Marine Biology 29, 99-103 (1975)

The Effect of Heavy Metals on Photosynthesis and Loss of Cell Potassium in Two Species of Marine Algae, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*

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Abstract

The effects of various heavy metals on light-induced oxygen evolution and on net potassium release were studied in short-term experiments using the unicellular marine algae *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. Heavy metals, except for copper and 3,(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) did not cause loss of potassium at concentrations similar to or less than those required for inhibition of photosynthesis. At the concentrations used, no significant loss of potassium was observed for Zn^{2+}, Cd^{2+}, Tl^{+}, Pb^{2+} nor DCMU. With Cu^{2+}, potassium release occurred at a marginally lower concentration than did inhibition of photosynthesis. The extreme sensitivity of light-induced oxygen evolution to inhibition by cadmium and lead found previously by the author (Overnell, 1974) with *Chlamydomonas reinhardii* was not found with the strains used here.

Introduction

It has been known for some time that the toxicity of heavy metal ions is associated with loss of potassium from cells. Thus, Joyce *et al.* (1954) showed that heavy metal ions caused net K^{+} loss from rabbit red blood cells in the order $Pb^{2+}>Au^{+}>Hg^{2+}$. Similarly, Passow and Rothstein (1960) found that Hg^{2+} caused complete loss of cellular K^{+} (accompanied by cellular anions) and Cu^{2+} caused loss of K^{+} from baker's yeast, but neither Zn^{2+} nor Pb^{2+} at similar concentrations had any effect.

McBrien and Hassall (1965), studying the effects of copper on *Chlorella vulgaris*, found that when a large quantity of Cu^{2+} was added to a suspension of the cells, and the supernatant analysed for cations other than Cu^{2+}, only K^{+} was found in large amounts. It was concluded that the potassium was released by the graded response of a barrier, normally of low permeability, due to increasing amounts of bound copper. This increase of permeability of the cells was considered to be the primary toxic effect of copper. Potassium-depleted cells were still viable if subsequently transferred to a copper-free medium.

Shieh and Barber (1973), working with *Chlorella pyrenoidosa*, found that 80μM $HgCl_2$ caused a stimulation of K^{+} exchange and a net loss of K^{+} after not more than 20 min. This same concentration of $HgCl_2$ after a pre-incubation of 10 min gave rise to an increase in respiration and light-induced evolution of oxygen.

In an experiment of 19 h duration on *Chlorella pyrenoidosa*, Kamp-Nielsen (1971) found that photosynthesis inhibition and K^{+} excretion induced by Cu^{2+} and Hg^{2+} followed similar dose-response curves, and it was concluded that the primary effect of bound metals (Cu and Hg) is to cause some destruction of a diffusion barrier causing an outflow of potassium.

Some 60 enzymes or enzyme activities are now known to be dependent on univalent cations (Suelter, 1970), and for most of these potassium is the preferred ion. Thus, loss of potassium would be expected to cause cell death. Since potassium is invariably concentrated against a chemical gradient, however, cell death would be expected to cause potassium loss.

With plant cells, both K^{+} loss and also abolition of photosynthesis are known results of heavy metal poisoning. It therefore seemed to be of interest to examine the relative sensitivity of photosynthesis (as measured by light-induced evolution of oxygen) and potassium loss after exposure to copper, mercury and other heavy metals, under identical conditions of short-term exposure in an attempt to identify the primary site of action.

Materials and Methods

Chemicals

Methyl mercuric chloride was obtained from Alpha Inorganics and was dissolved in ethanol. All other heavy-metal salts were AnalaR grade and were used as aqueous solutions. Mercury was used as mercuric chloride. DCMU was obtained from Pfaltz and Bauer and recrystallized before use. Morpholinopropanesulphonic acid (MOPS) and N-2-hydroxyethylpiperazine-N'-3-propanesulphonic acid (HEPPS) were the buffers used in this study and, because of their chemical similarity to those studied by Good *et al.* (1966), would be expected to show very small or negligible binding constants with metals.

Experimental Organisms

Dunaliella tertiolecta (Butcher) was obtained from the Culture Collection of Algae at Indiana Univer-

sity, Department of Botany, Bloomington, Indiana 47401 (USA), strain no. 999. This was freed of bacteria by treatment with benzylpenicillin and streptomycin, and a bacteria-free culture has been deposited with the Culture Collection of Algae and Protozoa, 36 Storey's Way, Cambridge, CB3 ODT (England). *Phaeodactylum tricornutum* (Bohlin) was kindly donated by Dr. M.R. Droop, Scottish Marine Biological Association, Dunstaffnage Marine Research Laboratory, Oban, Argyllshire, Scotland.

