The Cupric Ion as an Inhibitor of Photosynthetic Electron Transport in Isolated Chloroplasts[1]

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ABSTRACT

Strong inhibition of uncoupled photosynthetic electron transport by Cu^{2+} in isolated spinach chloroplasts was observed by measuring changes in O_2 concentration in the reaction medium. Inhibition was dependent not only on the concentration of the inhibitor, but also on the ratio of chlorophyll to inhibitor. Binding of Cu^{2+} to the chloroplast membranes resulted in removal of Cu^{2+} from solution. When chloroplasts were exposed to preincubation in light, there was increased inhibition as a result of Cu^{2+} binding to inhibitory sites. Preincubation in the dark resulted in Cu^{2+} binding to noninhibitory sites and decreased inhibition. The degree of inhibition was lower at low light intensities than at high light intensities.

When the photosystems were assayed separately, photosystem I was more resistant to inhibition than photosystem II. The most sensitive site to the inhibitor was the oxidizing side of photosystem II.

The role of copper in photosynthetic organisms depends greatly on its concentration. Copper, as cupric ion, in trace amounts is an essential micronutrient for algae and higher plants (17, 20) and is an essential constituent of several enzymes such as polyphenol oxidase (3) and plastocyanin (8), a component of photosynthetic electron transport. Concentrations higher than 1 μM are increasingly toxic to algal and higher plant tissues (4, 13). Cupric sulfate has been extensively used as an algaecide since the beginning of the century (12). The cupric ion has been shown to be an inhibitor of photosynthesis in algal cells (4, 11, 16) and to inhibit photosynthetic electron transport in isolated chloroplasts (5, 10).

The mechanism by which the Cu^{2+} inhibits the photosynthetic apparatus has been only partially elucidated thus far. The data on inhibition of photosynthesis in isolated chloroplasts gives an incomplete picture of the specific sites of inhibition in the electron transport chain. Macdowall (10) using isolated Swiss chard (*Beta vulgaris*) chloroplasts found that indophenol dye

reduction was inhibited 50% by approximately 10 μM $CuSO_4$. He studied the effect of light intensity on inhibition and concluded that the light reactions were directly affected by Cu^{2+}. In addition, Haberman (5) using chloroplasts from *Phytolacca americana* showed that both the Hill and Mehler reactions were inhibited by Cu^{2+} but that the Mehler reaction was inhibited by lower concentrations of the ion.

In contrast to Macdowall, Haberman concluded that Cu^{2+} was inhibiting a dark reaction and did not alter the reactions associated with the photoacts. She observed that Mn^{2+} added at a concentration of 0.5 mM reduced the inhibition of chloroplasts by Cu^{2+} and postulated that the Cu^{2+} was affecting the site of manganese function in O_2 evolution. Recently exogenous Mn^{2+} has been shown to donate electrons after the water oxidation site of photosystem II of isolated chloroplasts (2), suggesting that, at high concentrations, manganese functions in a way other than its primary role in the water oxidation act.

This work was conducted with the purpose of resolving the sites of Cu^{2+} inhibition in photosynthetic electron transport. Since previous work and our preliminary experiments pointed to the fact that photosystem II is preferentially inhibited by Cu^{2+}, emphasis was given to finding the specific sites of inhibition within this photosystem.

MATERIALS AND METHODS

Chloroplasts were prepared from market-grown spinach (*Spinacia oleracea* L.) as described by Robinson and Stocking (14). $MnCl_2$ was excluded from the grinding and resuspending media in the preparations where Mn^{2+} was used as an electron donor. Chlorophyll was determined by the method of Arnon (1). Ferredoxin was isolated by the method of San Pietro (15) as modified by Swader and Jacobson (18). The O_2 evolving capacity of the chloroplasts was destroyed by a mild heat treatment as described by Hinkson and Vernon (6). The chloroplasts were uncoupled by adding ammonium ions following the principle discovered by Krogman et al. (9).

The normal reaction media for studying O_2 evolution had the following composition in a total volume of 2 ml: 50 mM HEPES, pH 7.6; 10 mM KCl; 5 mM $MgCl_2$; 1 mM $(NH_4)_2SO_4$; 1.5 mM NADP; and saturating amounts of ferredoxin. A temperature of 25 C was maintained during all assays.

Catalase, NADP, and the MES and HEPES buffers were purchased from Calbiochem. The sodium salt of DCIP[2] and ascorbic acid were obtained from the J. T. Baker Chemical

[2] Abbreviation: DCIP: 2,6-dichloroindophenol.

