+ and Na^+ Concentration in Salt-Grown Cells. Batch cultures of *Synechococcus* 6311 exposed to a growth medium containing 0.3 and 0.6 M NaCl were analyzed regularly during the log phase of growth for K^+ and Na^+ content. Internal K^+ concentration (Fig. 4) in control cells was approximately 170 mM, and remained constant during 88 h of growth. The K^+ content increased in cells exposed to 0.3 M NaCl and remained constant after 16 h of growth at approximately 250 mM. Cells grown in 0.6 M NaCl also showed an enhanced K^+ concentration until they contained 330 mM after 40 h of growth. The internal Na^+ content (Fig. 5) of control cells was approximately 8 mM and remained constant during 88 h of growth. The Na^+ content increased markedly in salt-grown cells. In cells grown in 0.3 M

NaCl, the Na^+ content was 240 mM after 30 min of exposure to NaCl, decreased to 90 mM during the first 4 h of growth, and thereafter remained at the same level as in control cells after 40 h of growth. The Na^+ content of cells grown in 0.6 M NaCl increased to 550 mM after 30 min of NaCl exposure, decreased to 200 mM after 4 h of growth, and remained at a constant level of 40 mM after 40 h of growth.

Osmoregulatory Balance in Salt-Grown Cells. The accumulation of soluble sugars in response to NaCl-induced stress in *Synechococcus* 6311 has been quantitated (3). A comparison of the major solutes in salt-grown *Synechococcus* 6311 cells is given in Figure 6. Cells grown in 0.3 M NaCl (0.55 Os/kg) contained 225 mM sucrose, 250 mM K^+, and 8 mM Na^+ after 88 h of growth, which represents an osmotic pressure of 0.5 Os/kg. Cells grown in 0.6 M NaCl, equivalent to 1.1 Os/kg, contained 580 mM sucrose, 140 mM glucose, 330 mM K^+, and 40 mM Na^+, which altogether represents a total osmotic pressure of 1.1 Os/kg. No information was obtained about identities and concentration of anions.

DISCUSSION

The data obtained in this investigation strongly suggest that the very rapid NaCl entry into the cell triggers the adaptive response of the cyanobacterium *Synechococcus* 6311 to salt and that both organic and inorganic osmoregulatory mechanisms are involved in this process.

Osmoregulation can be almost completely accounted for by the intracellular concentrations of sucrose, glucose, K^+, and residual Na^+ ions. For 0.6 M NaCl-grown cells, there is a relatively rapid accumulation of sucrose (within 16 h, Fig. 6) followed by a more gradual accumulation of K^+ (40 h, Fig. 6), suggesting that the initial osmoregulatory response of the cells is the accumulation of sucrose, which is a 'compatible' solute, *i.e.* a solute which is not disruptive of macromolecular interactions (5, 13).

It has been suggested that Na^+ may have a specific effect in triggering the events that lead to the adaptation of another cyanobacterium *N. muscorum* to higher NaCl concentrations (1). Therefore, the permeability of *Synechococcus* 6311 cells to NaCl was studied by ESR monitoring of the kinetics of their volume changes during adaptation to different NaCl concentrations. *Synechococcus* 6311 has been shown previously to respond to variations in the osmotic pressure of the environment by cytoplasmic volume (plasmolysis-deplasmolysis) changes (3). When cells were exposed to increasing salt concentrations, they showed no volume changes up to 1.0 M NaCl (Fig. 2). At higher NaCl concentrations, the cells lost volume (plasmolyzed). Salt-grown cells required higher NaCl concentrations than controls before they began to show such volume losses. When cells that underwent more than 50% plasmolysis were transferred to lower NaCl concentrations, they did not recover their initial volume. This inability to recover initial volume suggests the occurrence of cell membrane damage. An alternative explanation is that the TEMPONE signal is quenched by uptake of quenching agents during transient membrane disruption. However, this is unlikely, inasmuch as the quenching agents were added to cell suspensions after 30 min of equilibration with the different NaCl concentrations. Our results suggest that membrane permeability to NaCl is altered when cells are grown in high salt concentrations. Since quencher was present during the rapid mixing experiments, and full recovery of cell volume occurred, membrane disruption during the osmotic shock and subsequent quencher penetration into the cells did not occur.

Cyanobacteria have been shown to possess an efficient mechanism for discriminating between Na^+ and K^+. *Anacystis nidulans* (7) and *Anabaena variabilis* (11) were shown to accumulate K^+, while maintaining a low intracellular Na^+ concentration. Na^+ extrusion is an active process driven by a Na^+/H^+ antiport system (9). The necessary H^+ gradient is generated by a proton translocating ATPase located in the plasmalemma (9). It has been suggested that an active mechanism for K^+ uptake accounts for K^+ accumulation in *A. variabilis* (11). K^+ ions may play an osmotic role in fresh water cyanobacteria as suggested previously (7).

The internal K^+ concentration was found to be constant in *Synechococcus* 6311 during the log phase of growth. These values are in agreement with those reported for other fresh water cyanobacteria (7, 11). Therefore, the increase in intracellular K^+ concentration with an increase in NaCl in the growth medium indicates that K^+ accumulation is a mechanism for osmoregulation in the adaptation of *Synechococcus* 6311 to salt. Although the steady state intracellular Na^+ concentration is maintained at a low level of 8 mM in control cells, when the NaCl concentration of the medium is abruptly increased, the cells shrink within 300 ms but then NaCl rapidly penetrates the plasmalemma and the cells regain their initial volume within 15 s (Fig. 3). Subsequently, the cells slowly (in a matter of hours) pump out Na^+ probably via a Na^+/H^+ antiporter (8) while they take up K^+ and synthesize sugars for osmoregulation. It appears that osmotic shock increases the permeability to NaCl. Moreover, it is very likely that osmotic shock inhibits the sodium pump and the ATPase that powers it (6). Therefore, after osmotic shock, permeability of the plasmalemma to NaCl would be anticipated to be too great for the sodium pump to overcome, resulting in a net uptake of NaCl. Following shock, the membrane recovers and becomes less permeable to NaCl (Fig. 3). This may be brought about by recovery of the sodium pump and ATPase from shock; Na^+ is pumped out, and Cl^- exits passively.

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