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Reversal of Copper Inhibition in Chloroplast Reactions by Manganese[1]

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Abstract. In the Mehler reaction, a Hill reaction utilizing molecular oxygen as the electron acceptor, rates of net oxygen uptake are stimulated by added manganous ions. Both whole cell photosynthesis and the Mehler reaction are inhibited by copper. Copper inhibition of the Mehler reaction can be reversed by manganese salts. Glutathione, which alone has no effect on Mehler reaction rates, enhances the effect of manganese in reversing copper inhibition. The effects of added Cu^{2+}, Cu^{2+} and Mn^{2+}, or Cu^{2+}, Mn^{2+}, and glutathione exhibit no induction phenomena when measured manometrically. Furthermore, the order of addition of these factors is unimportant: final rates are dependent only on the composition of reaction mixtures. Compared to the Mehler reaction, conventional Hill reactions are less sensitive to copper poisoning, while certain chloroplast mediated photoxidations (*e.g.* the photoxidation of diketogulonic acid) are far more sensitive. In all of the chloroplast mediated photoreactions tested, manganese is effective in reducing the sensitivity to copper poisoning.

Copper is an inhibitor of many enzymes and its toxic effects on photosynthetic organisms have long been recognized (5, 14). Although copper inhibits photosynthesis, it is present in at least 1 essential component of the electron transport system of chloroplasts, the blue copper-protein, plastocyanin (13).

The mechanism of copper inhibition has interested us since we discovered that copper is a specific poison for the flavin sensitized photoxidation of diketogulonic acid[2], a reaction that requires manganese (8, 11, 12). The photoxidation of DKGA can also be mediated by illuminated chloroplasts (9). The chloroplast system, like the flavin sensitized reaction, is poisoned by copper; like the Mehler reaction (7, 16), it is stimulated by added manganous ions (9, 10). The chloroplast mediated photoxidation of DKGA is less sensitive to copper poisoning when added manganese is present in the reaction mixture. This apparent protective action of manganese led us to test other chloroplast reactions for possible protective effects and for reversal of copper inhibition by manganese. In all of the reactions studied (Quinone-Hill reaction, Mehler reaction, and DKGA photoxidation) added manganous ions reduce sensitivity to copper inhibition. In the Mehler reaction, copper inhibition can be reversed by adding manganese. Glutathione (GSH), which alone does not affect Mehler rates, enhances the effects of manganese (*i.e.*, still higher concentrations of copper must be added to inhibit the reaction).

The effects of added manganese on copper inhibited chloroplast reactions suggest that manganese may provide a natural protective mechanism against levels of copper which might otherwise be toxic. There is evidence that such an antagonism may exist in nature. Forster (3) has found that excess manganese accumulates in the tops of crops grown in soils having an unusually high copper content.

Materials and Methods

Chloroplasts, prepared as previously described (6) from *Phytolacca americana* collected in the field, were stored at −85° until used. The components of reaction mixtures were prepared at the concentrations listed below and stored at −20° or at −85°: *p*-Benzoquinone (purified by sublimation, 0.02 M), dehydroascorbic acid (DHAA, from Mann Research Laboratories, New York, 0.02 M), GSH (from Nutritional Biochemicals Corporation, 0.02 M), cysteine (from Nutritional Biochemicals Corporation, 0.02 M), flavin mononucleotide (FMN, 0.001 M). Catalase (sterile solution, 30,000 e.u. per ml, from Nutritional Biochemicals Corporation) was used without dilution. The following salts were used as sources of metal ions: $CuSO_4$, $FeSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $MgCl_2$, and $ZnCl_2$. The composition of reaction mixtures is described with individual experiments.

