On the Role of Manganese in Photosynthesis

KINETICS OF PHOTOINHIBITION IN MANGANESE-DEFICENT AND 3-(4-CHLOROPHENYL)-1,1-DIMETHYLUREA-INHIBITED *EUGLENA GRACILIS*[1]

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ABSTRACT

Euglena gracilis (Klebs) cultures were grown under conditions where limitation in supply of manganese limited chlorophyll content much more than growth. Although the initial rates of photosynthetic oxygen evolution were not affected by the level of manganese, photoinhibition in high intensity light was markedly influenced. All cultures showed first order kinetics for photoinhibition, with the half-time exponentially related to the Mn concentration in the medium. Treatment with 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) also increased the rate of photoinhibition. Manganese-deficient cells were also more sensitive to CMU inhibition of photosynthesis. The similar effects on photoinhibition of manganese deficiency and of CMU treatment and the protective action of manganese against photoinhibition and CMU poisoning are interpreted to indicate a site of action of manganese on the reducing side of photosystem II, close to the CMU-sensitive site. This manganese-affected site may represent a secondary structural or metabolic consequence of manganese deficiency, not necessarily involved in quantum yields of oxygen.

The requirement of manganese for photosynthesis is well documented (*cf.* citations in 5, 6), but its role and site(s) of action have not been established unequivocally. Photoinhibition (of photosynthesis) has been defined as the debilitating effect of high intensities of visible light upon the photosynthetic capability of green organisms (22). No systematic examination of the effect of manganese supply on photoinhibition has been described. We report experiments on the relationship of manganese nutrition of *Euglena* to photoinhibition and effects of CMU[3] on the same phenomenon. The influence of manganese supply on CMU inhibition of photosynthetic oxygen evolution is also described, as well as modifying effects of the quality of light used for growing the cells. We in-

cluded CMU as a reference point since there is ample evidence that the CMU-sensitive site involved in inhibition of oxygen evolution is located between the two recognized photoreactions (3, 10, 14, 19). Throughout this work we have assumed the general correctness of the scheme of two photoacts connected in series as described by Duysens and Amesz (9) and outlined in more detail in a review by Hind and Olson (16).

After our work was completed (Nikos A. Gavalas, Ph.D. thesis, Rutgers University, May, 1968), the effects of manganese on the action of DCMU were reported (6, 7). Several publications describing other effects of manganese also appeared in which Anderson and Thorne (1) suggested a site of action for manganese at the reducing end of photosystem II, whereas Cheniae and Martin (7), Heath and Hind (15), Homann (17), and Itoh *et al.* (18) presented evidence for a site between the oxidation of water and photoact II.

MATERIALS AND METHODS

Euglena gracilis (Klebs), Z strain (Pringsheim), was used as the experimental organism, and the culture medium was the one used by Price and Vallee (26) without sucrose or ethanol and with varying concentrations of manganese. To avoid transfer of appreciable amounts of manganese with the inoculum, a first inoculation was made to a flask containing minus-Mn medium. Inoculum for the experiments was taken from this culture after 5 to 6 days of growth (1 ml of inoculum to 100 ml of medium in 250-ml Pyrex Erlenmeyer flasks). The cultures were shaken at 25 C under continuous illumination with either cool-white fluorescent tubes that gave an intensity of about 1500 ft-c (white-grown cultures) or neon tubes (red-grown cultures).

Red-grown cultures were much more sensitive to photoinhibition at all levels of Mn and were used in experiments where an increased sensitivity was desirable.

Metal-free water prepared by passage of distilled water through a mixed bed ion exchange resin was used, and the flasks were initially soaked for 4 weeks in a 1:1 mixture of nitric acid and water. After use they were returned to the nitric acid treatment until required again (3–5 weeks). Stock solutions of $ZnSO_4$, $FeSO_4$, $CuSO_4$, and $MnSO_4$ were prepared from Johnson, Matthey & Company Specpure salts and the macronutrient stocks of $Ca(NO_3)_2$, $MgSO_4$, K_2HPO_4-KH_2PO_4 buffer and ammonium glutamate were purified by three successive extractions with 1 % (w/v) 8-hydroxyquinoline in chloroform after adjustment to pH 7.5 to 8.5 (13). Three additional extractions with chloroform were made to remove 8-hydroxyquinoline from the aqueous phase. The solutions were subsequently aerated for 2 days in the cold, autoclaved and aerated again for 2 days to remove traces of chloroform. The stock solution of DL-malate was purified by passage through a column of AG 50W-X8 resin (100–200 mesh; H^+ form). The HCl used was metal-free prepared according to Thiers (31), and the ammonia solution was redistilled from reagent grade ammonia.

