BBA 45668

SITE OF MANGANESE FUNCTION IN PHOTOSYNTHESIS

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(Received December 11th, 1967)

SUMMARY

Manganese-deficient Scenedesmus cells, showing no marked changes in chloroplast lamellar structure and respiratory activity, were used in an analysis of the effect of the deficiency upon flash yield of O_2 and quantum yields of photosynthesis and quinone photoreduction. It was shown that, with increasing deficiencies, loss of chloroplast-bound Mn led to decreased flash yield of O_2 (measure of E, the photochemical substrate of Photosystem II) and increasing quantum requirement of photosynthesis and quinone photoreduction. The decreased amount of E as a result of manganese deficiency is reflected in an increased sensitivity to DCMU.

From measurements of flash yield of O_2 and quantum yield of photosynthesis with cells containing a determined amount of chloroplast-bound Mn, it was shown that approx. 8 Mn occur for each E equivalent. The amount of bound Mn was linear with amount of E and not quantum yield.

It was concluded that manganese functions specifically in the photochemical complex E of Photosystem II.

INTRODUCTION

Perhaps the least understood aspects of photosynthesis are the reactions within Photosystem II which result in evolution of O_2. Past studies concerning mineral requirements for autotrophic growth of O_2-evolving photosynthetic organisms have indicated an absolute requirement for manganese[1–4]. Studies concerning fluorescence yield[5], light re-emission[5], Hill reaction[6–9] or photosynthesis[6–9] in Mn-deficient autotrophic cultures revealed a deleterious effect of Mn deficiency on these processes.

KESSLER[8,10] showed that photoreduction of CO_2 by H_2 in hydrogen-adapted algae was not adversely affected. These results, as well as the effects of Mn growth deficiencies in higher plants and algae on some partial reactions of chloroplasts[7,11–14] became more understandable in the concept of two photosystems, which yielded the conclusion that Mn was a catalyst in Photosystem II. As yet no evidence has been presented for a direct function of Mn in a specific photosynthetic reaction, and the possibility is not excluded that Mn deficiency, established in long-term growth experiments, might affect the fragile Photosystem II in an indirect manner. For instance

Abbreviations and nomenclature: v_{max}, rate at saturating intensity; V, quantum yield; E, photochemical substrate of photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, N-methylphenazonium ion (phenazine methosulfate); Chl, chlorophyll.

it has been observed[14,15] that prolonged, extreme Mn deficiency leads to virtually complete disorganization of the lamellar structure of spinach chloroplasts.

In the work reported below, an attempt has been made to minimize possible indirect effects of Mn deficiency by using Scenedesmus cells which showed marked Mn deficiency within 48 h after inoculation into a Mn-free medium. Even at extreme deficiencies the chloroplast lamellar structure of Scenedesmus was essentially maintained and in general the cell structure remains remarkably similar to that of non-deficient cells. This material has been used in the work reported here to more specifically localize the site of Mn action in photosynthesis.

MATERIALS AND METHODS

Culture of alga

The alga Scenedesmus was cultured autotrophically at 30° in Roux bottles containing 600 ml of medium described by NORRIS, NORRIS AND CALVIN[16]. *Anacystis nidulans* was cultured on medium C of KRATZ AND MYERS[17] at 35° as described previously[29]. Both cultures were aerated with 3 % CO_2 in air and illuminated with a bank of cool-white fluorescent lights providing 1200 ftcandles incident. The sterile medium for Scenedesmus was inoculated, under aseptic conditions, with 10 μl packed cell volume. For packed cell volume determination suitable aliquots of Scenedesmus were centrifuged at 1100 $\times$ *g* for 10 min in hematocrit tubes. Preliminary experiments with non-deficient Scenedesmus cells indicated that maximum efficiency of photosynthesis could be observed if the algae were harvested 48 h after inoculation. Cells, therefore, were harvested by centrifugation at this time, resuspended in the appropriate growth medium and used directly in studies of O_2 evolution in photosynthesis or photoreduction of quinone. A portion of the Scenedesmus cells was retained for chloroplast particle preparation[18], used for Hill-reaction assays and for determination of manganese by either a slight modification of the formaldoxime procedure[19], or by atomic absorption, or by ^{54}Mn radioactivity.

All glassware for growth of algae and experimentation was vigorously cleaned by detergent, rinsed, then cleaned with approx. 4 M HCl. Water used in subsequent cleaning and for growth media was obtained by distillation from an all-glass still, followed by passage in series through two columns of ion-exchange resin Ilco-Way Research Model.

For Scenedesmus two levels of deficiency were used routinely. One level was obtained by using reagent grade chemicals for both macro and micro-elements with omission of $MnCl_2$ (Medium I). Extreme levels of deficiency were obtained by using Spec-Pure chemicals (Jarrell-Ash and Co.) as a source of the micro-elements (Medium II). The principle source of Mn contamination in reagent grade chemicals was in the iron salts. In Medium II, growth was sub-optimal unless both Co and vanadium were supplied (0.1 ppm). In experiments concerning the flash yield of O_2 as affected by Mn deficiency, additions of Mn were made to Medium II and extremely deficient cells were used for inoculation. Following 3–4 transfers the cells reached an equilibrium deficiency.

Rate measurements and flash yields of O_2

Unless otherwise stated, all measurements were made with a Pt–AgO_2 electrode covered with 0.5 mil Type A teflon FEP membrane (DuPont). The covered electrode

was encased in a lucite vessel thermoregulated at 30° unless otherwise stated. The volume of the reaction chamber was 1.00 ml. For absolute quantum yield determinations, a similar vessel with a 1-cm light path was used. Rapid stirring was provided with a magnetic stirrer. Light from a 750-W projection lamp was filtered through 12 inches water, 1 inch 0.05 M potassium ferricyanide solution and focussed with suitable condensers through an OG-3 (Schott) filter onto the front of the vessel. Intensity was varied using suitable neutral density filters.

Measurements of the yield of O_2 resulting from brief saturating flashes of light were made at 15° with the apparatus and technique of JOLIOT, JOLIOT AND KOK[20]. After constant phase was obtained, the cells (300 μg of chlorophyll per ml) were kept in darkness for 10 min to assure complete deactivation. Flashes (approx. 8-μsec duration) of white light were presented intermittantly every 4 sec. Maximum relative yield of O_2 was obtained with the third flash.

Absorption measurements of the cell or particle suspensions were made in a double-beam integrating sphere using the same interference and cut-off filters employed in rate measurements for quantum yields. In general, the percent absorption of cell suspensions used in quantum yield measurements was less than 25 %. Light intensity was measured with a calibrated thermopile. Intensity was varied using neutral density filters, such that a 2-fold increase in intensity yielded a 2-fold increase in rate.