Reactions were followed manometrically in rectangular Warburg vessels (14 cm^2 bottom area, *ca.* 17 ml volume). Temperature was 20° and the gas phase for all experiments was air. Illumination was provided by a bank of 300 w reflector flood lamps. Light quality was controlled by means of Plexiglas and cellophane filters. Light intensity was varied by changing lamp voltage (Fisher Powerstat, 20 amp with voltmeter). Spectral distribution of incident

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[2] Abbreviations used in the text are: DKGA, diketogulonic acid; DHAA, dehydroascorbic acid; FMN, flavin mononucleotide; GSH, glutathione.

light energy was measured with an ISCO Model SR Spectroradiometer using the remote probe suspended at the position of the vessels in the water bath. Chlorophyll concentration was determined by Arnon's method (1).

Results

Inhibition of DKGA Photoxidation by Copper. The first indication that sensitivity to copper inhibition could be influenced by added manganese was obtained from studies of the effects of Cu^{2+} on rates of chloroplast mediated photoxidation of DKGA. This reaction is essentially the same as the chloroplast mediated photoxidation of manganese studied by Bachofen (2) except that DKGA serves as an electron donor for oxidized manganese. Our present view of the mechanism of this photoxidation is that 2 reducing equivalents per DKGA reduce 2 atoms of Mn^{3+} generated by photosystem II. Electrons from photosystem II are transferred *via* photosystem I to flavin. Autoxidation of flavin results in the uptake of 1 mole of oxygen per mole DKGA and formation of 1 mole of H_2O_2 (10). There are 3 variations of the chloroplast mediated photoxidation of DKGA. The "endogenous" system has the following components: chloroplasts, catalase, ethanol, and DHAA as beginning substrate. The "plus Mn^{2+}" system contains components of the "endogenous" system plus Mn^{2+}; the "complete" system contains components of the "endogenous" system plus Mn^{2+} and FMN. Under the experimental conditions used there is a rapid and spontaneous conversion of DHAA to DKGA. The time course of light dependent oxygen uptake is essentially the same in all 3 systems: an induction period of slow oxygen uptake, a period of rapid oxygen uptake (oxidation of DKGA to oxalate and threonate) and a final slow oxygen uptake. Addition of Mn^{2+}, or of Mn^{2+} and FMN, increases rates and decreases the time required for induction (9, 10). "Far red" light as defined in ref. 10, with incident light predominantly at wavelengths longer than 700 nm, was used. Under these conditions rates of DKGA photoxidation were high relative to the corrections made for Mehler reaction rates (measured in the absence of DKGA).

The data in Fig. 1 show the differences in sensitivity to copper between reaction mixtures containing added manganese (the "plus Mn^{2+}" and