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[3] Abbreviations: CHL: chlorophyll; CMU: 3-(4-chlorophenyl)-1,1-dimethylurea; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; R_0: initial photosynthetic rate; $t_{1/2}$: half-time of decay of photosynthetic rate under high light intensity.

Chlorophyll was determined according to MacKinney (24) (methanol extraction), and the density of the cultures was measured with a Klett-Summerson photometer (filter transmitting maximally at 540 nm) after dilution 1:10 with water. This dilution brought the Klett reading below 40 so that the K_c values obtained were a linear measure of cell number (26).

All measurements of oxygen exchange including experiments on photoinhibition were made with a Yellow Springs Instrument Company biological oxygen monitor (model 53) employing the YSI 5331 oxygen probe, standardized with air at 25 C. Changes in oxygen concentration in the solutions were calculated from recorder tracings and converted to micromoles of oxygen released or absorbed per test sample per unit time.

Illumination of the sample in the YSI oxygen monitor during the period when photoinhibition was measured was provided by a 1000-w projection lamp (GE DPT), and the light was filtered through 5 or 9.5 cm of water to remove infrared radiation. At the distance of the sample the light intensity was about 8000 ft-c (measured with a Weston illumination meter).

Aliquots from cultures of cells grown in Erlenmeyer flasks were diluted with distilled water to a final volume of 3 or 5 ml and with chlorophyll content in the range of 8 to 20 μg/3 ml. These samples were then placed in the oxygen monitor. This practice gave repeatable results. The oxygen evolution rates were unaffected by dilution in the range of 10 to 30 μg of CHL/3 ml, and the cells kept their photosynthetic ability unimpaired for long periods of time in the dilute culture medium. Before each measurement, 5% CO_2 in N_2 was bubbled into the sample in the YSI oxygen monitor until the oxygen level fell to 30 to 40% of air saturation. Respiration was measured for 3 to 5 min before the light was turned on. A brief induction period (2–4 min) was observed after the light was turned on before a maximal photosynthetic rate was obtained. This maximal rate either remained constant or declined with time (photoinhibition). All photosynthetic rates reported are corrected for dark respiration. Oxygen evolution rates were determined for as many consecutive 2- or 3-min periods as possible until oxygen levels in the test solutions approached equilibrium with air (approximately 230 μM O_2 concentration).

The values obtained were used for calculation of the regression of photosynthetic rate on time, of the initial photosynthetic rate, R_0 (extrapolation of the regression line to zero time), and of the half-time of decay of oxygen evolution $(t_{1/2})$. All calculations were made with the IBM 7040 computer at Rutgers University.

The change in photosynthetic rate closely followed first order decay kinetics as described by Kok (22), Kok et al. (23), and Jones and Kok (20, 21). The general phenomenon is expressed by the equation $R_t = R_0 \cdot e^{-k_i t}$ where R_0 = photosynthetic rate at 0 time, R_t = rate at time t, and k_i is a constant for photoinhibition. The half-time $(t_{1/2})$ was selected as the most convenient index of photoinhibition. Since $t_{1/2} = \ln 2/k_i$, it is inversely proportional to the photoinhibition constant.

It should be pointed out, however, that the reported half-times refer to the curve representing the initial rapid decline in photosynthetic rates. At a later time, usually beyond the respiration-compensating point, the rate of photoinhibition has been observed to decrease. A mathematical model to fit this second phase has been proposed by Kok (22), but no attempt to study this second phase was made in the present work.

The validity of extrapolating the observed photosynthetic rates to zero time has been established by Kok (22). Table I shows the reproducibility of the measurements of $t_{1/2}$ and of R_0.