Reaction mixtures

The reaction mixtures for the following reactions for whole cells are given in μmoles per vessel (1.13 ml): (1). Photosynthesis: 0.1 M $NaHCO_3$–0.1 M Na_2CO_3 (pH 9.4), saturated with 5 % CO_2 in air, 100, with 20 μg chlorophyll. Under these conditions maximum rates were obtained with no deviation of rate over the period of time measured (4–6 min) at 30°. (2). Quinone photo reduction: Potassium-phosphate buffer (pH 6.5), 50; freshly sublimed 1,4-benzoquinone, 10; and 20 μg chlorophyll. This relatively high amount of quinone was required, independent of KCN concentration (10^{-3} M) to sustain maximum rates over the period of time measured (approx. 3 min). Addition of ferricyanide (5 μmoles) reduced the concentration of quinone required but did not significantly change results obtained with quinone alone.

Assay of Hill activity of Scenedesmus particles was done at 20° with the following reaction mixtures given in μmoles per vessel (1.13 ml). (1). Quinone Hill reaction (particles): phosphate buffer (pH 6.5), 50; NaCl, 20; freshly sublimed 1,4-benzoquinone, 0.2; KCN, 0.1; and particles of Scenedesmus containing 20 μg of chlorophyll. (2). Ferricyanide Hill reaction (particles): Tris–HCl (pH 7.2), 50; NaCl, 20; ferricyanide, 1.0; with 20 μg of chlorophyll. (3). Methyl viologen Hill activity (particles): phosphate buffer (pH 6.8), 50; methyl viologen, 0.1; KCN, 0.1; with 20 μg of chlorophyll. (4). Cytochrome *c* Hill activity (particles): phosphate buffer (pH 6.8), 20; $MgCl_2$, 7.5; NaCl, 16; cytochrome *c* (Type III, Sigma), 0.4; KCN, 2; PMS, 0.2; with 20 μg of chlorophyll.

Preparation of Scenedesmus particles

Cells were harvested by centrifugation and washed twice with 0.02 M phosphate (pH 6.5), containing 0.001 M EDTA, then once with the phosphate buffer alone.

Chloroplast particles were then prepared essentially as described by KOK AND DATKO[18]. Resuspension of particles was made in 0.4 M sucrose–0.05 M Tris maleate–0.01 M NaCl (pH 6.7). Particles stored under liquid N_2 in this medium retained Hill activity (v_{max} and quantum yield) for at least 3–4 months.

Chlorophyll measurements

For chlorophyll determinations on Scenedesmus cells, the cells were extracted at 63° for 3 min with 5 ml of 2.5 % methanolic KOH containing 5 % Triton X-100, then centrifuged to remove the pellet. Absorptions were read at peak absorption of 645 mμ. Spinach chloroplasts with a known amount of chlorophyll (determined following 80 % acetone[21] extraction) were used for standardization purposes. This procedure, a modification of that reported by MILNER *et al.*[22] routinely gave complete extraction of the chlorophylls.

Chlorophyll determinations for Scenedesmus particles and Anacystis cells were made following extraction with 80 % acetone[21].

Manganese and total phosphate determinations

Chloroplasts of higher plants and Scenedesmus particles were washed twice at 4° with 0.02 M phosphate (pH 6.75) containing 0.001 M EDTA before analyzing for Mn. Following wet digestion with HNO_3–$HClO_4$ (see ref. 19) the Mn content was determined by either the formaldoxime method[19] or by atomic absorption. With a 2.5-cm light path cuvette, the absorbance (450 mμ) in the formaldoxime method was linear with Mn concentration up to at least 5 μg Mn.

For determinations of Mn by radioactivity cells were uniformly labeled (48 h) with ^{54}Mn of known specific activity. Following isolation of washed particles the radioactivity was determined in a NaI scintillation well counter, calibrated for the ^{54}Mn emission.

Total phosphate determinations were made[23] using the Fiske–SubbaRow reagent[24].

Electron microscopy

Samples for electron microscopy were fixed with 3 % glutaraldehyde in culture medium and post-fixed in chrome–osmium[25]. The pellet was then embedded in 2 % agar, dehydrated in graded ethanol and embedded in Fluka Araldite epoxy resin. The sections, cut on an LKB ultratome, were stained with lead citrate[26]. Photographs were made in a Siemens Elmiskop Ia.

RESULTS

General effects of manganese deficiency

Structure: Figs. 1 and 2 (Mn) illustrate the cellular and chloroplast structural changes associated with the extremely Mn-deficient cells described in later sections. Cells moderately deficient were indistinguishable from normal cells except for a diminished amount of starch granules. Comparison with non-deficient cells (Figs. 3 and 4) reveals that the general cellular structure remains essentially unaltered. The thickness of the cell wall is decreased, the number and size of starch granules become vestigial, and the chloroplast lamellae are separated into narrow bundles in the Mn-

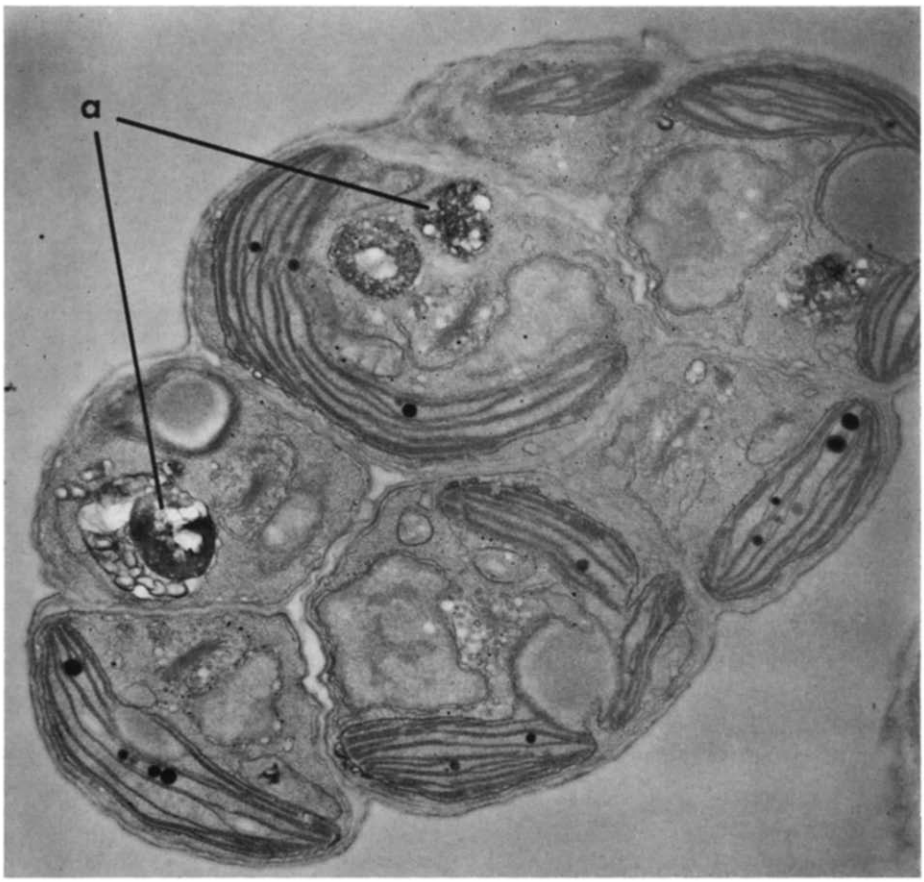


Fig. 1. Extremely deficient cell. The bodies labeled "a" are considered to be embedding artifacts, since they were found in both normal and deficient cells. Magnification: 15000 ×.