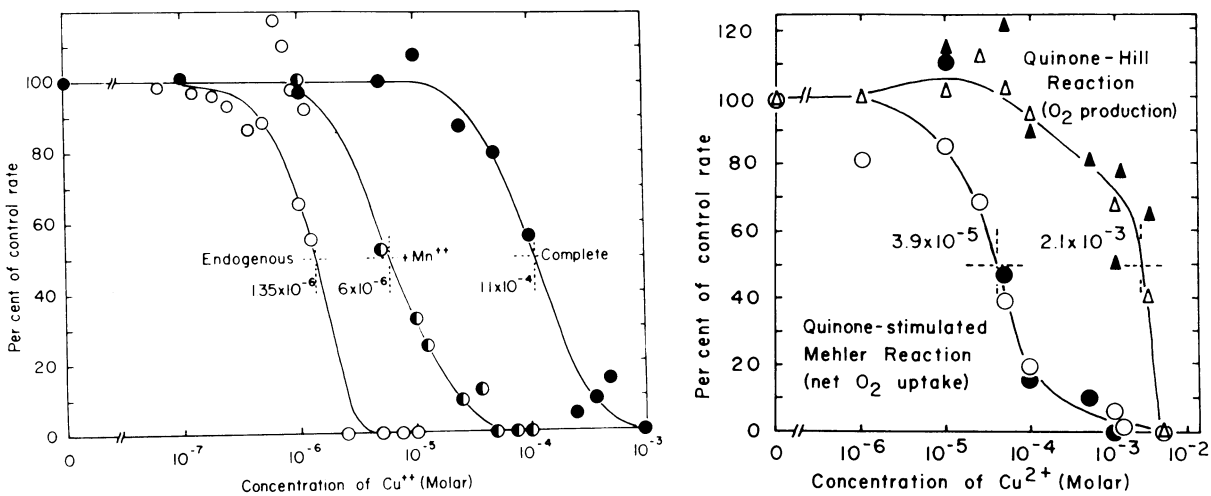


FIG. 1. (left) Effect of Cu^{2+} concentration on rates of DKGA photoxidation. Illumination for all reactions: far red light (202 μw/cm^2, wavelengths of incident light > 700 nm). -○-○- "Endogenous" system. Vessel contents: *Phytolacca* chloroplasts (1.13 mg chl), 2.5 ml phosphate buffered sucrose (0.5 M in M/15 phosphate buffer, pH 6.8, 0.005 M in KCl), 0.3 ml catalase solution, 0.1 ml 50 % ethanol 10 μmoles DHAA, Cu^{2+} concentration as indicated, in a total volume of 4 ml. Control rate: 0.5 μl O_2/min per vessel. Half closed circles—"Plus Mn^{2+}" system. Vessel contents: as in "endogenous" system plus 2 μmoles Mn^{2+}. Control rate: 1.9 μl O_2/min per vessel. -●-●- "Complete" system. Vessel contents: as in "endogenous" system (except chloroplasts with 1.43 mg chl), plus 4 μmoles Mn^{2+} and 0.1 μmole FMN. Control rate: 3.1 μl O_2/min per vessel.

FIG. 2. (right). Effects of Cu^{2+} concentration on rates of oxygen production in the Quinone-Hill reaction and on subsequent rates of net oxygen uptake in the quinone-stimulated Mehler reaction. Illumination: "high" intensity red light (2697 μw/cm^2, wavelengths of incident light> 600 nm); "low" intensity red light (768 μw/cm^2). Vessel contents for all reactions: *Phytolacca* chloroplasts (0.73 mg chl), 2.5 ml phosphate buffered sucrose, 0.3 ml catalase solution, 0.1 ml 50 % ethanol, 10 μmoles quinone, copper as indicated, in a total volume of 4 ml. -△-△- Quinone-Hill, "high" light intensity. Control rate: 10.3 μl O_2/min per mg chl. -▲-▲- Quinone-Hill, "low" light intensity. Control rate: 4.6 μl O_2/min per mg chl. -○-○- Quinone-stimulated Mehler, "high" light intensity. Control rate: —4.5 μl O_2/min per mg chl. -●-●- Quinone-stimulated Mehler, "low" light intensity. Control rate: —2.4 μl O_2/min per mg chl.

"complete" systems) and the "endogenous" system which contains no added manganese.

Copper Inhibition of Quinone-Hill and Quinone-stimulated Mehler Reactions. Reaction mixtures for these studies contained chloroplasts, catalase, ethanol and quinone. Oxygen was evolved (Quinone-Hill reaction) during the early part of the light period. Following quinone reduction, net oxygen uptake (Mehler reaction) began. In the Mehler reaction there is simultaneous uptake of molecular oxygen (which reacts with the reductant generated by photosystem I) and evolution of oxygen from the oxidized product of photosystem II. Added catalase and ethanol remove the hydrogen peroxide formed in the reduction of molecular oxygen. Mehler reaction rates are considerably higher than control rates following photoreduction of quinone but there is no difference in the stoichiometry of oxygen consumption and production between control and quinone-stimulated Mehler reactions (6). Fig. 2 summarizes experiments in which copper concentration was varied in reactions run at 2 different light intensities. The Hill and Mehler reactions differ significantly in their susceptibility to copper poisoning (2×10^{-3} M *vs.* 4×10^{-5} M needed for 50 % inhibition). It may be significant that those reactions most susceptible to copper inhibition are those stimulated by added manganese. Other experiments on the effects of light intensity showed an essentially constant ratio between control and copper inhibited rates over the entire range of light intensities used (up to saturation). Thus it appears that copper affects a dark reaction and not the photoacts *per se*.