There is no indication whatsoever that the photoinhibition observed with Euglena grown at various levels of manganese is different from the phenomenon studied in detail by Kok and his collaborators (20–23) with Chlorella and spinach chloroplasts. Our preliminary experiments showed that photoinhibition in Euglena does not depend on molecular oxygen, that it in-

Table I. *Reproducibility of Measurements of Initial Photosynthetic Rates* (R_o) *and of Photoinhibition* $(t_{1/2})$

Replication	R_0	$t_{1/2}$
	μmoles O_2/mg CHL·hr	min
1	268	29.26
2	282	31.66
3	277	31.70
Average	276	30.87
SD	7.1	1.40

Table II. *Growth and Chlorophyll Content of Euglena gracilis Cultures Grown under White (Fluorescent) and Red (Neon) Light at Different Mn^{2+} Concentrations in the Culture Medium*

Light for Growth	Age of Culture	Mn^{2+} Concn	Chlorophyll Content	Density	$\dfrac{\mu g\ CHL}{ml \cdot K_c} \times 10^2$
	days	M	μg/ml	K_c^1	
Red	6	5.0×10^{-7}	3.40	230	1.48
		7.5×10^{-7}	6.62	260	2.55
		1.0×10^{-6}	9.37	300	3.12
Red	5	2.5×10^{-6}	13.59	350	3.88
		5.0×10^{-6}	14.64	350	4.18
		1.0×10^{-5}	18.57	390	4.76
Red	8	7.5×10^{-7}	7.18	265	2.71
		1.0×10^{-6}	9.63	290	3.32
		1.0×10^{-5}	19.33	430	4.50
White	5	5.0×10^{-7}	5.94	215	2.76
		7.5×10^{-7}	8.37	270	3.10
		1.0×10^{-6}	15.06	270	5.58
		2.5×10^{-6}	24.98	390	6.41
		1.0×10^{-5}	30.12	425	7.09

[1] K_c = corrected Klett units (index of growth).

creases with increasing light intensity, that it is partially reversible in the dark (with reversibility higher when light treatments are shorter), and that no bleaching of chlorophyll is involved.

Two experiments demonstrating absence of significant photobleaching can be cited. After illumination periods of 45 or 47 min the chlorophyll content was 97.0 and 100.5% of that present before illumination. Within these same intervals, rates of oxygen evolution decreased to 34.3 and 21.7%, respectively, of the original rates before photoinhibition.

RESULTS

Effects of Manganese Deficiency on Growth, Chlorophyll Content, and Initial Rates of Photosynthesis. Manganese deficiency reduced chlorophyll content much more than growth, as shown in Table II. Even at the lowest levels of Mn employed, deficiency was not severe enough to inhibit growth. With appreciable amounts of carbon sources in the medium, growth was probably somewhat heterotrophic, especially during the first days of culture, even under light.

Constantopoulos (8) has reported reduction in chlorophyll content with Mn deficiency of Euglena when grown photoheterotrophically, and Richter (27) showed less chlorophyll in Mn-deficient Anacystis nidulans. In several instances, however, growth of other algae has been limited more than chlorophyll content when Mn was deficient (11, 25).

Precise measurements of oxygen evolution were not possible with cultures grown at Mn levels lower than 5×10^{-7} M because the chlorophyll content was then extremely low and the

correction for dark respiration with its inherent uncertainty became relatively high. Therefore, Mn levels used in most of the reported experiments were 7.5×10^{-7} M and higher.

The level of Mn in the cultures had no appreciable influence upon the initial rates of evolution of oxygen (R_0) per mg of chlorophyll, even at the lowest concentrations of Mn (Table III).

Dependence of Photoinhibition on Manganese Level of Culture. We thought that a detailed examination of the relationship of manganese supply to photoinhibition could produce valuable evidence concerning the site(s) affected by manganese.

In several experiments *Euglena* cultures were grown at different Mn concentrations, and their sensitivity to photoinhibition was determined after 5 to 8 days of growth. Figure 1 shows the results of a representative experiment involving seven levels of Mn^{2+} from 7.5×10^{-7} to 10^{-4} M in the culture medium. The observed close dependence of photoinhibition on the Mn status of the culture over such a wide range of concentrations suggests a primary role of this metal in photoinhibition, though a secondary effect cannot be excluded.

It is noteworthy that the protective effect of Mn against photoinhibition extends considerably beyond the level of this element (10^{-5} M) required for normal growth of *Euglena*.