deficient cells. Despite separation of lamellar bundles, the individual lamellae appear to remain normal and similar to those of the non-deficient cells. Total chlorophyll content of the normal and deficient cells, on a dry weight basis, was 5.4 and 4.9 %, respectively. Thus the deficiency resulted in only a 9 % decrease of total chlorophyll, which strengthens our supposition that the lamellae as such remain unaltered despite the separation from the typical closely packed bundle structure. Some earlier reports on the effect of extreme Mn deficiency on the structure of spinach chloroplast[14,15] described a nearly complete loss of chloroplast structure. However, HOMANN[14] observed that at least in one higher plant the chloroplast structure was maintained despite extreme Mn deficiency. This variation of the observed effects on chloroplast structure in higher plants may result mainly from procedural differences used to establish deficiency—prolonged exposure to suboptimal conditions resulting from the deficiency, thereby inducing secondary effects due to the primary loss of Photosystem II. A direct comparison among these seemingly conflicting results, relating effects of deficiency to structure, is impossible unless made on some comparable basis such as the amount of bound Mn/Chl. However, despite the variation in chloroplast structure of higher

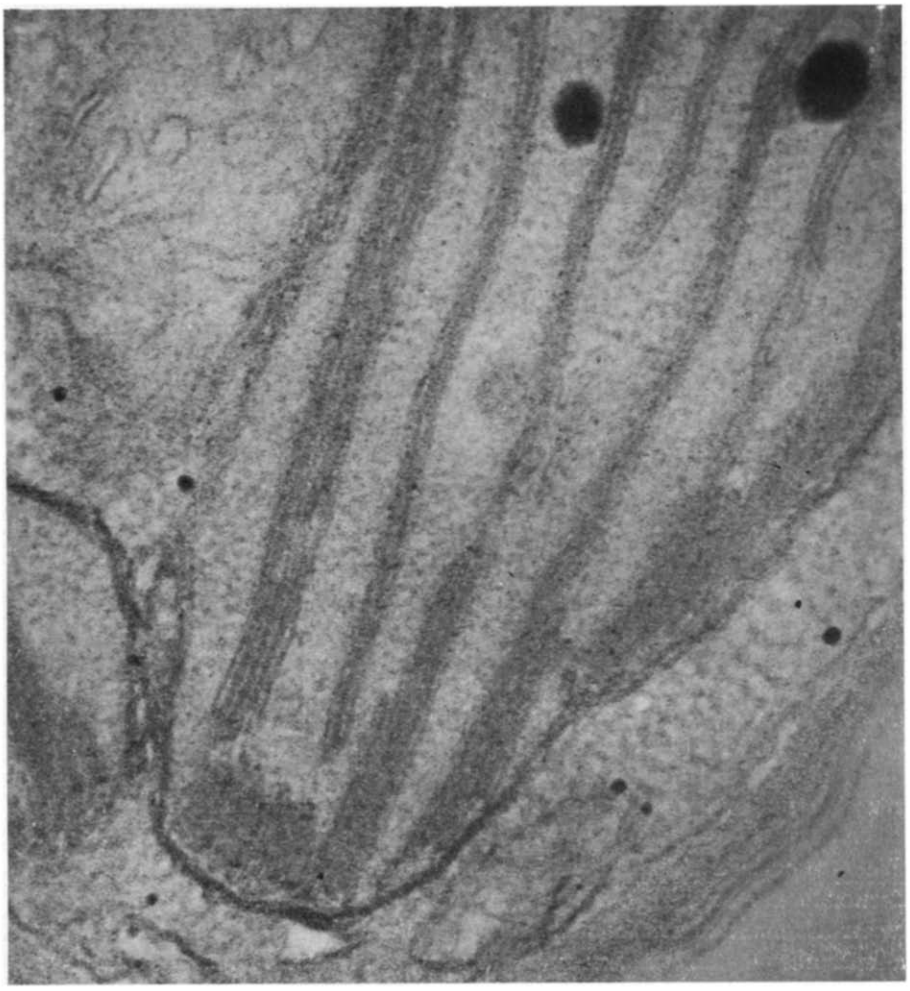


Fig. 2. Extremely deficient cell. Magnification: 102000 ×.

plants the chlorophyll *a*/*b* ratio in both higher plants[11] and Scenedesmus[7] remains relatively constant.

In contrast, the pigments of Anacystis are altered markedly by Mn deficiency[4,9]. As shown in Fig. 5 extensive loss of chlorophyll *a* (expressed on a dry weight basis) is observed even when deficiencies are obtained in 24 h. The extent of loss of chlorophyll *a* has been dependent upon the degree of deficiency and the temperature during cultivation. At 30° the extent of the loss is much smaller than at 38°.

This shift in phycocyanin/chlorophyll *a* with loss of System II is similar to the effect on pigment ratio produced by culturing Anacystis in far-red light[27]. In the latter instance a slight increase of phycocyanin and a marked decrease in chlorophyll *a* is observed. These inverse changes in accessory pigment and chlorophyll *a* possibly represent adaptive effects serving to maintain some balance between the pigments associated with Photosystem I and Photosystem II and thereby adjusting the rate