The Protective Action of Added Manganese in Reducing the Extent of Copper Inhibition. The large differences in the effects of Cu^{2+} on the 3 types of chloroplast mediated photoxidation of DKGA suggested that manganese, alone or in combination with FMN, protects against copper inhibition. To determine whether added manganese has the same protective effect on conventional Hill reactions, Quinone-Hill and quinone-stimulated Mehler reaction rates were measured with and without added copper over a range of concentrations of added manganese. The general effect of added manganese is a reduction in the extent of inhibition caused by a given amount of copper (Fig. 3).

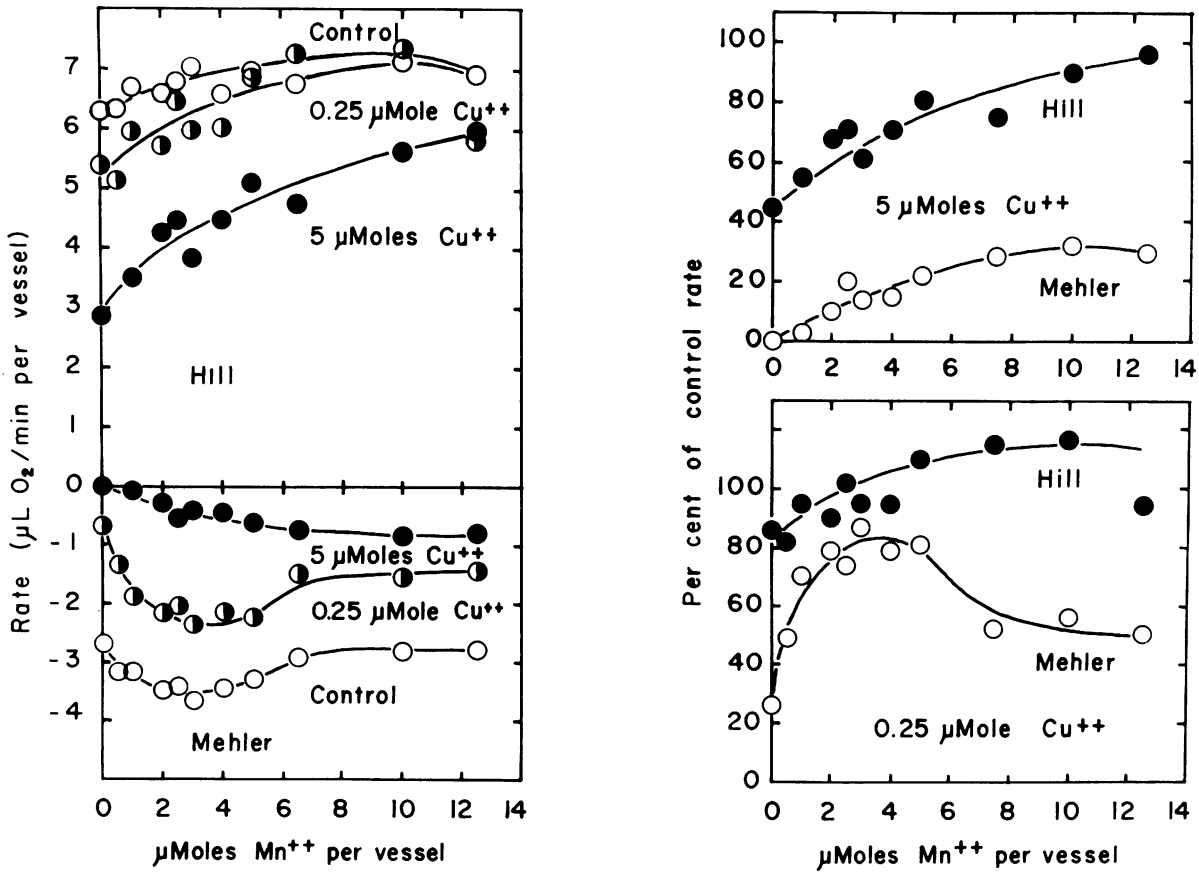
In another experiment the effects of manganese and FMN, alone and in combination, were compared. Added FMN (without added Mn^{2+}) had no effect on copper inhibited Hill or Mehler reaction rates. FMN and Mn^{2+} combined had approximately the same effect as Mn^{2+} alone.

