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Absorption of Cu^{++} by Long-term Cultures of *Dunaliella salina, D. tertiolecta,* and *D. viridis*

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Algae vary widely in their resistance to heavy metal toxicity, such as to copper (Erickson et al. 1973). Some organisms are inhibited at concentrations that are within the environmental range (Davey et al. 1973). Dunaliella, a unicellular green algae, exhibits high tolerance to copper concentrations (Pace et al. 1977; Bentley-Mowat and Reid 1977). A variety of mechanisms have been proposed to account for algal resistance to heavy metal toxicity, including extracellular complexing to organic substances secreted by the cells (Hellebust 1974). Alternate proposals suggest exclusion of the heavy metal by the cell surface or cell wall (Bentley-Mowat and Reid 1977). However, since Dunaliella lacks a cell wall, the latter mechanism cannot be the one responsible (Eyden 1975). As Zmiri and Ginzburg (1983) reported with sodium, the heavy metals ions may be incorporated in non-essential intracellular sites, and thereby excluded from vital metabolic locations (Hawkins and Griffiths 1982). Previous uptake studies have indicated that Dunaliella does not exclude heavy metals from the cell (Parry and Hayward 1973; Hawkins and Griffiths 1982).

By means of atomic absorption analysis, we measured the effect of salinity on the absorption of copper. We used long term cultures of Dunaliella in order to determine if alterations in plasmalemma permeability occur as a result of adaptation to different osmotic conditions. Since copper appears to affect the mechanisms involved in osmotic resistance with Dunaliella, we hoped to elucidate some of the characteristics of permeability.

MATERIALS AND METHODS

Dunaliella viridis, D. tertiolecta, and three ecotypes of D. salina were grown on (0.2u) millipore filtered artificial seawater medium of 3.2%S, pH 7.8, considered to be isotonic to seawater (Provasoli et al. 1957). The cultures were tested periodically and were free of bacterial contamination. Transfers were made to media of 2.0%S (hypotonic) and 6.0%S (hypertonic). Glassware was acid washed and sterilized. It was filled with 50 ml of medium

and incubated 24 hours prior to inoculation. Cell counts and calculations were made to establish an inoculum of 10^6 cells. Cultures were maintained in an incubator at 22-25°C, continuously illuminated with 1720 lux from GE cool white 15 watt fluorescent bulbs.

Copper was supplied as $CuSO_4 \cdot 5H_2O$ from 1% stock solution to achieve theoretical concentrations of 0, 10 and 50 ppm. These values were tested by atomic absorption analysis of uninoculated samples. The concentration and solubility of Cu^{++} is determined by pH and chelation. In addition to buffering pH, TRIS (hydroxy-methyl-amino-methane) chelates copper. It regulates Cu^{++} activity so that it remains constant throughout the course of the experiment at a value determined by the total amount of copper added to the medium.

Since Cu^{++} delays log phase, it was determined that cells were in log phase at 14 days after inoculation. Samples were removed from the cultures and counted, using a Spencer hemocytometer. The samples were centrifuged at 1500Xg for 10 minutes. The pellet was washed twice in PO_3 buffer, pH 7.8, containing 3% NaCl.

The cells were then filtered using fine grain filter paper through a glass filter. The filter paper and residue were digested in 1 ml each of conc H_2SO_4, conc HNO_3 and conc $HClO_4$ in a boiling water bath for 45 minutes. The liquid samples of uninoculated media were analyzed without digestion or dilution. However, the samples were then diluted where the concentrations were too high and did not fall in the straight line portion on the Beers law curve. All samples were analyzed on a Perkin-Elmer 5000 atomic absorption instrument. Results of copper analyses were calculated on the basis of ppb for 10^6 cells.

RESULTS AND DISCUSSION

The investigations showed that for all samples, the assigned copper value of the medium was not significantly different from the actual value, as determined by atomic absorption analysis (Table 1). For this reason, the theoretical value was used. It should be noted that the medium contained a trace of copper, so that "0 ppm" is actually 0.79 ± 0.34.

Our results indicate that Dunaliella does not exclude copper from the cell. This is similar to results achieved by Hawkins and Griffiths (1982) and is in accordance with similar reported studies of other heavy metals (Parry and Hayward 1973). As the extracellular concentration of Cu^{++} increases, the intracellular accumulation rises proportionately; so that the intracellular $[Cu^{++}]$ is greater at 50 ppm than in the controls. The resistance of Dunaliella to heavy metals does not appear to be due to exclusion from the cell (Figure 1).

Table 1. Comparison of theoretical and actual [Cu^{++}].

Theoretical ppm	Actual ppm	Salinity %
0	0.64±0.25	2.0
0	0.82±0.25	3.2
0	0.92±0.50	6.0
10	8.96±0.35	2.0
10	9.18±0.34	3.2
10	9.08±0.33	6.0
50	55.14±4.67	2.0
50	55.92±2.40	3.2
50	55.72±3.21	6.0

On the other hand, salinity has a significant effect on the amount of copper accumulated by the algal cells. Cultures grown at hypotonic 2%S absorb significantly more copper than those of isotonic 3.2%S. Hypersaline 6% cultures show significantly less copper absorbed than either of the other salinities. This effect is demonstrable at 10 ppm and to a greater extent at 50 ppm Cu^{++}. Control cultures, with no added copper, do not show a decrease in intracellular copper as salinity is increased. We also found that copper produced greater lethality in hypotonic medium than in hypertonic (Table 2).