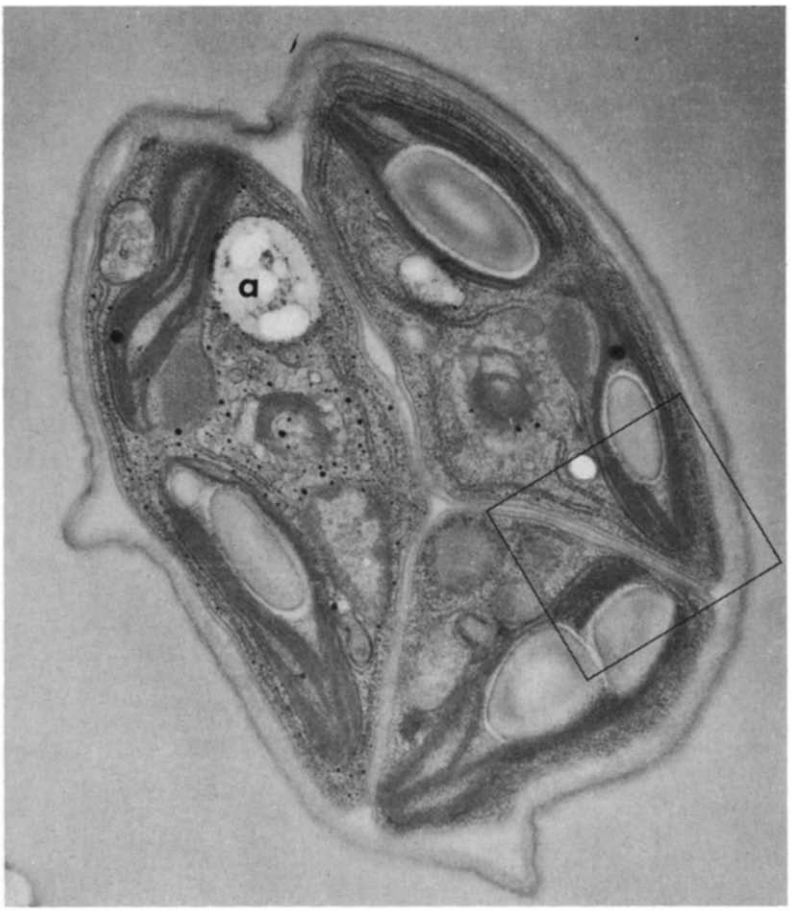


Fig. 3. Normal cell. a, embedding artifact. Magnification: 20000 ×.

attainable by System I to that attainable by System II. From this argument it would be predicted that similar pigment changes would occur in cells cultured in white light in the presence of suitable concentrations of DCMU.

Effects on respiration and enzyme levels in Scenedesmus: On a chlorophyll basis the typical respiratory rate without added substrate for normal and extremely deficient Scenedesmus cells was 0.0985 μmole O_2 per mg Chl per min and 0.0967 μmole O_2 per mg Chl per min respectively. On a mg dry weight basis the respiratory rates were 1.82 μmoles O_2/min and 1.99 μmoles O_2/min for the normal and extremely deficient cells. These results with Scenedesmus agree well with those previously reported for other green algae.

In addition the deficiency resulted in only small decreases (15–36 % and 5–20 %, respectively) in the specific activity of isocitrate dehydrogenase (*threo*-D$_S$-isocitrate: NADP oxidoreductase (decarboxylating), EC 1.1.1.42), a Mn-requiring enzyme, and ribulose-P_2 carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39), an enzyme of the Calvin cycle whose activity is maximally enhanced[28] by Mg^{2+} but to an appreciable extent also by Mn^{2+}. Thus the markedly

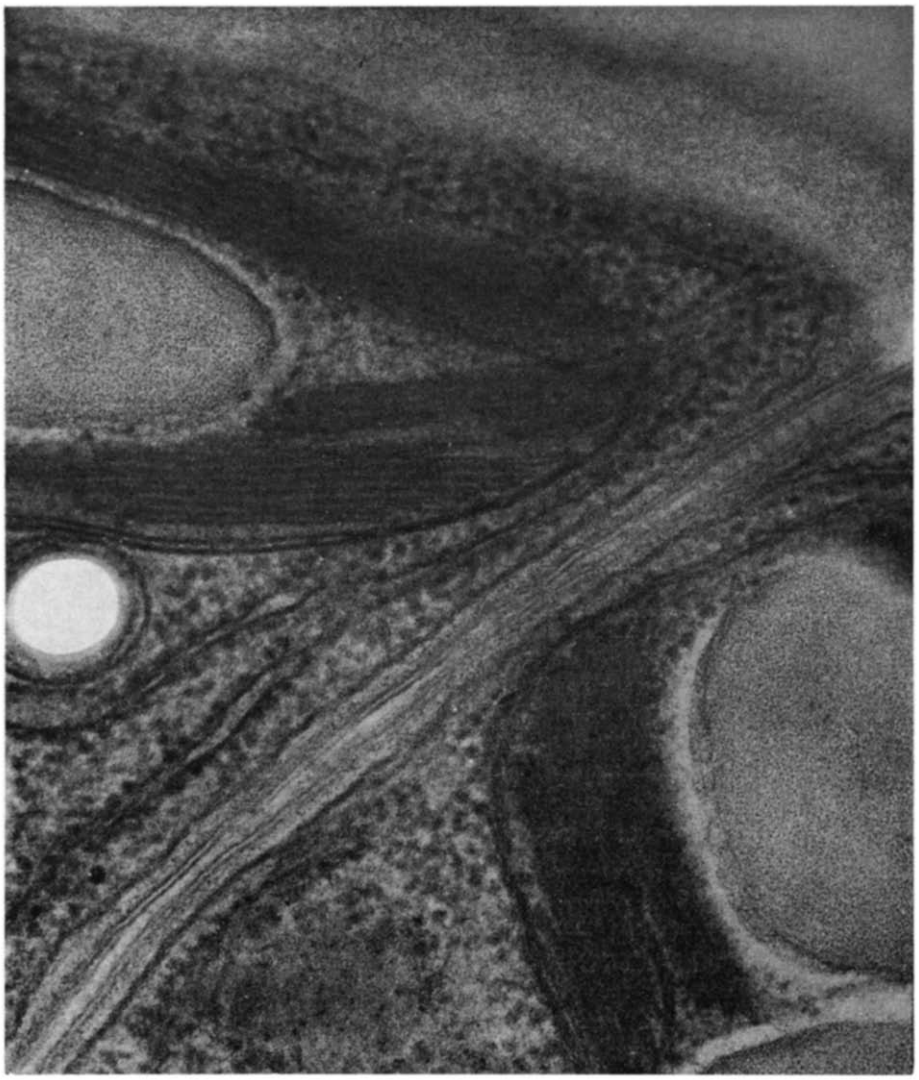


Fig. 4. Enlargement of indicated part of Fig. 3. Magnification: 102000 ×.

reduced rate of photosynthesis at saturating intensity is not the result of a rate limitation of this enzyme of the Calvin cycle.

From this and other data[7,29] we conclude that Mn deficiency, obtained in short-term culture does not result in a general disorganization of cellular structure and metabolism but limits the photosynthetic capacity through at least one specific effect within Photosystem II.

Specific effects of manganese deficiency

Effect of deficiency on quantum yields and flash yields: Table I presents the autotrophic growth yield of Scenedesmus as affected by two levels of manganese deficiency

and the resulting content of bound Mn (ref. 7) in isolated chloroplast particles. These data represent averages obtained from many determinations.