Culture Conditions

Both organisms were grown in the S50 medium of Droop (1958) in a semi-continuous culture apparatus having a 1-l growing vessel which was stirred magnetically and a 5-l reservoir. The culture vessel was filled with 800 ml of medium and illuminated with a single 20 W warm-white fluorescent tube. Air containing 5% carbon dioxide was bubbled through. When vigorous growth had been established, 400 ml of the culture was harvested once a day and the culture diluted to 800 ml with fresh medium. From time to time the culture was plated onto nutrient agar plates (Oxoid Ltd., 20 Southwalk Bridge Road, London SE1), and also examined under the phase-contrast microscope to test for sterility. Contaminated cultures were discarded.

Assay Procedures

The harvested cells were centrifuged at 3000 revs/min (1000 xg) for 10 min and washed rapidly with and resuspended in 0.01 M morpholinopropanesulphonic acid (MOPS), 1.5% NaCl pH 7.0. The conditions were initially chosen to allow comparison of the inhibition results with those obtained previously using the fresh-water alga *Chlamydomonas reinhardii* (Overnell, 1974). Some results were later checked using the major ions of the S50 medium (Droop, 1958), i.e., Na^+, Mg^{2+} and Ca^{2+} and 0.01 M hydroxyethylpiperazinepropanesulphonic acid (HEPPS) pH 7.9. For studies of the effects of washing with potassium-free medium alone, the starting cell suspensions were washed and made-up in the following buffer: 0.01 M MOPS, 1.5% NaCl, 0.04% KCl pH 7.0. Chlorophyll *a* was determined using an 80% acetone extract employing the parameters of Ziegler and Egle (1965). It was found that 80% acetone did not extract all the chlorophyll from *Phaeodactylum tricornutum*, and so for chlorophyll *a* determination these cells were disrupted in a French press before extraction. To facilitate routine assays a calibration curve was constructed of A_{674} for whole cells against the weight of chlorophyll *a*/ml determined from the disrupted cells. Using a cuvette holder near the photomultiplier, a straight-line graph was obtained.

Light-Induced O_2 Evolution

The standardized solution of cells was diluted to 5µg of chlorophyll *a*/ml using the required buffer containing 2mM HCO_3^-, and put into a transparent-jacketed 5-ml vessel maintained at 20°C having an integral Clark electrode. The dark respiration was first measured for 5 min and then the vessel was illuminated with saturating red light using a 100 W quartz halogen lamp, a red filter with 50% transmission at 595nm, and a 100-ml round-bottomed flask filled with water as a focussing lens and heat filter. The Clark electrode had a 2.5kΩ load, and the output was recorded on a strip-chart recorder having a sensitivity adjustable between 1 and 10 mV for full-scale deflection. The amount of photosynthesis was taken as the algebraic difference between the slopes of the dark and light curves.

Heavy-Metal Incubation

The oxygen tension measurements were preceded by a 10-min incubation in the dark with the required quantity of the heavy metal in the absence of bicarbonate which was added at the start of the measurements.

Heavy Metals and Potassium Loss in *Dunaliella tertiolecta*

The suspension of the cells was washed twice with 0.01 M MOPS, 1.5% NaCl pH 7.0, and then made up to 5µg of chlorophyll *a*/ml as described above. 20-ml aliquots of this suspension were then put in plastic centrifuge tubes and incubated for 15 min in the dark with varying amounts of heavy metals. At the end of this time they were centrifuged for 5 min at 3000 revs/min (1000 xg), the supernatants discarded, and the pellet washed with a further 10 ml of buffer. The pellet was then transferred to a plastic screw-capped tube, and heated on a boiling water bath for 15 min with 3 ml of water. The tubes were centrifuged and decanted into 5-ml volumetric flasks and CsCl added to give a final concentration of 7.5×10^{-3}M. The potassium concentration was then determined using a flame photometer (Varian Techtron).