The presence of TRIS in the medium would act to chelate Cu^{++} and maintain its concentration at a steady level throughout the experiment. Interaction with other ions in the medium would thereby be minimized. It would serve to limit the binding effect of extracellular exudates as well. This does not appear to be a factor since glycerol, which is the fundamental osmoregulatory compound produced by Dunaliella, has been shown to be excreted more readily in hypotonic than hypertonic media (Kessly and Brown 1981). Extracellular binding to glycerol would not be important in explaining the lower absorption in hypertonic solutions.

Copper binds to the cell surface and therefore causes alteration of transport systems (Gavis et al. 1981). Riisgard (1979, 1980) suggested that Dunaliella marina was unable to regulate its volume in anisotonic media in the presence of Cu^{++}. In hypotonic medium the cells remained swollen, while in hypertonic they failed to shrink. Permeability and transport mechanisms are thought to be different in hypotonic than hypertonic media. Our results give support to this theory. We have shown greater permeability in hyposaline medium than in hypersaline, as is indicated by the increased absorption of copper into the 2%S cultures than the 6%S. The increased lethality in hypotonic medium may be due to increased water influx or to greater permeability of copper into the critical regions of the cell.

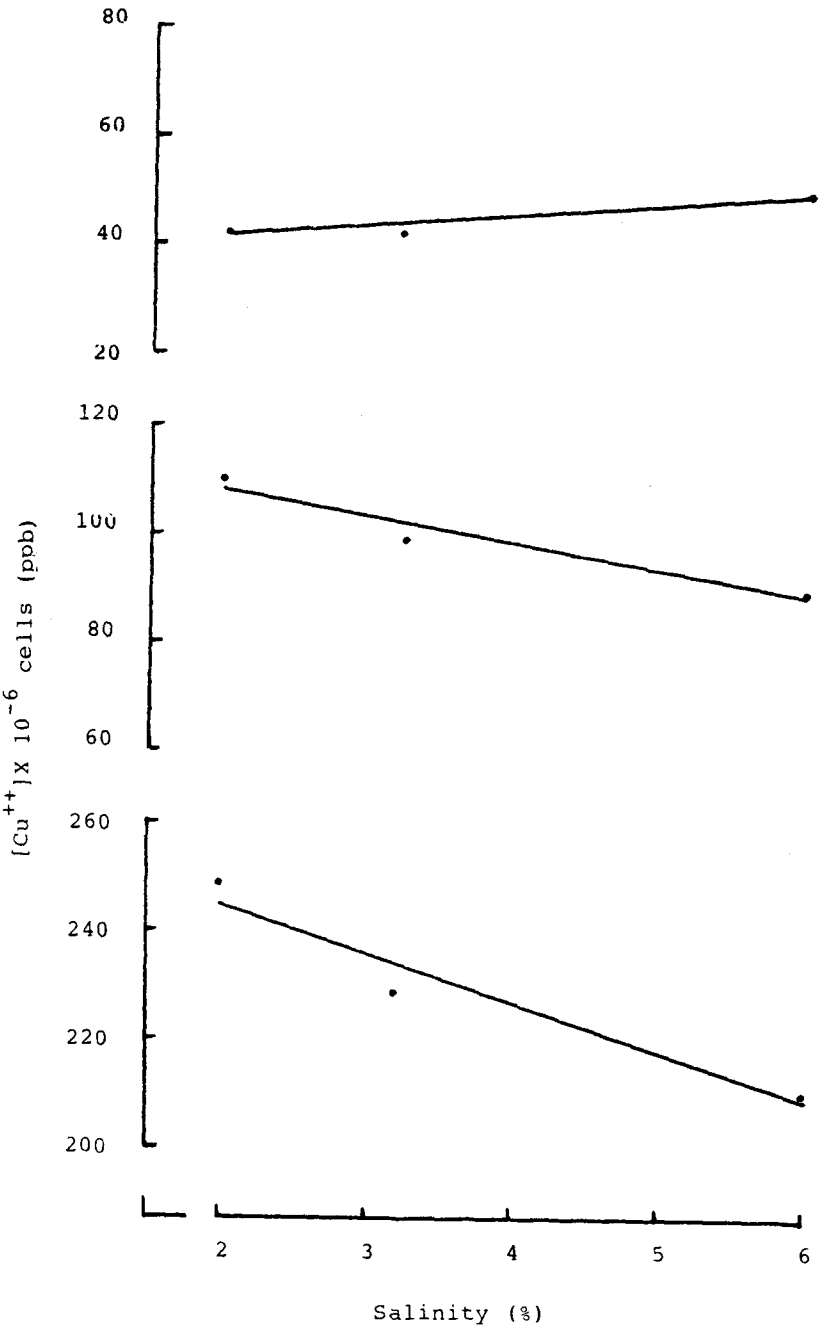

Figure 1. Effect of salinity on absorption of copper in 14 day cultures of Dunaliella. The top graph represents the control with no additional copper.

Table 2. Effect of Copper on Dunaliella cultures at 14 days

$[Cu^{++}]$ ppm	Cells/ml X10^6	Salinity %
0	3.31±0.45	2.0
0	3.16±0.73	3.2
0	3.08±0.74	6.0
10	2.27±0.80	2.0
10	2.37±0.64	3.2
10	2.69±0.54	6.0
50	1.54±0.54	2.0
50	1.60±0.44	3.2
50	1.86±0.34	6.0

The inverse relationship of salinity and copper permeability may apply to other heavy metal ions as well. This may help to explain the resistance of Dunaliella to heavy metal toxicity.

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