The amount of bound Mn was independent of the isolation medium employed (Table II), the method of analysis (Table III) and the total phosphate content of the Scenedesmus particles. This independence upon total phosphate is pertinent

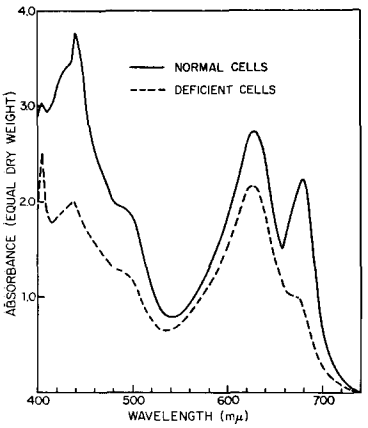


Fig. 5. Effect of extreme Mn deficiency on absorption spectra (equivalent dry weight) of *Anacystis nidulans*. Suitable aliquots of cells were suspended in 30% bovine serum albumin[42] and their absorptions were recorded on a Cary Model 14 spectrophotometer. After determination of chlorophyll *a* the resulting pellet was resuspended in 30% bovine serum albumin for determination of phycocyanin. Both curves are adjusted to 1 mg dry weight of cells per cm^2 of light path. Dry weight determinations were made after drying cells at 110° then over P_2O_5. Phycocyanin content of normal and deficient cells for two experiments was 35.2 and 32.4% and 23.0 and 22.6%, respectively. Chlorophyll *a* content was 2.55 and 2.85% and 0.67 and 0.75%, respectively.

TABLE I

EFFECT OF MANGANESE DEFICIENCY ON GROWTH YIELD OF SCENEDESMUS AND ON MANGANESE CONTENT OF CHLOROPLAST PARTICLES

Level of deficiency	*Growth yield*		*Chloroplast Mn content*	
	ml packed cell volume	%	*Mn (g atoms) per 50 Chl (moles)*	%
None	2.24	100	1.08 ± 0.10	100
Moderate	1.32	59	0.22 ± 0.05	22
Extreme	0.18	8	<0.05	<5

because in mitochondria, chromatophores and chloroplasts certain cations are known to be precipitated as their phosphates. As shown in Table III, the concentration (about 1 Mn/50 Chl) found in non-deficient chloroplast particles was observed also in whole (or broken) spinach chloroplasts. A similar value has been reported earlier for spinach chloroplasts[14,38], and a higher value (about 1 Mn/26 Chl) for tomato chloroplasts[13]. In the tobacco mutant chloroplasts, expressed on a chlorophyll basis, about a 2-fold higher value than for spinach was observed. However, on a dry weight basis the values for spinach and tobacco were essentially equal since the percent chlorophyll of dry weight for spinach and tobacco mutant was 16 and 6.2, respectively.

TABLE II

EFFECT OF ISOLATION PROCEDURE AND PHOSPHATE CONTENT OF SCENEDESMUS PARTICLES ON THE ABUNDANCE OF MANGANESE IN PARTICLES

Decrease in phosphate content of particles was obtained by resuspension of uniformly labeled cells into a low phosphate (2 % of normal amount) culture medium for 16 h. The specific activity of ^{54}Mn was maintained in this medium.

Isolation medium	*Cell treatment*	*Mn/Chl (counts per min per mg)*	*Phosphate/Chl (μmoles/mg)*
0.02 M Tris–HCl (pH 7.0)	None	$81 \cdot 10^3$	4.6
0.02 M Tris–HCl (pH 7.0)	Lyophillized	$80 \cdot 10^3$	4.8
0.02 M Tris–HCl (pH 7.0)	Phosphate starved	$84 \cdot 10^3$	1.9
0.02 M potassium phosphate (pH 6.5)	None	$80 \cdot 10^3$	—
0.02 M potassium phosphate (pH 6.5) containing 1 mM EDTA	None	$80 \cdot 10^3$	—

TABLE III

ABUNDANCE OF MANGANESE IN CHLOROPLASTS

Source of chloroplasts		*Mn (g atoms) per 50 Chl (moles)*		
		Chemical analysis	*Atomic absorption analysis*	*Analysis by labeling (^{54}Mn)*
Spinach*	(a)	1.01	—	—
	(b)	1.01	—	—
	(c)	0.78	—	—
	(d)	1.07	—	—
	(e)	0.86	0.82	—
Scenedesmus particles	(a)	0.78	—	0.90
	(b)	1.07	—	1.10
	(c)	1.14	1.20	1.44
	(d)	1.07	—	—
Tobacco mutant**	(a)	2.07	1.90	—

* Isolated by the procedure of SCHWARTZ[41]. Spinach was obtained from greenhouse and local markets.

** Seed of consolation, yellow green obtained from Dr. G. R. NOGGLE, North Carolina State University, Raleigh, N.C. Broken chloroplasts, obtained by resuspension in water, were used to eliminate massive amounts of starch granules. By repeated fractional centrifugations broken chloroplasts were separated from starch granules before Mn and dry weight determinations. The chloroplasts, on a chlorophyll basis, had a Hill reaction specific activity (saturating light) twice that of spinach chloroplasts (B. KOK, unpublished). For other details see text.

On a chlorophyll basis the saturation rate for tobacco chloroplasts was 2-fold greater than that of spinach, perhaps indicating a 2-fold increase of trapping centers per chlorophyll.

Using intact Mn-deficient Scenedesmus cells, as described in Table I, the effect of deficiency upon the rate of photosynthesis and the photoreduction of quinone was examined in various intensities.

Fig. 6 shows reports of typical data obtained plotted as rate (R) *versus* rate over

intensity (R/I). These will be linear for light curves which fit a rectangular hyperbola. Obviously this hyperbola does not describe the rate curve for the non-deficient cells, since in weak light the rate is a linear function of intensity. Nevertheless such a plot (R *vs.* R/I) is convenient for computing the two parameters v_{max} and v/I ($v/I = V/I$ for $V \to 0$), the maximum velocity and quantum yield, respectively.

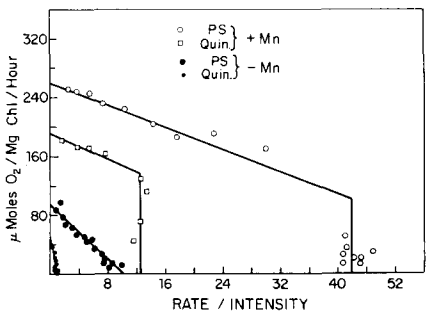


Fig. 6. The effect of extreme manganese deficiency on photosynthesis (PS) and photoreduction of quinone (Quin.) plotted as rate *vs.* rate/intensity. v_{max} was computed as the intercept with the ordinate, and v/I, the quantum yield, was obtained as the intercept with the abscissa. Data given in Table IV were obtained by such computation.

TABLE IV

EFFECT OF MANGANESE DEFICIENCY ON QUANTUM YIELDS AND SATURATION RATES OF PHOTOSYNTHESIS AND QUINONE PHOTOREDUCTION OF SCENEDESMUS CELLS

Numbers in parentheses are percentages of controls (non-deficient cells).