Results

The inhibition curves for the light-induced oxygen evolution (Fig. 1A) and the curves for the potassium release (Fig. 1B) are plotted as a function of the heavy metal concentration for *Dunaliella tertiolecta*. Light-induced oxygen evolution is more sensitive to Hg^{2+} and Tl^+ than is potassium leakage, but for Cu^{2+}, K^+ leakage occurs over a similar range of concentration, and is perhaps marginally more sensitive. The case of methyl mercury is interesting, since the effect on potassium leakage is limited and constant, giving a loss of only about 40% of the control concentration over a methyl mercury range of 5×10^{-7} to 4×10^{-5} M (the highest concentration tested).

Other metals were tested for their ability to induce K^+ loss, and the results of Zn^{2+}, Cd^{2+},

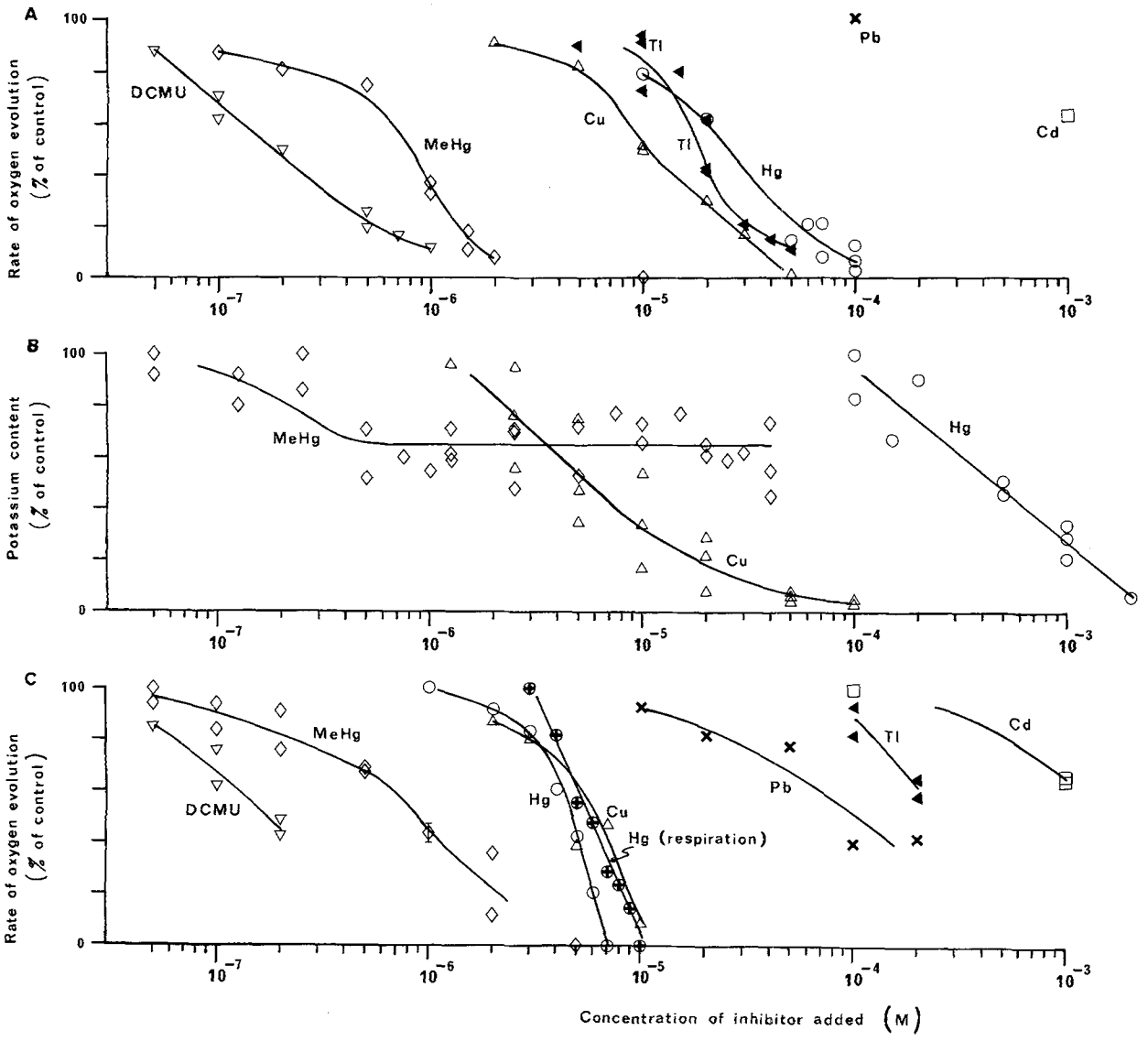

Fig. 1. (A) (B) *Dunaliella tertiolecta*. (A) Effect of DCMU, $MeHg^+$, Cu^{2+}, Tl^+, Hg^{2+}, Cd^{2+} and Pb^{2+} on light-induced oxygen evolution of whole cells; 5µg chlorophyll *a*/ml in 0.01 M MOPS, 1.5% NaCl, 2mM $NaHCO_3$, pH 7.0 in saturating red light, as a function of molarity of heavy metal. (B) Effect of $MeHg^+$, Cu^{2+} and Hg^{2+} on potassium content of cells; 5µg chlorophyll *a*/ml in 0.01 M MOPS, 1.5% NaCl, pH 7.0, as a function of molarity of heavy metal. (C) *Phaeodactylum tricornutum*. Effect of DCMU, $MeHg^+$, Cu^{2+}, Hg^{2+}, Pb^{2+}, Tl^+, and Cd^{2+} on net light-induced oxygen evolution