Plant Physiol. (1970) 46, 183–185

Observations on the Mechanism of Copper Damage in *Chlorella*[1]

Received for publication January 12, 1970

R. E. GROSS,[2] P. PUGNO,[3] AND W. M. DUGGER
Department of Life Sciences, University of California, Riverside, California 92502

ABSTRACT

Addition of excess copper to nongrowing cells of a normal, green *Chlorella* caused a reduction in total pigments and a blue shift of chlorophyll absorption, concurrent with the inhibition of photosynthesis. Chlorophylless yellow and white mutant strains of the same alga showed a rise in nonspecific absorption (*i.e.*, change in light scatter) within 5 to 10 minutes after the addition of $CuSO_4$; concomitantly a lowering of packed cell volume and a rise in respiration occurred. Glutathione prevented all copper-induced changes, whereas $MnCl_2$ protected only partially. Selective inhibition of some responses to copper was observed when O_2 was absent or an antioxidant present.

The basis for copper toxicity in plants is largely unknown. It is well established that copper is a required micronutrient for algae; in *Chlorella* culture deficiency symptoms begin when the copper level is lower than 0.1 μM (15). Greenfield (2) has reported that photosynthesis in *Chlorella* was inhibited by copper concentrations higher than 0.1 μM. Recently, it was reported that the effect of copper on photosynthesis and respiration of *Chlorella* is magnified when copper is applied under anaerobic conditions (1).

The reduction of a redox dye by isolated chard chloroplasts was shown to be sensitive to 10 to 100 μM $CuSO_4$ (10). Haberman (7) inhibited the Mehler reaction of chloroplasts of *Phytolacca americana* L. with copper and then reversed this inhibition with manganous salts. Glutathione had no effect alone, but enhanced the reversal by manganese. Heavy metals are well known inhibitors of enzymes and copper binds strongly with sulfhydryl groups of proteins (13).

The object of the present study is to examine some of the effects of copper on *Chlorella* cells which occur immediately upon exposure to the metal ion. The early effects and their alleviation through chemical additions are compared with the more familiar damage to photosynthesis and respiration.

MATERIALS AND METHODS

A culture of *Chlorella* isolated from Pacific tidal waters was designated strain A and served as the wild type for this study. A chlorophylless, yellow mutant clone, A^1, and a colorless clone, A^{11}, were isolated from ultraviolet-treated WT[4] cultures.

All cultures were grown on glucose and acetate media (3) in dim light at room temperature, 25 C $\pm$ 1, in cotton-plugged Erlenmeyer flasks on a reciprocating shaker. Routinely, cells were harvested by centrifugation at 2000*g*, washed once, resuspended in a 0.02 M potassium phosphate buffer, and shaken in a constant temperature water bath at 30 $\pm$ 1 C. The method of pigment analysis was previously reported (4). Respiration and photosynthesis were measured polarographically (5).

Absorption of whole cells and cell extracts was measured in a Coleman junior spectrophotometer model 6A and a Cary 15 spectrophotometer, which for whole cell measurements was equipped with a ground glass filter (14).

Ethoxyquin (Santoquin-primarily ethoxyquin) was obtained from Monsanto, β-carotene was obtained from Matheson Coleman & Bell, and glutathione was obtained from Nutritional Biochemicals Co.; the salts used were reagent grades supplied by Mallinckrodt Chemical Co.

RESULTS

Preliminary observations showed the inhibition or stimulation of respiration is a function of copper concentration. At the highest treatment levels, <80 μM Cu^{2+}, respiration was severely inhibited and the yellow A^1 cells lost their color and became indistinguishable from the white A^{11} cells.

Net photosynthesis rates of WT cells in $CuSO_4$ concentrations from 10 to 100 μM showed the expected inhibition, which was essentially complete at the upper end of this range. Full protection by equimolar concentrations of GSH and partial recovery in the presence of $MnCl_2$ were also observed. Whole cell absorption spectra suggested a correlation. The unprotected cells with the most copper-inhibited photosynthesis showed the greatest reduction in chlorophyll absorption, together with a blue shift of the absorption pattern.

Pigment destruction induced by copper in the yellow mutant cells at pH 6.3 showed a biphasic change in absorbance at 480 nm (Fig. 1, curve a). A rise in absorbance for several minutes was followed by a reduction which continued until the cells were nearly colorless. The second phase was prevented in an atmosphere of N_2 (Fig. 1, curve e); however, upon the addition of air the second phase was greatly accelerated, and the cells bleached within 5 min (not shown on graph). If the pH level of the buffer was above 7, several hours of exposure to copper were required to reduce the pigments effectively. The presence or absence of light during the copper treatment had no effect on the rate of pigment breakdown.