Expt. No.	*Deficiency*	*Photosynthesis*		*Quinone photoreduction*	
		v_{max}	v/I	v_{max}	v/I
I	None	178	33.7	200	10.8
	Moderate	188 (105)	21.0 (62.3)	162 (81.0)	4.2 (38.8)
II	None	195	32.7	190	11.2
	Moderate	195 (100)	21.0 (64.2)	185 (83.2)	5.3 (47.3)
III	None	219	40.0	180	9.5
	Extreme	62 (28.3)	7.0 (17.5)	34 (18.9)	0.6 (6.3)
IV	None	260	44	185	22.6
	Extreme	94 (36.2)	10 (22.7)	50 (27.0)	1.0 (4.4)
V	None	50*	50*	210	14
	Extreme	34* (68.0)	15.3* (30.6)	58 (27.6)	1.8 (12.9)

* Assayed in 0.08 M $NaHCO_3$. For other details see METHODS.

Table IV shows the effect of deficiency upon these parameters. Moderate levels of deficiency (a 5-fold lowering of the Mn content from its normal value of 1 Mn/50 Chl to approx. 1 Mn/250 Chl) exert no effect on v_{max} in photosynthesis and only a 20 % decrease of v_{max} in the photoreduction of quinone. However, at the same time the quantum yields of photosynthesis and quinone photoreduction were decreased 40 % and 50–60 %, respectively. This means firstly that the effect of Mn deficiency is not

on the "dark" rate-limiting reaction but on the quantum conversion process. A similar effect has been observed with low concentrations of DCMU[30].

At the higher degrees of deficiency (approx. 1 Mn/1000 Chl, 0.05 of normal) the quantum yield of photosynthesis and quinone photoreduction was decreased further (to 20–30 % and 10–15 %, respectively). In this case, as expected, the severe loss of quantum yield was accompanied by a significant decrease of v_{max}.

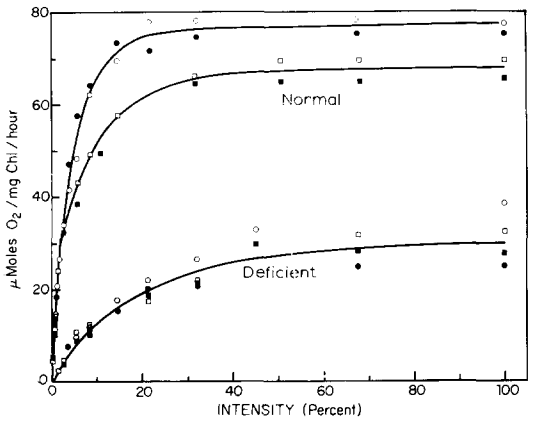


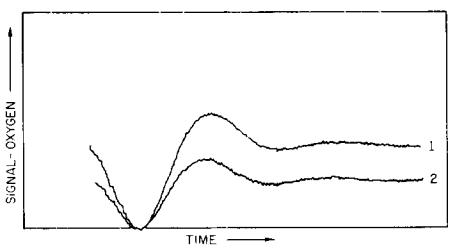
Fig. 7. Hill reaction activity of particles from normal and extremely deficient Scenedesmus cells (Medium II). ○, cytochrome *c*; ●, methyl viologen; □, benzoquinone; ■, ferricyanide. Note absence of any difference in quantum yield with different acceptors or with use of low concentration of quinone.

High quinone concentrations (10 μmoles/ml) "magnified" the effect of Mn deficiency. However, with normal cells the quantum requirement for quinone photoreduction was high (40–50 *hν* per O_2), decreasing to 19 *hν* per O_2 with a low concentration of quinone (0.5 μmole/ml), in comparison to the quantum requirement for photosynthesis (10–16 *hν* per O_2). These difficulties with the use of quinone and whole algae, which have been encountered earlier also by some workers[31–33], precluded assessment of the significance of "magnification" of the deficiency effects in quinone reduction.

Chloroplast particles prepared from normal (quantum requirement for particles of 16–20 *hν* per O_2) and deficient cells reflected the effect of Mn deprivation on v_{max} and v/I in much the same manner as whole cells. With cell free particles a low concentration (0.2 μmole/ml) of quinone could be used, and as illustrated in Fig. 7 in this case no difference in efficiency could be discerned between quinone and the other acceptors (ferricyanide, cytochrome *c* and viologen).

Yield of O_2 in short flashes: The direct effect of manganese deficiency upon the quantum yield of Photosystem II leads to the supposition that this element is operative in the early conversions in this photosystem. These presumably take place in conversion centers which occur in low abundance, a measure for this abundance being the yield of O_2 which can be evolved in a single brief flash. This flash yield will be maximal if (a) the System II trapping centers are in the oxidized state, which can be brought about either by preincubation in darkness or by preillumination with far-red light, and if (b) they are in the activated state[34]. This activation presumably requires one quantum per trapping center, so that the maximum yield of a short flash will be

observed after the trapping centers have been brought into an oxidized state, then activated with a subsequent activating flash. In our measurements of flash yield, the maximum yield was actually obtained with the third flash. A complete description of the measurement technique which allowed the measurement of relative quantum yield simultaneously with the flash yield is given by JOLIOT, JOLIOT AND KOK[20]. Fig. 8 shows that the O_2 signal produced by a flash varied in amplitude but not in



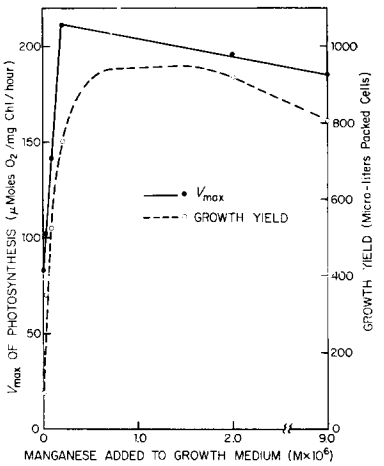


Fig. 8. Wave forms of oscilloscope traces at different sensitivity settings (2-fold increase in sensitivity for Curve 2 compared to Curve 1) of the O_2 signals observed after a brief flash given to a normal and Mn-deficient sample of Scenedesmus cells. Note similarity of signal shape despite a 3.2-fold difference of signal height.

Fig. 9. Growth yield and v_{max} of photosynthesis as a function of Mn concentration in growth medium. Total volume of medium was 300 ml.

shape with the degree of Mn deficiency. This result ascertained that the amplitude of the signal was a true index of the related amount of oxygen evolved per flash.

For our study of the effect of deficiency upon flash yield we used cells harvested from parallel cultures grown under various degrees of deficiency. Fig. 9 shows the growth yields and the rates of photosynthesis (v_{max}) observed with these batches. Fig. 10 shows the relative quantum yield and relative flash yields observed with the five different batches of cells. Cells from a parallel culture which was provided with abundant manganese (2 μM $MnCl_2$) were used as control in respect to which the observed data were normalized. As was shown by JOLIOT AND JOLIOT[35] due to photon transfer between photosynthetic units the relative quantum yield increases in a non-linear fashion with the concentration of active photochemical reaction centers E. The relation between V and E, measured by JOLIOT AND JOLIOT[35], is plotted in Fig. 11 as a full line, whereas the dots in this figure represent the data of Fig. 10. The observations clearly fit with the assumption that Mn deficiency leads to a decrease of active reaction centers. The data of Figs. 8–11 thus indicate that, without grossly affecting either the probability of photon transfer between units or the reaction time of O_2 evolution, Mn deficiency decreases the number of reaction centers in the O_2 evolving photosystem

Plotted also in Fig. 11 is the relation between v_{max} to V and E. The comparison shown between v_{max} and V obtained by Mn deficiencies is very similar to that obtained by GINGRAS AND LEMASSON[30] using DCMU. From the relation of the v_{max} curve to V and E it can be ascertained that a considerable loss of V (50–60 %) and E (60–70 %) can occur without a decrease of v_{max}. Additional reference to this relationship is made in the next section.