of whole cells; 5µg chlorophyll *a*/ml in 0.01 M MOPS, 1.5% NaCl, 2mM $NaHCO_3$, pH 7.0 in saturating red light, as a function of molarity of heavy metal; a curve of the inhibition of dark respiration is included; 15-min dark preincubation with heavy metal in all the above cases

Hg^{2+}, $MeHg^+$, Tl^+, Pb^{2+}, Cu^{2+} and (as a control) DCMU are illustrated in Fig. 2. Clearly only copper and methyl mercury were having a pronounced effect at the levels tested.

The presence of calcium in the medium is known to reduce the loss of potassium from *Porphyra perforata*, as shown by Eppley and Cyrus (1960), and so the dose-response curves for the effect of copper on light-induced oxygen evolution and potassium loss were repeated using the major cations of the growth medium, again omitting potassium (i.e., Na^+, Mg^{2+}, Ca^{2+}) and buffered at pH 7.9 with HEPPS. The results are shown in Fig. 3, from which it can be seen that the 50% inhibition value for light-induced oxygen evolution is ca. 4×10^{-5}M, while that for K^+ loss is ca. 2×10^{-5}M. Determination of the values from statistical curve-fitting techniques was not considered worthwhile because much

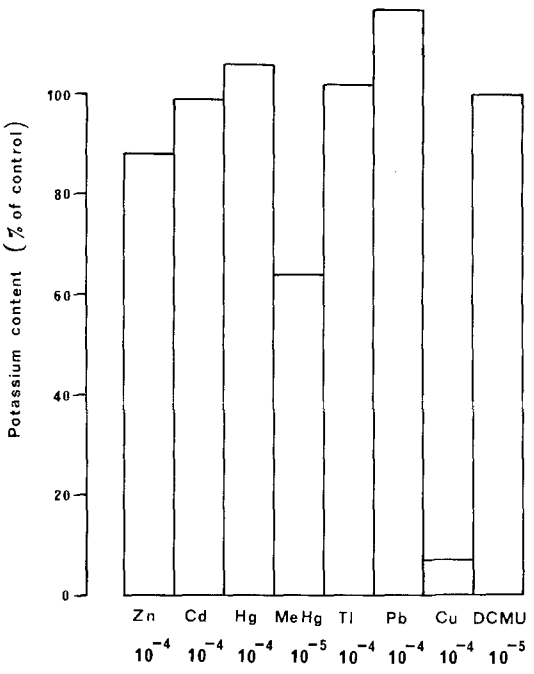

Fig. 2. *Dunaliella tertiolecta*. Potassium content of cells in suspension of 5µg chlorophyll *a*/ml after 15-min dark incubation with Zn^{2+}, Cd^{2+}, Hg^{2+}, $MeHg^{+}$, Tl^{+}, Pb^{2+}, Cu^{2+} and DCMU at concentrations shown (in molarity)