Table III. *Initial Photosynthetic Rates (R_0) for Euglena gracilis Cultures Grown at Different Mn^{2+} Concentrations*

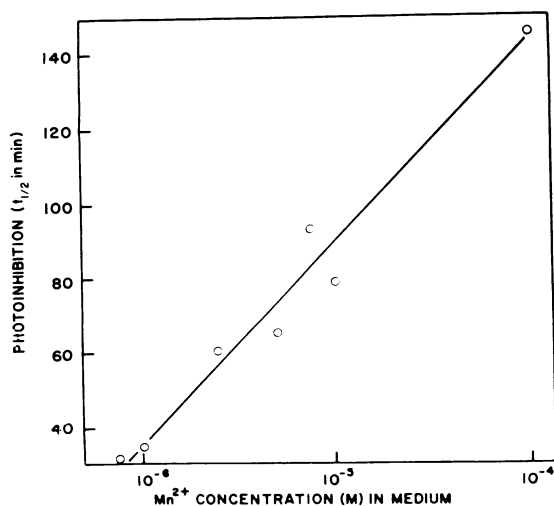| Age of Cultures	Mn^{2+} Concn	R_0
days	*M*	*μmoles O_2/mg CHL·hr*
8	7.5×10^{-7}	252
	1.0×10^{-6}	212
	2.5×10^{-6}	224
	5.0×10^{-6}	201
	7.5×10^{-6}	242
	1.0×10^{-5}	240
	1.0×10^{-4}	239
6	7.5×10^{-7}	194
	1.0×10^{-6}	217
5	2.5×10^{-6}	240
	5.0×10^{-6}	245
	1.0×10^{-5}	220
8	7.5×10^{-7}	226
	1.0×10^{-6}	226
	1.0×10^{-5}	203

FIG. 1. Dependence of observed photoinhibition ($t_{1/2}$) in *Euglena gracilis* cells on the Mn^{2+} concentration of the culture medium. Coefficient of determination (r^2) = 0.964.

Table IV. *Effect of Interchange of Media on Photoinhibition*

Manganese Concn[1]		R_0	$t_{1/2}$
Growth media	Test media[2]		
M	*M*	*μmoles O_2/mg CHL·hr*	*min*
10^{-6}	10^{-6}	190	59.2
10^{-6}	10^{-5}	230	36.6
10^{-5}	10^{-5}	181	...[3]
10^{-5}	10^{-6}	217	184.1

[1] 10^{-6} M was deficiency level; 10^{-5} M was normal.

[2] All measurements of photosynthesis made with cells in media in which normal or deficient cells had already been grown.

[3] Photosynthetic rate constant for duration of measurements.

To exclude the possibility that the factor responsible for the observed stronger photoinhibition under Mn deficiency was extracellular (differences in Mn concentration in the medium, differences in pH, accumulation of an inhibitor in the culture medium) cells were used in which measurements of photoinhibition were made both in their original media and after interchange of the latter. Older cultures than usual were used since the effect of an accumulating inhibitor should be more pronounced. The cells were separated by low speed centrifugation and resuspended with a Vortex mixer in the same final volume but with medium from the other culture.

No evidence of the existence of an extracellular factor was shown (Table IV). Regardless of the test medium, $t_{1/2}$ values show that cells grown in a deficient medium were sensitive to photoinhibition while the normal cells were relatively resistant to photoinhibition. The increase in R_0 values for either culture when shifted to the other medium is probably due to a dispersion of clumped cells after resuspension (clumping is characteristic of older cultures of *Euglena*).

Effect of CMU on Photoinhibition. On the basis of the hypothesis that photoinhibition involves damage to the trapping centers of photoact II (23), we thought that any treatment partially blocking electron flow towards photosystem I would lead to an increased photoinhibition.

To test this prediction, low concentrations of CMU were added to aliquots from *Euglena* cultures grown with 10^{-5} M manganese. Cultures grown under white fluorescent light were used to avoid any possible complications associated with the inherently greater sensitivity of red-grown cells. At all levels of Mn, without CMU, red-grown cells had shown much more photoinhibition. In one experiment, e.g., with cells grown with 7.5×10^{-7} M Mn, the $t_{1/2}$ for red-grown cells was 31.5 min as compared with 77.6 min for white-grown cells.

When CMU was added, an increased sensitivity to photoinhibition was found as predicted by the working hypothesis, as well as the usual inhibitory effect of the substituted urea on photosynthetic oxygen evolution. Results are shown in Figure 2. The rate of photoinhibition was increased with increasing CMU concentrations up to the level of 0.84 μM. A decrease was observed at the next higher level (1.68 μM). This experiment was easily reproducible, and the reversal of the photoinhibitory effect at higher concentrations of CMU was always present, for which no explanation can be offered.