The Effects of Other Metal Ions. The responses of chloroplast reactions to added copper and manganese resemble those of a plant flavokinase described by Giri *et al.* that is activated by Mg^{2+}, Zn^{2+}, and Mn^{2+} and inhibited by Hg^{2+}, Fe^{2+}, and Cu^{2+} (4). The effects of these metal ions on the Quinone-Hill and Mehler reactions were compared to find out whether the responses of chloroplast reactions parallel those of the plant flavokinase. As expected (5, 14), Hg^{2+} is a more potent inhibitor than copper. While Fe^{2+} inhibits the Quinone-Hill reaction, it enhances rates of the quinone-stimulated Mehler reaction. Although Zn^{2+} and Mg^{2+} have slightly stimulating effects, neither is as effective as Mn^{2+} in reversing copper inhibition. It seems appropriate to conclude that resemblances between chloroplast reactions and the plant flavokinase are superficial and do not suggest any common mechanism of inhibition or of reversing inhibition.

Enhancement of Manganese Effects by GSH. The generally inhibitory effects on the photosynthetic apparatus of heavy metal ions suggest that inhibition may be due to competition for sulhydryl groups with a resulting displacement of other metals needed for activity or changes in protein conformation. If such changes do occur, then added cysteine or GSH might decrease the inhibiting effects of copper. In testing GSH, control Mehler reaction mixtures that had not previously reduced quinone were used. The effects of adding components to reaction mixtures can be seen in the representative time course data shown in Fig. 4. These data reveal several characteristics of the reactions that are apparent only when Cu^{2+}, Mn^{2+}, or GSH are added during the light period. First, rates are determined by the composition of the reaction mixtures and not on the order in which components are added. Second, inhibition of the Mehler reaction by copper and reversal of copper inhibition by manganese exhibit no apparent time lags when measured manometrically. Third, GSH has no effect on Mehler rates without added manganese; yet manganese and GSH together are more effective in reversing copper inhibition than manganese alone (see Fig. 4a, b and c).

The effects of Mn^{2+} and GSH, alone and in combination, on control and copper inhibited Mehler reaction rates are summarized in table I. In contrast to the experiments with Quinone-Hill and quinone-stimulated Mehler reactions already described, where the addition of Mn^{2+} had negligible stimulating effects on rates, in the experiments summarized in table I dual effects of both Mn^{2+} and GSH become evident. Manganese both stimulates rates and decreases sensitivity to copper poisoning. GSH depresses rates and this small inhibition makes the apparent protective action of GSH against copper poisoning unconvincing. In spite of the difficulties of interpretation, a consistent pattern of decreased susceptibility to copper poisoning in the presence of added manganese is apparent. Even the lowest concentration of added Cu^{2+} (10^{-6} M) significantly inhibits Mehler rates. With added Mn^{2+}, concentrations of Cu^{2+} of the order of 10^{-5} M are needed for observable inhibition. With Mn^{2+} plus GSH, concentrations of Cu^{2+} in excess of 10^{-4} are needed for inhibition.

Cysteine, which like GSH has no stimulatory effect on Mehler rates in the absence of Mn^{2+}, does not exhibit such synergism with manganese.