Co. and 2-anthraquinone sulfonic acid (sodium salt) from East-man Organic Products. $CuSO_4$ was used as the source of Cu^{2+} and 1,5-diphenyl carbazide obtained from Mallinckrodt was recrystallized from ether.

The oxygen concentration of the assay media was determined using a Gilson Medical Electronics Oxygraph model KM with a vibrating platinum electrode. The chart was calibrated to obtain full scale readings of 0.25 μmole of oxygen per ml of solution in the reaction cell. In those experiments where O_2 evolution was studied, N_2 was bubbled through the reaction media. In assays where 2-anthraquinone sulfonic acid was the electron acceptor, catalase and ethanol were added to the assay media. This allowed the conversion of the H_2O_2 formed during the Mehler reaction to acetaldehyde and water so that a net O_2 uptake could be measured.

Samples were illuminated with two 300-w, 120-V Ken-Rad reflector lights. Each bulb provided 2200 ft-c measured at the surface of the reaction cell. Infrared radiation was removed with water jackets. Intensity of light was varied by means of wire screens of known transmission.

DCIP photoreduction was measured at 590 nm with a Zeiss spectrophotometer model PMQ II, adapted for illumination. Samples were illuminated as described previously but only one light was used. All reactions were measured in light and dark, and the data presented are the differences between the rate in the light and the rate in the dark.

RESULTS

Cu^{2+} inhibits uncoupled photosynthetic electron transport to NADP at very low concentrations, as shown in Figure 1. The extent of inhibition depends not only on the concentration of Cu^{2+} but also on the ratio of Cu^{2+} to chloroplasts; the higher the amount of chl, the greater the amount of copper required to cause the same degree of inhibition. This fact suggests that the cupric ions are bound by chloroplast membranes.
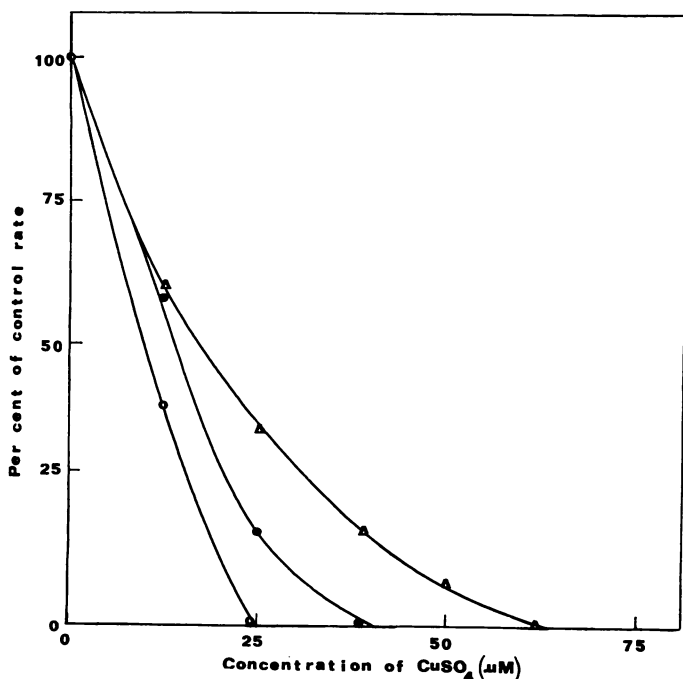

FIG. 1. Effect of Cu^{2+} on oxygen evolution by isolated chloroplasts with NADP as the electron acceptor. Reaction mixture as described in "Materials and Methods" with chloroplasts at a chl concentration of: O: 42 μg/2 ml; ●: 86 μg/ml; △: 169 μg/2 ml. Control rate: 590 μeq O_2 evolved/mg chl·hr.
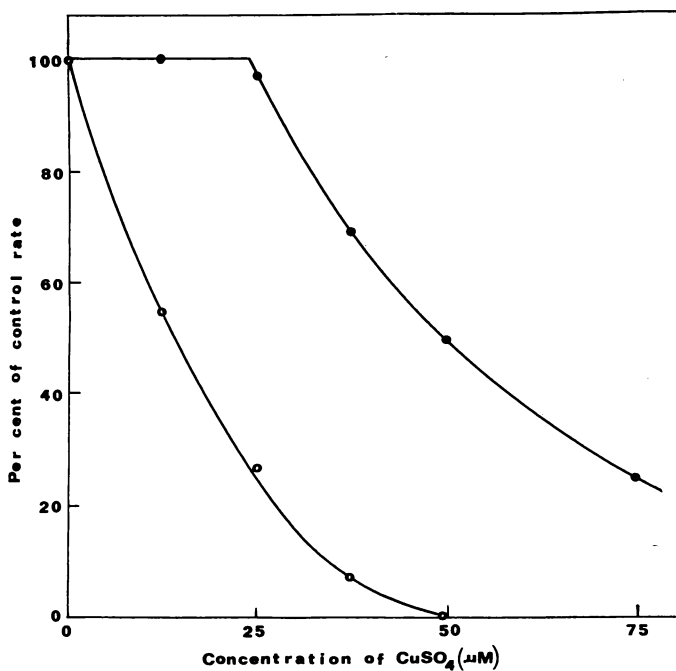