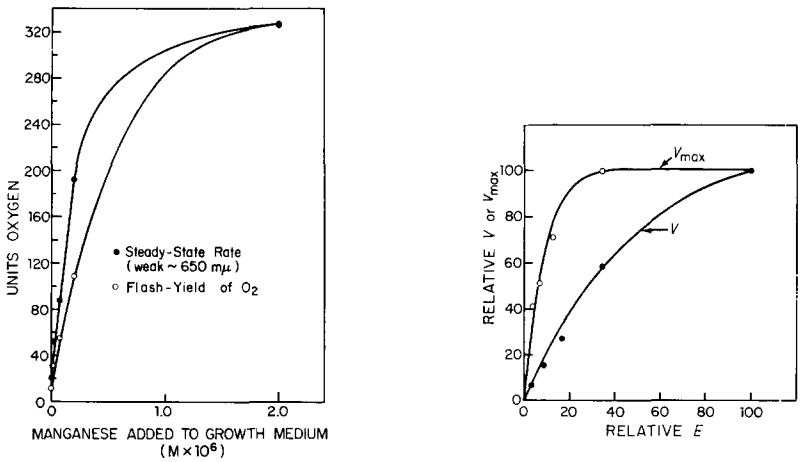


Fig. 10. Flash yield of O_2 and relative quantum yield of photosynthesis as a function of Mn in growth medium. Results obtained from each type measurement were normalized to the concentration of Mn (2 μM) giving maximum values. At 9 μM Mn, flash yields were reduced by approx. 30 %.

Fig. 11. Plot of V and v_{max} *vs.* E. The curve labeled V was replotted from JOLIOT AND JOLIOT[35]. The v_{max} curve was obtained from data of Figs. 9 and 10. Solid circles on V curve and data obtained (Fig. 10) with Mn-deficient cells.

DCMU inhibition: Inactivation by DCMU occurs reversibly by a binding of the inhibitor (I) with a trapping center (C) to form a complex between inhibitor and center (C–I). Thus for a decreased number of reactive conversion centers due to low [Mn] one would predict, for $K_i\,[(C\text{–}I)/(C)\,(I)]$ to remain constant, that a decreased amount of DCMU is required to evoke a given degree of inhibition. This supposition was tested as shown by the data in Fig. 12 obtained with Scenedesmus particles and spinach chloroplasts. The low quantum yield of Mn-deficient particles necessitated assay of v_{max} as the index of photochemical activity to insure adequate precision.

Mn deficiency indeed proved to bring about an increased sensitivity to DCMU. Irrespective of the source of chloroplast material 50 μg chloroplast chlorophyll proved to absorb irreversibly and inactivate $4.4 \cdot 10^{-11}$ moles of DCMU without this having an effect upon the rate. This corresponds to the irreversible binding of about 1 DCMU per 900 chlorophyll molecules, a value very similar to that predicted by the data of IZAWA AND GOOD[36]. Thus the three inhibition curves given in Fig. 12 can be corrected for the same amount of "inactivated" DCMU to arrive at a meaningful value for the effective DCMU concentration in the suspension needed for *e.g.* 50 % inhibition (arrows on upper abscissa). In this manner we computed for the concentrations giving 50 % inhibition: $5.3 \cdot 10^{-8}$ M or 1 DCMU per 420 Chl for non-deficient algae particles or spinach chloroplasts and $2.4 \cdot 10^{-8}$ M or 1 DCMU per 1073 Chl for deficient particles.

It should be noted that the same value (1 DCMU per 420 Chl) was obtained with spinach chloroplasts and normal Scenedesmus particles despite a difference in v_{max} of approx. 3-fold. In terms of quantum requirement, however, the two preparations differed only slightly: 16–20 *hν* per O_2 for normal Scenedesmus particles and 10–14 *hν* per O_2 for the spinach chloroplasts.

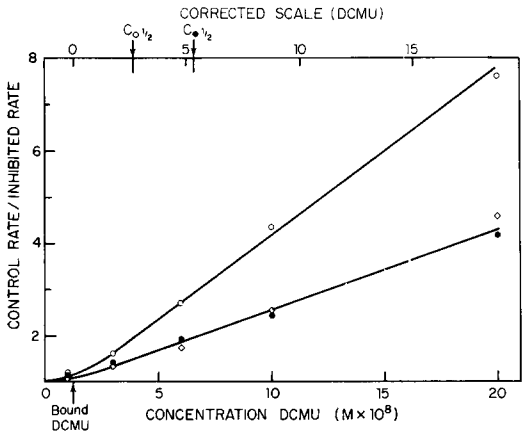


Fig. 12. DCMU inhibition of ferricyanide Hill activity of normal Scenedesmus particles (●), spinach chloroplasts (◇) and deficient Scenedesmus particles (○). Assay was made at saturating white light for 2 min at 20° followed by determination of ferrocyanide as described by AVRON AND SHAVIT[43]. Reaction mixtures contained for spinach chloroplasts: Tris buffer (pH 7.5), 0.05 M; methylamine–HCl, 0.03 M; and ferricyanide, 1.6 mM with 20 μg chlorophyll in a 1-ml reaction. Specific activity for spinach chloroplasts without inhibitor was 1100 μmoles ferricyanide reduced per mg Chl per h. For Scenedesmus particles the reaction mixture was the same except the ferricyanide concentration was 0.6 mM. Specific activity was 352 and 172 μmoles ferricyanide reduced per mg Chl per h for normal and deficient particles, respectively.

Deficient particles as used in experiment Fig. 12 generally showed quantum requirements 3–4 times greater than the normal particles. From a figure (not shown), reconstructing the data of IZAWA AND GOOD[36] to relate v_{max} and V to full E in chloroplasts, we would predict an 80 % loss of E and a 2.7-fold increase in quantum requirement (V) corresponding to a 2-fold decrease of the maximum rate (v_{max}). Thus the observations are in reasonable agreement with the prediction and clearly reflect an increased sensitivity of the Mn-deficient particles to DCMU. The approximate 3-fold and 1.5-fold difference of v_{max} and V, respectively, observed between spinach chloroplasts and normal Scenedesmus particles does not seem relevant for the above present evaluation. A critical extension of these observations should reveal the relation between the number of Mn sites and the number of DCMU sites. By such an extension of the observations reported here, an insight into the significance and mechanism of "partitioning"[36] of System II poisons in chloroplasts possibly may be obtained.