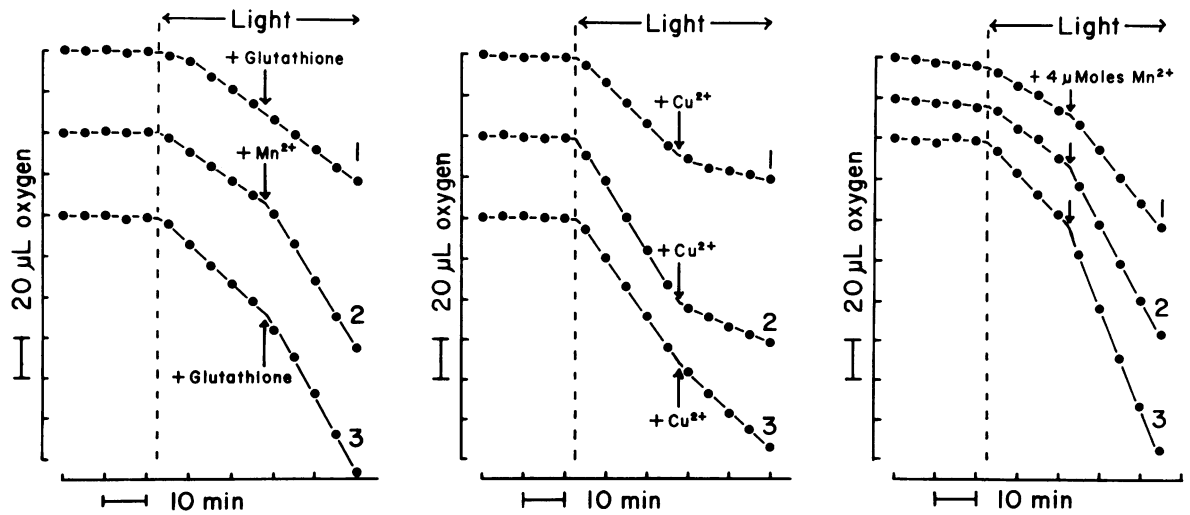


FIG. 3. (top). Protective action of Mn^{2+} in reducing the extent of Cu^{2+} inhibition of the Quinone-Hill and the quinone-stimulated Mehler reactions. (Left) Rates of Quinone-Hill and subsequent quinone-stimulated Mehler reactions. Control (no added Cu^{2+}), 0.25 µmole Cu^{2+} per vessel (0.62×10^{-4} M) and 5 µmoles Cu^{2+} per vessel (1.25×10^{-3} M) with 0 to 12.5 µmoles added Mn^{2+}. Vessel contents: *Phytolacca* chloroplasts (0.72 mg chl), 2.5 ml phosphate buffered sucrose, 0.3 ml catalase, 0.1 ml 50 % ethanol, 10 µmoles quinone, Cu^{2+} and Mn^{2+} as indicated, in a total volume of 4 ml. (Right) Rates expressed at percent of control rates (no added Cu^{2+}).

Table I. *Effect of Mn^{2+} and GSH on Control and Cu^{2+} Inhibited Mehler Reaction Rates*

Vessel contents were *Phytolacca* chloroplasts (1.0 mg chlorophyll), 0.3 ml catalase, 0.1 ml 50 % ethyl alcohol, 2.5 ml phosphate buffered sucrose, Mn^{2+}, GSH, and Cu^{2+} as indicated in a total volume of 4.0 ml. Illumination was red light (2697 $\mu w/cm^2$). The data are the mean ± std. dev. of 2 to 20 experiments.