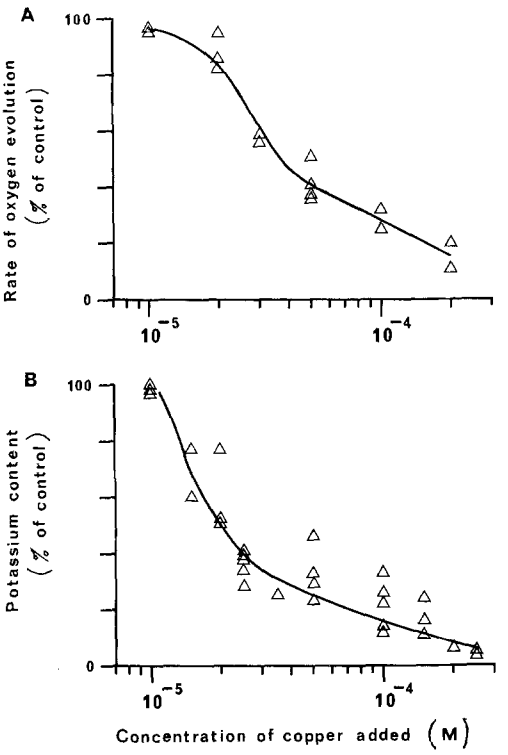

Fig. 3. *Dunaliella tertiolecta*. Effect of Cu^{2+} on light-induced oxygen evolution (A) and potassium content (B) of cells; 5µg chlorophyll *a*/ml in 0.01 M HEPPS, 1.5% NaCl, 0.25% $MgCl_2.6H_2O$, 0.05% $CaSO_4.2H_2O$, pH 7.9, as a function of molarity of heavy metal; 15-min dark incubation

of the scatter was due to variation of results from day to day, and the results were not repeated for a statistically significant number of days. The apparent two-fold concentration difference may not be significant.

It was hoped to extend this work to the diatom *Phaeodactylum tricornutum* and the light-induced oxygen-evolution inhibition curves were determined as before (Fig. 1C). Treatment with potassium-free medium alone, however, without heavy metals, had the effect of removing most of the potassium from the cells.

When *Phaeodactylum tricornutum* was treated with Hg^{2+} it was found that the mercury also inhibited respiration over a similar range to that required for inhibition of photosynthesis; this is also illustrated in Fig. 1C. When the mercury concentration was increased above 10^{-5}M the respiration rate in the dark was zero, but in the light there was an apparent light-induced respiration such that at a concentration of 10^{-4}M Hg^{2+} it was equal to the value for the dark respiration of the untreated cells. This light-induced oxygen uptake was not only seen with buffer and mercury, but was shown to an even greater extent with cells killed by boiling. High concentrations of Cu^{2+} also gave rise to light-induced oxygen uptake, but the effect was much less pronounced than with Hg^{2+}.

Discussion

The results reported here for *Dunaliella tertiolecta* show that for Cu^{2+}, potassium loss (presumably associated with membrane damage) occurs over a similar and perhaps lower Cu^{2+} concentration range than does inhibition of oxygen evolution. It was expected that the presence of Ca^{2+} ions in the medium would give some protection against potassium loss (Eppley and Cyrus, 1960). When this was investigated using a buffer containing the major ions of the half strength sea-water medium, the concentration of copper needed for 50% loss of potassium rose from 5×10^{-6} to 2×10^{-5}M and that for 50% inhibition of light-induced oxygen evolution from 10^{-5} to 4×10^{-5}M.

It would appear that mercury and thallium may be transported through the outer membrane of the cell to the chloroplast, where photosynthesis is inactivated at concentrations which cause no damage to the cell membrane as reflected in potassium leakage. Indeed, thallium can be actively taken up (presumably by the potassium pump) (Solt *et al.*,

1971). These results are in general agreement with those obtained for *Chlamydomonas reinhardii* by Overnell (1974), who examined the effect of heavy metals on the inhibition of Photosystem I and Photosystem II reactions and on light-induced oxygen evolution of whole cells. It was found there that $MeHg^{+}$, Hg^{2+} and Cd^{2+} appeared to be transported to the sites of the light reactions of photosynthesis with some degree of specificity.

The results reported here are consistent with the suggestion of McBrien and Hassall (1965) that the primary toxic effect of copper is to increase the permeability of the cell. The work reported here on *Dunaliella tertiolecta* does not accord with conclusions of Kamp-Nielsen (1971) on another chlorophycean alga, *Chlorella pyrenoidosa*, for which he suggested that the primary effect of mercury was to induce leakage through the diffusion barrier, causing an outflow of potassium. This may represent a species difference.

The limited effect of methyl mercury on K^{+} leakage is very interesting, and presumably reflects a very selective action on the membrane. This limited leakage also occurs at concentrations which cause complete inhibition of oxygen evolution. The results are similar to those found by Shieh and Barber (1973) with *Chlorella pyrenoidosa*, in that only limited leakage of potassium was observed.