FIG. 2. Irreversible binding of Cu^{2+} into the membrane system of isolated chloroplasts. Reaction mixture as described in "Materials and Methods" with chloroplasts at a chlorophyll concentration of 61 μg/2 ml. Control rate: 744 μeq O_2 evolved/mg chl·hr. The reaction was measured in the dark and light, chloroplasts were centrifuged from the reaction media, and the supernatant was used as reaction media with fresh aliquots or chloroplasts, NADP, and ferredoxin added. O: Original; ●: supernatant.

Binding of Cu^{2+} by the chloroplast membranes should remove cupric ions from the media. Therefore, supernatants obtained by centrifuging the chloroplasts out of the samples exposed to Cu^{2+} were used as assay media to determine the amount of remaining Cu^{2+}, according to the technique of Izawa and Good (7). If cupric ions are removed by the chloroplasts, then inhibition by supernatants on fresh chloroplasts would be greatly reduced. The resulting curves for inhibition (Fig. 2) were the same shape for both originals and supernatants, but the curve for supernatants was displaced by more than 24 μM $CuSO_4$ from the normal inhibition curve. Therefore, 24 μM $CuSO_4$ was totally removed by the chloroplasts. This corresponds to approximately 0.8 μmole of the inhibitor removed per mg of chl.

In preliminary experiments a reduction in the inhibitory effects of Cu^{2+} was observed upon leaving the samples for short periods of time in the dark. It appeared that in the dark Cu^{2+} was being irreversibly bound to noninhibitory sites in the membranes, which prevented it from binding with inhibitory sites in the light. The results obtained on preincubating samples in dark and light on the inhibitory effects of Cu^{2+} are shown in Table I. Preincubation in the dark for 5 and 10 min reduced the inhibition whereas preincubation in light showed the inhibition developed rapidly, since most of the inhibition occurred during the time required to run the assay. Little additional inhibition occurred during the 5 more min of illumination. Copper apparently reaches its site of inhibition much more rapidly and to a greater extent in the light.

Preincubation in the dark with 50 μM $CuSO_4$ shows that the noninhibitory binding appeared to be complete after 5 min since very little decrease in inhibition occurred from 5 to 10 min in the dark. At 25 μM $CuSO_4$, however, some further binding occurred from 5 to 10 min in the dark. In addition, if

after preincubation in the dark for 10 min with 50 μM CuSO$_4$, the chloroplasts were separated and suspended in fresh media, very little or no inhibition was obtained (data not shown). In the dark nearly all the copper removed from solution is irreversibly bound at noninhibitory sites, and this binding occurs more rapidly at higher Cu^{2+} concentrations.

The role of light in the inhibitory action of Cu^{2+} was studied further. The relationship between light intensity and the inhibitory effect of Cu^{2+} is shown in Table II. There is a reduction in inhibition at light intensities below 1400 ft-c. At high light intensities the degree of inhibition remains relatively constant. This was also observed in experiments in which higher or lower concentrations of the inhibitor were used. Macdowall (10) also observed reduction of the degree of inhibition at low light intensities. This reduction in the degree of inhibition could be related to our observation that light stimulated the binding of Cu^{2+} to inhibitory sites.