The addition of copper to both the yellow and the white mutant cells caused an initial rise in absorbance at 600 nm where nonspecific absorption takes place. This was followed by a gradual decline to the original absorbance. Table I summarizes the observed changes in absorbance at 480 nm, packed cell volume, and rates of respiration. The maxima in absorption were always accompanied by a decline in packed cell volume.

Figure 1, curve d, shows the complete protection of the cells by GSH from the copper-induced change in absorbance. Ethoxy-

[1] This work was supported in part by Grant AP-40 from the National Center for Air Pollution Control, United States Public Health Service.

[2] Present address: Purdue University, Indianapolis Campus, Indianapolis, Indiana 46205.

[3] United States Public Health Service undergraduate research trainee.

[4] Abbreviations: WT: wild type.

quin, an antioxidant, protected the cells from the loss in pigmentation, but the initial absorbance changes occurred as in the unprotected copper treatment (Fig. 1, curve c). Also shown (Fig. 1, curve b) is a partial protection by $MnCl_2$ from the initial rise in absorbance and a slower rate of decline due to pigment breakdown. The concentrations of ethoxyquin, GSH, and $MnCl_2$ used did not cause changes in absorbance.

In an attempt to approximate *in vitro* the copper-induced pigment breakdown *in vivo*, pure β-carotene dissolved in 90% methanol and 10% water (v/v) was treated with copper salts. This system was relatively stable, and the decrease in absorbance at 470 nm was very gradual. Addition of copper sulfate under aerobic conditions accelerated carotene breakdown; however, anaerobic conditions did not alleviate the final loss of pigment. Ethoxyquin protected the pigments as observed previously with whole cells. Unlike the protection in whole cells, $MnCl_2$ greatly enhanced the effect of copper on the pigment.

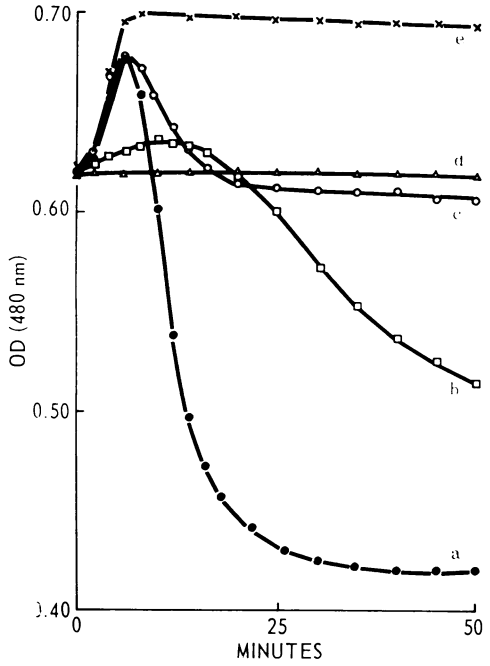

FIG. 1. Protection from copper-induced absorption changes in *Chlorella* A^1, a yellow mutant. Cells were suspended in buffer, pH 6.3, and shaken in room light for 8 hr. Protective compounds were added to the cells 3 min before copper, which was added at 0 time. a: 0.1 mM $CuSO_4$ aerobic; b: 0.1 mM $CuSO_4$ + 0.2 mM $MnCl_2$; c: 0.10 mM $CuSO_4$ + 0.10 mM ethoxyquin; d: 0.10 mM $CuSO_4$ + 0.10 mM GSH; e: 0.10 mM $CuSO_4$ + anaerobic (N_2); untreated control equivalent to curve d is not shown.

Table I. *Changes in Cell Structure and Function in the Presence of Copper Sulfate*

Cells of *Chlorella* strain A^1 (yellow) and A^{11} (white) were suspended in buffer pH 6.3, and shaken in room light for 8 hr before exposure to 10^{-1} mM $CuSO_4$ in darkness at 0 time.