Attempts to confirm the results obtained with *Dunaliella tertiolecta* using *Phaeodactylum tricornutum* revealed the peculiar sensitivity of this latter organism to the external potassium concentration; this will be the subject of a subsequent report.

The extreme sensitivity of *Chlamydomonas reinhardii*'s light-induced oxygen evolution to cadmium and lead (50% inhibition at 1.5×10^{-6}M) found by Overnell (1974) was not found for either of the species reported here. Indeed, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* were most insensitive to these ions. Although *C. reinhardii* is a fresh-water species, the assay conditions only differed in that those for the marine species contained 1.5% sodium chloride. It would thus appear that the differences in sensitivity are primarily due to species differences.

The apparent light-induced respiration which is induced in a suspension of *Phaeodactylum tricornutum* cells with a Hg^{2+} (or Cu^{2+}) concentration in excess of 10^{-5}M was unexpected. This is presumably due to some decomposition of the chloroplast lamellae to liberate forms of chlorophyll capable of promoting the photosensitized oxidation of reducible substrates present in the cells (cf. Warburg and Schocken, 1949). The fact that dark respiration is blocked under these conditions suggests a considerable disruption of the internal constitution of the cell. In this context, it is interesting to note that Nielsen and Kamp-Nielsen (1970) and Nielsen and Wium-Andersen (1971) found that copper leads to a loss of cellular organic material in the case of two species of diatom, but not in the case of *Chlorella pyrenoidosa*.

Literature Cited

Droop, M.R.: Requirement for thiamine among some marine and supra-littoral protista. J. mar. biol. Ass. U.K. *37*, 323-329 (1958)

Eppley, R.W. and C.C. Cyrus: Cation regulation and survival of the red alga *Porphyra perforata*, in diluted and concentrated sea water. Biol. Bull. mar. biol. Lab., Woods Hole *118*, 55-65 (1960)

Good, N.E., G.D. Winget, W. Winter, T.N. Connolly, S. Izawa and R.M.M. Singh: Hydrogen ion buffers for biological research. Biochemistry, N.Y. *5*, 467-477 (1966)

Joyce, C.R.B., H. Moore and M. Weatherall: The effects of lead, mercury and gold on the potassium turnover of rabbit blood cells. Br. J. Pharmac. Chemother. *9*, 463-470 (1954)

Kamp-Nielsen, L.: The effect of deleterious concentrations of mercury on the photosynthesis and growth of *Chlorella pyrenoidosa*. Physiologia Pl. *24*, 556-561 (1971)

McBrien, D.C.H. and K.A. Hassall: Loss of cell potassium by *Chlorella vulgaris* after contact with toxic amounts of copper sulphate. Physiologia Pl. *18*, 1059-1065 (1965)

Nielsen, E.S. and L. Kamp-Nielsen: Influence of deleterious concentrations of copper on the growth of *Chlorella pyrenoidosa*. Physiologia Pl. *23*, 828-840 (1970)

- and S. Wium-Andersen: The influence of Cu on photosynthesis and growth in diatoms. Physiologia Pl. *24*, 480-484 (1971)

Overnell, J.: The effect of some heavy metal ions on photosynthesis in a freshwater alga. Pestic. Biochem. Physiol. (1974). (In press)

Passow, H. and A. Rothstein: The binding of mercury by the yeast cell in relation to changes in permeability. J. gen. Physiol. *43*, 621-633 (1960)

Shieh, Y.J. and J. Barber: Uptake of mercury by *Chlorella* and its effect on potassium regulation. Planta *109*, 49-60 (1973)

Solt, J., H. Paschinger und E. Broda: Die energieabhängige Aufnahme von Thallium durch *Chlorella*. Planta *101*, 242-250 (1971)

Suelter, C.H.: Enzymes activated by monovalent cations. Science, N.Y. *168*, 789-795 (1970)

Warburg, O. and V. Schocken: A manometric actinometer for the visible spectrum. Archs Biochem. *21*, 363-369 (1949)

Ziegler, R. und K. Egle: Zur quantitativen Analyse der Chloroplastenpigmente. I. Kritische Überprüfung der spektralphotometrischen Chlorophyll-Bestimmung. Beitr. Biol. Pfl. *41*, 11-37 (1965)

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Date of final manuscript acceptance: October 14, 1974. Communicated by J.H.S. Blaxter, Oban