Specific sites of inhibitory action of Cu^{2+} were studied by assaying photosystems I and II. Photosystem II was assayed by the technique employed by Vernon and Shaw (19) where light-dependent electron flow from 1,5-diphenyl carbazide to DCIP was used. Photosystem I was assayed by using a far red filter (no transmission of wavelength less than 700 nm and 80% transmission of wavelengths greater than 700 nm) and by using DCIP-ascorbate as the electron donor with 2-anthraquinone sulfonic acid as the electron acceptor (Fig. 3). Both photosystems are inhibited by Cu^{2+}, but photosystem I is more resistant to inhibition than photosystem II. Thus, photosystem II appears to be the site of inhibition at low concentrations of the inhibitor.
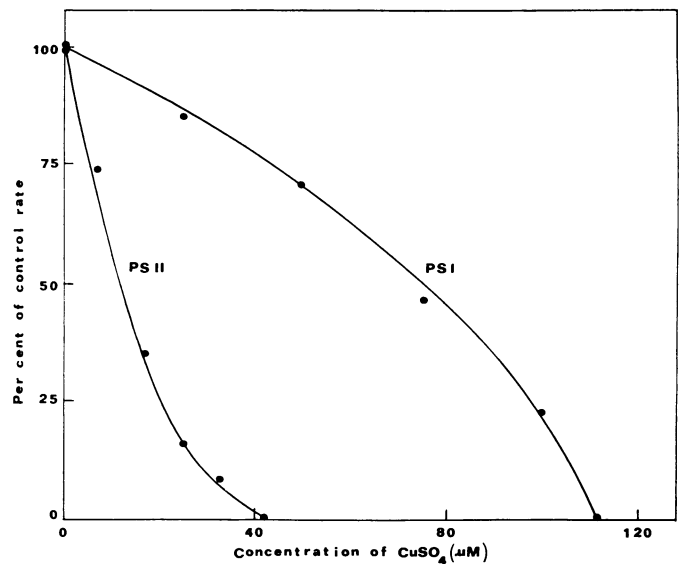
As a means of exploring where within photosystem II in-

FIG. 3. Effect of Cu^{2+} on uncoupled electron transport in photosystems I and II of isolated chloroplasts. Photosystem II assay, reaction mixture (3 ml) contained: 30 mM K$_2$HPO$_4$, pH 7.6, 0.25 M sucrose, 1 mM (NH$_4$)$_2$ SO$_4$, 0.67 mM 1,5-diphenyl carbazide, 0.33 mM DCIP, and chloroplasts (65 μg chl.). Control rate was 108 μmoles DCIP reduced/mg chl·hr. Photosystem I assay, reaction mixture (2.0 ml) contained: 50.0 mM HEPES, pH 7.6, 50 μm 2-anthraquinone sulfonic acid, 0.25 mM DCIP, 1 mM ascorbate, 1 mM (NH$_4$)$_2$ SO$_4$, 2.5% ethanol, catalase (74,000 e.u.), and chloroplasts (61 μg chl). Control rate was 317 μeq of O$_2$ uptake/mg chl·hr.

Table I. *Effect of Light and Dark Preincubation on Cu^{2+} Inhibition of Photosynthetic Electron Transport*

Reaction mixture as described in "Materials and Methods" containing chloroplasts at a chl concentration of 44 μg/2 ml. Preincubation consisted of incubating the chloroplasts plus inhibitor in 50 mM HEPES, pH 7.6, in dark or light (2200 ft-c) at 0 C.

CuSO$_4$	Rate of O$_2$ Evolution				
	Light		Dark		
	0 min	5 min	0 min	5 min	10 min
μM	μeq/mg chl·hr				
0	763	799	763	741	683
25	230	173	230	284	426
50	22	0	22	226	208

Table II. *Effect of Light Intensity on Cu^{2+} Inhibition of Photosynethetic Electron Transport*

Reaction mixture as described in "Materials and Methods" with chloroplasts at a chl concentration of 94 μg/2 ml and 15 μM CuSO$_4$.

Light Intensity	Rate of O$_2$ Evolution		Control Rate
	Control	Cu^{2+}	
ft-c	μeq/mg chl·hr		%
350	68	62	91
700	124	99	81
1400	299	198	66
2800	419	258	62
4400	454	301	63

Table III. *Effect of Several Photosystem II Electron Donors on the Cu^{2+} Inhibition of Electron Transport in Isolated Chloroplasts*

Reaction mixture (2 ml) contained 50 mM HEPES, pH 7.6; 50 μM 2-anthraquinone sulfonic acid; 2.5% ethanol; catalase (74,000 e.u.); chloroplasts (85 μg chl); and, where required, 20 mM MnCl$_2$ or 2 mM ascorbate. The chloroplasts were heat-treated where Mn^{2+} or ascorbate was used as electron donor. Control rate of O$_2$ uptake for each electron donor in microequivalents/mg chl· hr. were: Mn^{2+}, 637; H$_2$O, 735; and ascorbate, 516.