Time	A^1 (Yellow)			A^{11} (White)	
	Absorbance at 480 nm	Packed cell volume in suspension	Respiration	Absorbance at 480 nm	Packed cell volume in suspension
min		*per 10 ml*	*μl O_2/mg dry wt·hr*		*per 10 ml*
0	0.60	0.039	3.6	0.44	0.045
6	0.67	0.034	7.9	0.50	0.040
20	0.45	0.035	3.8	0.47	0.042
60	0.39	0.034	1.3	0.43	0.042

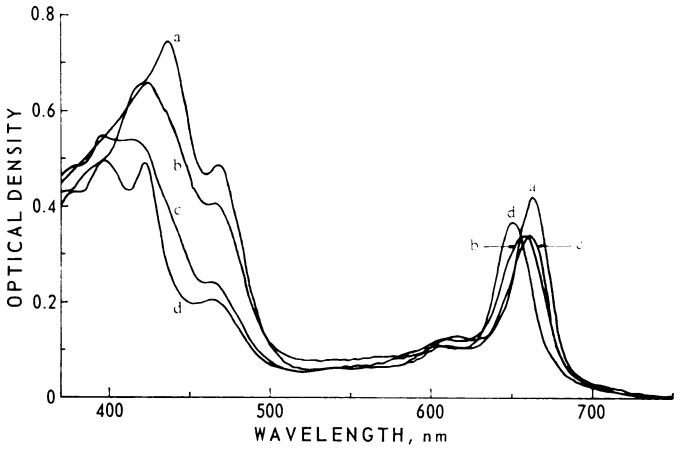

FIG. 2. Absorption changes of pigments from *Chlorella* A (WT) in methanol-water induced by $CuSO_4$. Pigments were dissolved in methanol-water (90:10, v/v). Conditions were: 30 C, darkness, shaken aerobically. Final concentrations and times of exposure: a: No copper added; b: 1 mM $CuSO_4$, 60 min; c: 1 mM $CuCl_2$, 10 min; d: 1 mM $CuCl_2$, 60 min.

After pigments of the yellow mutant (A^1) were extracted and saponified, carotenes were isolated from xanthophylls by phase separation (1), and the two fractions were transferred to a solution of methanol-water. Carotenes behaved identically to the pure β-carotene. The xanthophylls, although more stable as an untreated control, produced a cloudy suspension upon the addition of copper or other compounds.

Extracts of WT cells containing all the pigments were transferred to the methanol-water system. Addition of copper caused a reduction in absorbance as well as a 10 to 20 nm blue shift of the chlorophyll peaks (Fig. 2). This is in good agreement with the observations of whole cells. In addition it was observed that copper-treated chlorophyll does not fluoresce. The addition of $CuCl_2$ to this system was more effective in bleaching pigments than $CuSO_4$. Not shown on the graph is an increased $CuSO_4$ activity when NaCl was added to the system.

DISCUSSION

The exact role of copper in the development of cellular damage is not known, but several model systems are available. Gurd (6) showed that copper ions catalyze the oxidation of sulfhydryl groups and formation of disulfide bridges. Krivis and Rabb (9) reported that the enhancement of antitubercular activity of Isoniazid by Cu (II) may actually be due to the formation of a Cu (I) complex. Virtually all toxicity studies use Cu (II), since Cu (I) is highly insoluble. In addition to chelation and participation in organic redox reactions, copper ions probably hydrate or form hydroxides, which introduce further complications.

The results reported here suggest that the mechanism of action may be influenced by the structure of the cell, with the rupture of membrane barriers the first step in the damage sequence. The initial absorbance rise is concomitant with a drop in packed cell volume (Table I), and it is tempting to compare this observation with known effects of light on chloroplast membranes resulting in a similar combination of changes (12). The experiments *in vitro* showed no initial rise upon the addition of copper. Anaerobiosis cannot forestall the rise in absorbance in whole cells, but it prevents any decline (Fig. 1). In view of the inability of anaerobic conditions to protect the pigments *in vitro*, it seems reasonable to assume that two steps must be completed *in vivo* before copper can act on the pigments, and by blocking the second

step with N_2 pigment loss is prevented The second step is possible in the presence of the antioxidant (Fig. 1, curve c), but no subsequent loss in carotenoids ensues. GSH affords complete protection (Fig. 1, curve d) from any absorbance changes, and this is reflected in its protective action in photosynthesis, suggesting a neutralization of the copper ion before it can reach the cell membrane. The addition of $MnCl_2$ dampens the copper effects *in vivo*, yet $MnCl_2$ enhanced the action of copper on β-carotene *in vitro*. It was shown, however, that the Cl^- ion was the enhancing factor in the latter case.

The stability of the green color observed after the initial blue shift and reduction in chlorophyll absorption may be related to the observations made by Hill (8). He suggested that chlorophyll was metastable with reference to Mg, and the green Cu pheophytin was very stable. Perhaps a replacement of the ionically bound Mg by a Cu atom took place, but an oxidation of chlorophyll or some other reaction cannot be excluded with the present evidence.

Chlorophyll changes and carotenoid destruction accompany the cessation of photosynthesis and respiration. The evidence presented points to another important cellular change in the presence of copper. The membrane integrity may be altered, and that could be the primary focal point of copper action.

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