The observed effect of CMU on photoinhibition could not be attributed to ethanol (the stock solution of CMU was in ethanol; 4 to 20 μl were added to 3-ml samples) since ethanol alone did not induce such a response.

Relationship between Manganese Status and CMU Inhibition. The similarity of the effect of CMU treatment and Mn deficiency on photoinhibition led us to consider the possibility
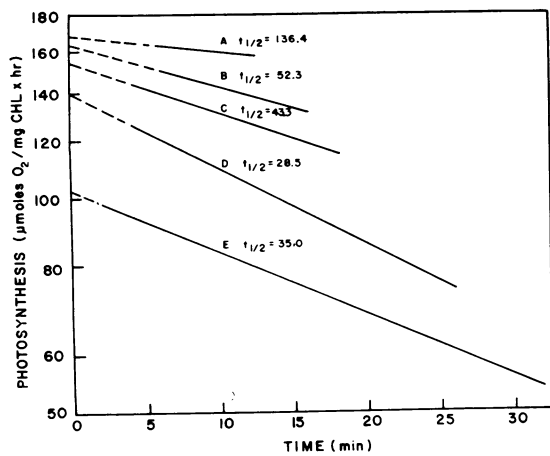
FIG. 2. Effect of concentration of CMU on photoinhibition ($t_{1/2}$) in *Euglena gracilis* cells. A: Control; B: 0.33 μM CMU; C: 0.5 μM CMU; D: 0.84 μM CMU; E: 1.67 μM CMU. Chlorophyll content of samples 19.83 μg/3 ml; Mn^{2+} concentration of medium, 10^{-5} M. The respective coefficients of determination (r^2) for A, B, C, D, and E, were 0.731, 0.985, 0.987, 0.984, and 0.993.
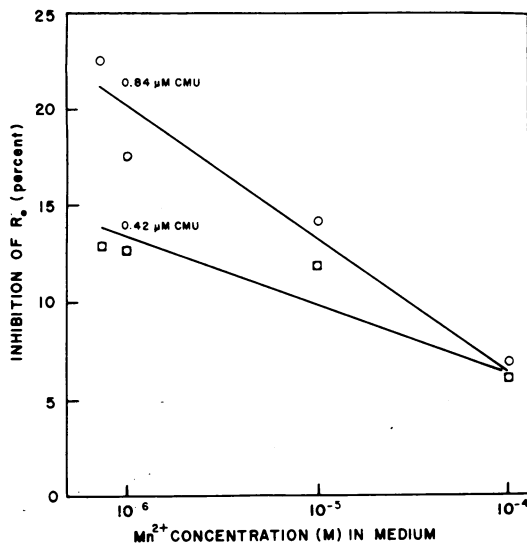

FIG. 3. Effect of concentration of Mn^{2+} in the medium on inhibition of initial photosynthetic rates (R_0) by CMU. The R_0 values of the uninhibited controls at levels of 7.5×10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M Mn were 236, 223, 185, and 162 μmoles O_2/mg CHL·hr. Chlorophyll contents of samples around 20 μg/3 ml. The coefficients of determination (r^2) were 0.927 and 0.845 for the upper and lower lines, respectively. Differences in slopes of lines statistically significant at the 10 to 20% level.

that the action of Mn was also at the reducing side of photosystem II. This prompted us to examine the degree of CMU inhibition of oxygen evolution in cultures grown at different Mn levels. The prediction was that if the CMU-sensitive site and the Mn-affected site are identical or close together, CMU sensitivity would be more pronounced under Mn deficiency. Bishop (4) has reported a synergistic effect of DCMU poisoning and Mn deficiency in hydrogen-adapted *Ankistrodesmus* as far as oxygen evolution and readaptation under intense light were concerned.

Cultures grown at four different levels of Mn were used, and the effect of two concentrations of CMU was determined. Results are shown in Figure 3. As predicted, low-manganese cells are more sensitive to CMU inhibition. Cheniae and Martin (6)

Table V. *CMU Inhibition of Initial Photosynthetic Rates in White- and Red-grown Euglena Cultures*

Photosynthetic rates (μmoles O_2/mg CHL · hr) of uninhibited control samples were: 302 at 10^{-6} M Mn^{2+} and 298 at 10^{-5} M Mn^{2+} for red-grown cultures; 223 at 10^{-6} M Mn^{2+} and 185 at 10^{-5} M Mn^{2+} for white-grown cultures.