CuSO$_4$	Inhibition		
	Mn^{2+}	H$_2$O	Ascorbate
μM	%		
0.0	0	0	0
12.5	44	23	21
25.0	69	35	...
37.5	79	57	29
50.0	88	69	...
62.5	100	87	58
75.0	...	94	...
87.5	...	100	73

hibition occurs, further experiments were conducted with Mn^{2+} as electron donor and 2-anthraquinone sulfonic acid as electron acceptor. Mn^{2+} appears to donate electrons to the photo-act of photosystem II after the water oxidation act (2), permitting the use of heat-treated chloroplasts for the assays. The data in Table III show that inhibition was even greater when Mn^{2+} was used as electron donor than when water was used as electron donor. Thus, it becomes clear that electron donation within photosystem II occurring after the water oxidation act

is very sensitive to Cu^{2+}. Ascorbate, which can donate electrons to the oxidizing side of photosystem II, was more resistant to inhibition than either water or Mn^{2+}. This could have resulted from complex reactions between Cu^{2+} and ascorbate (21). The rates of electron transport when water or ascorbate was used as electron donor (data not shown) were increased by addition of Mn^{2+} in the presence or absence of Cu^{2+}.

DISCUSSION

In studying the effects of Cu^{2+} on electron transport of isolated chloroplasts it was found not only that the concentration of the inhibitor was important in determining the degree of inhibition, but that the ratio of chloroplasts to inhibitor, as well as preincubation, must also be considered—facts overlooked in previous studies (5, 10). Steemann-Nielsen et al. (16) studying the effect of Cu^{2+} on photosynthesis of whole algal cells noticed that the ratio of cells to inhibitor was one of the most important factors determining the degree of inhibition obtained. Our results show that when isolated chloroplasts were exposed to Cu^{2+} the ion was removed from solution by binding to the chloroplast membranes and the amount of the ion being bound was proportional to the amount of chloroplasts present. Binding is, therefore, one of the reasons why there has to be a certain minimal ratio between chloroplasts and inhibitor before inhibition is observed.

It was observed that exposure of the chloroplasts to the inhibitor for short periods of time in the dark reduced the degree of inhibition while exposure for short periods to light had opposite effects. Our evidence points to the fact that light is essential for the interaction of Cu^{2+} with electron transport mechanism of isolated chloroplasts. Steemann-Nielsen et al. (16) also observed that Cu^{2+} does not cause damage to the photosynthetic apparatus of whole algal cells unless the cells were illuminated during the whole exposure period. It appears that the mechanism of action of Cu^{2+} in whole algal cells is similar to that in isolated chloroplasts.

From studies of the effect of light intensity on chloroplast-mediated reactions which were inhibited by Cu^{2+} Macdowall (10) and Haberman (5) have expressed contrasting views regarding the type of reactions (whether dark or light) that were affected by the inhibitor. Our experiments on the effects of light intensity on inhibition using H_2O as the electron donor and NADP as the electron acceptor showed that at very low light intensities inhibition was lower than at high light intensities. However, since light stimulated and was apparently essential for Cu^{2+} damage, it appears that the relationship between light intensity and the type of reaction affected is more complex than as previously viewed.

Haberman (5) observed that exogenous Mn^{2+} reduced the degree of Cu^{2+} inhibition in several kinds of chloroplast-mediated reactions and postulated that Cu^{2+} affects the site of manganese action; however, this could be due to Mn^{2+} stimulating electron transport. In fact, her data show a marked increase in the rate of electron transport when Mn^{2+} was included in the reaction media. Ben-Hayyim and Avron (2) used Mn^{2+} as an electron donor and demonstrated that Mn^{2+} increased the

rate of electron transport in several chloroplast reactions. We have used chloroplasts with no O_2-evolving capacity for our experiments, from which it is evident that exogenous Mn^{2+} participates in reactions other than its primary role in O_2 evolution. Our results indicate that Cu^{2+} inhibits electron transport with Mn^{2+} as the electron donor.

The results of previous workers combined with the observations presented in this report indicate that the components on the oxidizing side of photosystem II are the most sensitive site to Cu^{2+} inhibition of photosynthetic electron transport.

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