Concentration of Cu^{2+} in reaction mixture	Mehler reaction rates					+4 μM Mn^{2+} and 4 μM GSH
	No additions	+ 2 μM Mn^{2+}	+ 4 μM Mn^{2+}	+2 μM GSH	+ 4 μM GSH	
M	$\mu l\ O_2/min$ per vessel					
0	1.99 ± 0.58	2.69 ± 0.32	3.29 ± 0.64	1.80 ± 0.14	1.64 ± 0.14	3.25 ± 0.46
10^{-6} M	1.34 ± 0.16	2.63 ± 0.05	3.48 ± 0.45	...	...	...
10^{-5} M	1.21 ± 0.21	2.08 ± 0.10	3.05 ± 0.56	1.42 ± 0.02	1.19 ± 0.02	3.63 ± 0.22
3×10^{-5} M	1.01 ± 0.21	1.63 ± 0.51	...	...	1.24 ± 0.22	3.33 ± 0.39
10^{-4} M	0.79 ± 0.22	0.86 ± 0.01	0.88 ± 0.21	...	1.25 ± 0.05	3.38 ± 0.04
3×10^{-4} M	0.41 ± 0.12	...	0.84 ± 0.02	...	...	1.78 ± 0.03
10^{-3} M	0.32 ± 0.05	0.38 ± 0.08	0.60 ± 0.02	...	0.26 ± 0.02	0.30 ± 0.04
Lowest Cu^{2+} conc. that inhibits	10^{-6} M	10^{-5} M	10^{-5} M	...	...	3×10^{-4} M

Discussion

In the photosynthetic apparatus, copper inhibition and the reversal of copper inhibition by manganese appear to be general phenomena, *i.e.*, they are apparent in all the chloroplast reactions tested. Light intensity has no effect on the extent of copper inhibition. Thus the site of inhibition appears to be a locus other than either of the photoacts. The generally accepted hypothesis about the role of manganese in photosynthesis is that it is involved in oxygen evolution, *i.e.*, in a reaction closely associated with photosystem II (2, 10, 15). The antagonism between copper and manganese in several kinds of chloroplast reactions suggests that copper may affect the site of manganese action. Competition between manganese and copper for the same site on an enzyme under conditions where the Cu-enzyme complex is inactive, while the Mn-enzyme complex is active, could explain both copper inhibition and the reversal of copper inhibition by manganese. If one were to assume further that the enzyme without added metal (or perhaps with some attachment sites unfilled) is active, but not as active as the enzyme-Mn complex, the stimulation of rates of some chloroplast reactions by added manganese is also understandable.

An equally plausible mechanism suggested by the apparent antagonism between copper and manganese is that these metal ions influence membrane permeability. The observed synergism between manganese and GSH supports this hypothesis. Although the data suggest possible mechanisms to account for the antagonism between copper and manganese, it is not possible to choose between these or other mechanisms on the basis of the experiments described here. However, a single basic phenomenon emerges from these diverse observations: added manganese has a protective effect against the generally inhibiting action of copper on the photosynthetic apparatus.

Acknowledgment

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FIG. 4. (bottom). Representative time course data showing effects of adding reactants to Mehler reaction mixtures. a) (left). Effects of added GSH and Mn^{2+} on Mehler reaction rates. (1) 4 μmoles GSH added to control Mehler. (2) 4 μmoles Mn^{2+} added with 4 μmoles GSH already present. (3) 4 μmoles GSH added with 4 μmoles Mn^{2+} already present. b) (center). Effects of added Cu^{2+} on Mehler reaction rates. (1) 1.2 μmoles Cu^{2+} (3×10^{-4} M final concn.) added to control Mehler. (2) Cu^{2+} added with 4 μmoles Mn^{2+} already present. (3) Cu^{2+} added with 4 μmoles Mn^{2+} and 4 μmoles GSH already present. c) (right). Effect of added Mn^{2+} on control and Cu^{2+} inhibited Mehler rates. (1) 4 μmoles Mn^{2+} added with 10^{-5} M Cu^{2+} in reaction mixture. (2) 4 μmoles Mn^{2+} added with 10^{-6} M Cu^{2+} in reaction mixture. (3) 4 μmoles Mn^{2+} added to control Mehler reaction. Vessel contents: *Phytolacca* chloroplasts (1 mg chl), 0.3 ml catalase solution, 0.1 ml 50 % ethanol, 2.5 ml phosphate buffered sucrose, Cu^{2+}, Mn^{2+} and GSH as indicated in a total volume of 4 ml. Illumination: red light (2697 $\mu w/cm^2$).

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