Mn^{2+} Concn	Inhibition			
	4.2×10^{-7} M CMU		8.4×10^{-7} M CMU	
	White	Red	White	Red
M	%	%	%	%
10^{-5}	9.97	18.07	13.44	25.68
10^{-6}	13.53	34.51	20.27	53.51

independently found with *Scenedesmus* cells that Mn deficiency increased sensitivity to DCMU.

It is noteworthy that a level of 10^{-4} M Mn, which is 10 times that required for normal growth of *Euglena*, was more effective in relieving CMU inhibition of oxygen evolution (R_0) than 10^{-5} M Mn. This result parallels the alleviating effect on photoinhibition of the same high concentration of Mn.

CMU Inhibition in Red-grown *Euglena* Cultures. Since the red-grown *Euglena* cells were much more sensitive to photoinhibition than white-grown cells, it was thought that the former should be more sensitive to CMU inhibition because the CMU block would be imposed on an already defective mechanism.

The effect of CMU on the photosynthetic rates of cultures grown under white light was compared to that of cultures grown under red light at two concentrations of manganese. Results are shown in Table V. As predicted, red-grown *Euglena* cells are more sensitive to CMU at both levels of Mn and of CMU.

DISCUSSION

We recognize that the phenomenon of photoinhibition is complex and may involve more than one site (28, 29). But high intensity visible light apparently affects photosystem II primarily (23). Under our conditions, the kinetics of photoinhibition closely followed those reported by Kok (22), Kok et al. (23) and Jones and Kok (20, 21). They interpret the phenomenon as an inactivation of a primary reaction center which participates in a rate-limiting dark step in photosynthesis. Kok et al. (23) favored the hypothesis that light inactivates a collector of charges from the trapping center rather than a collector of energy from the pigment bed. From the irreversible annihilation of the variable fraction of fluorescence it appears as if the quencher (Q) is short-circuited by the photodamage; Q is thus able to drain light from the sensitizing pigments but is unable to perform useful photochemistry. Kok et al. (23) interpreted the relieving effect of phenazine methosulfate on photoinhibition as the result of a decrease in the lifetime of the excited traps, and they offered as evidence the depression of the fluorescence yield by phenazine methosulfate. Essentially the same interpretation was offered by Forti and Jagendorf (12) and Avron (2) except that they used the terms "reduced" or "oxidized" traps, which seems more plausible. Because of the close dependence of the observed photoinhibition in our experiments on the Mn status of the cells, we consider that photosystem II is affected.

If slower electron flow between the two photoreactions enhances photoinactivation, and the photosensitive intermediate (or one of them [21]) is located at the reducing side of photosystem II, then the observed effect of Mn deficiency is suggestive of a Mn-affected site between the two photoreactions. The analogous effect of CMU on photoinhibition and the close relationship between CMU inhibition and the Mn status of the

cells further support this suggestion. Alternatively, it is also possible that a Mn deficiency lesion on the O_2 side of system II could increase the back reaction of reduced Q by increasing the lifetime of oxidized system II traps. However, it becomes more difficult to explain the close relationship of Mn and of CMU to photoinhibition if the two are considered to act at sites separated by a photochemical and/or several dark reactions.

Comparative fluorescence of normal and Mn-deficient chloroplasts has been used in attempts to locate the Mn site in photosystem II. Anderson and Thorne (1) suggested a Mn site between the primary and secondary reductants of photosystem II. On the other hand, the results of Cheniae and Martin (7), Heath and Hind (15), Homann (17) and Itoh *et al.* (18) are consistent with a functional Mn site between water oxidation and the system II photoact. As Heath and Hind (15) have suggested, Mn may have a structural role in addition to its functional one. We think it is more likely that such a role would be observed in plants made deficient by a lack of Mn in the growth medium rather than in chloroplasts where Mn has been extracted by various means (tris, heat inactivation, etc.), especially in view of the recent results of Teichler-Zallen (30) with *Chlamydomonas reinhardi* and of Constantopoulos (8) with *Euglena gracilis*. It is possible that our results and those of Anderson and Thorne (1) involve the structural effect postulated by Heath and Hind (15).

An adequate explanation of the greater sensitivity of red-grown than of white-grown *Euglena* cells to photoinhibition and to CMU inhibition of photosynthesis cannot be offered without more evidence on the effect of the conditions of growth on the composition of the cells, including types of chlorophyll present. We interpret our data to mean that the energy-converting processes of plants grown in red light function relatively better than the electron-transporting chain.

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