DISCUSSION

In this report several questions have been asked concerning the possible function of manganese in photosynthesis. These were: (1) Is the effect on photosynthesis of manganese deprivation during growth a direct effect on a specific reaction(s) or is it simply one of gross disorganization of Photosystem II? (2) What catalyst is

affected directly by manganese limitation? And (3) if Mn does participate in one or more reactions within Photosystem II, what amount is specifically required and how many sites of the action are involved?

To the first question we conclude from the lack of significant effects of the deficiency on chloroplast structure, whole cell respiration, certain respiratory enzymes, ribulose-P_2 carboxylase, chlorophyll content and the chlorophyll *a*/*b* ratio that Mn deprivation specifically affects the transfer of electrons from water to the reductant of System I. Previous work has established that System I activities (v_{max})[7,12,14] and quantum yield or v_{max} of Scenedesmus chloroplast particles[7] are not diminished by Mn limitation established by growth in deficient media or removal of Mn from particles by heat treatment. Thus the site(s) affected by Mn deficiencies is within System II or in the reactions linking the two photoacts.

The decreasing quantum yield of photosynthesis and quinone photoreduction by whole cells and of Hill activity of chloroplast particles with decreasing amounts of bound manganese suggested that photocatalyst *E* (ref. 34) of System II was primarily affected. The decreased flash yields of O_2 (measure of *E*) with increasing deficiencies confirmed this supposition and moreover showed that the resulting effect was identical to that produced by either DCMU or *o*-phenanthroline poisoning[37].

These data and those in which Mn deficiency (decrease of *E*) proved to yield an increased sensitivity to DCMU inhibition showed that the site affected by Mn deficiency is identical to the site of DCMU poisoning. It should be made clear that the term "site" is used here to indicate a unit of "O_2-evolving enzyme", in all likelihood a complex multi-enzyme system. Further elucidation of this enzyme might well reveal that the specific locus of attack is different for the various poisons and may not be the locus occupied by Mn itself.

The amount of bound Mn in chloroplasts, determined by a number of investigators[7,14,38], is about 1 Mn per 50 Chl. As shown here a decrease from this amount of bound manganese results in decreased flash yield of O_2. We must consider, therefore, the question of the size of the photosynthetic unit of Photosystem II that accommodates this amount of manganese.

The absolute amount of *E* in our Scenedesmus samples is unknown, our measurements being of relative amount. Generally in green algae flash yields of 1 O_2 per 1500–1800 chlorophylls have been reported[40] which implies that on an equivalent basis catalyst *E* occurs in a concentration of approx. 1 per 400 Chl_{total}. Many workers have estimated the unit to be of the order of 1 per 250–500 chlorophylls. From studies of DCMU inhibition of Hill activity of chloroplasts IZAWA AND GOOD[36] have concluded that only one unit is present for every 2500 chlorophylls. This conclusion is based, however, on the assumption that all *E* centers are active in their preparation and moreover that a given loss of *V* is a direct reflection in loss of traps. This latter assumption obviously is incorrect (*e.g.*, 20% loss of *V* is reflected in over a 40% loss of *E*).

As to the first assumption, we can compare directly our DCMU data to that of IZAWA AND GOOD[36]. In all aspects we corroborate their results. The chloroplasts used by us in corroborating their results show a quantum requirement for O_2 of 10–12, values 25–50% greater than the generally accepted theoretical value of 8. If we accept the theoretical value of 8, this implies a loss of 40–60%, respectively, of *E* centers. Measurements by KOK *et al.*[39] in this laboratory do indicate a loss of *E*

centers of this magnitude. Because of these considerations we question the conclusions of IZAWA AND GOOD[36] regarding the size of the unit of Photosystem II and would suggest that because of their assumptions they have underestimated the size of the units.

As yet we have not considered from quantitative aspects the effects on v_{max}, V or E resulting from a decrease of the normal amount of bound Mn. The concept of direct participation in the O_2-evolving system leads to the question how many Mn atoms are required per unit O_2-evolving enzyme "E".

POSSINGHAM AND SPENCER[13] have reported that v_{max} is linearly related to the amount of bound Mn. Their observations were limited to a 2- to 3-fold range in amount of the element. Moreover, their rate measurements with chloroplasts were for v_{max} only. In whole cells, with low quantum requirement, the relation for photosynthesis of v_{max} to V in relation to E is not parallel as can be deduced from data of GINGRAS AND LEMASSON[30] and as shown here (Fig. 11). In chloroplasts[36] the relation of v_{max} to E parallels (but does not superimpose) that of V to E. These differences between whole cells and chloroplasts in behavior of v_{max} to E thus make it impossible to reconcile the differences between our data and those of POSSINGHAM AND SPENCER. Because of the strong non-linear function of V (or v_{max}) *vs.* E it seems unlikely that manganese is linearly related to v_{max}. The results of POSSINGHAM AND SPENCER clearly show, however, a high order of dependency upon the considerable amount of bound Mn.

In contrast, EYSTER *et al.*[3] have reported that an amount of 1 Mn/500–600 Chl sufficed for maximum growth of Chlorella or "full" photosynthesis and Hill reaction. This value would appear to be closely related to E on an equivalent basis. Since, however, the rates of photosynthesis and Hill reaction as measured by EYSTER *et al.*[3] were made apparently at saturating intensities, we would conclude (Fig. 11) that such measurements would grossly underestimate the amount of Mn necessary for maximum E.

In the results reported here, with cells in which the bound Mn (as measured in isolated chloroplast particles prepared thereof) was decreased from the normal amount of 1 Mn per 50 Chl to 1 Mn per 250 Chl, the amount of photocatalyst E was decreased considerably (approx. 70 %). A decrease of bound chloroplast Mn to approx. 1 Mn per 1000 Chl was correlated with a 95 % decrease of the amount of active E. Assuming the concentration of E in our material to be the same as it occurs in other green algae, we can estimate the amount of bound Mn in E.

In normal Scenedesmus with approximately 1 E_{equiv} per 400 Chl_{total} a value of 1 Mn per 50 Chl_{total} was observed. Similarly with the deficient samples showing approximately 70 % and 95 % loss of E (approximately 1 E_{equiv} per 1330 Chl_{total} and 1 E_{equiv} per 8000 Chl_{total}, respectively) the amount of bound Mn measured was 1 Mn per 250 Chl_{total} and 1 Mn per 1000 Chl_{total}. Thus from this limited amount of data linearity between the amount of bound Mn and amount of E is observed with a value of approx. 8 Mn per E_{equiv}. These data are tabulated in Table V.

The ratio of 8 Mn per E_{equiv} may be compared to the ratio of the pool size of the O_2 gush (pool A) and the pool size of flash yield of O_2 (pool E). This ratio is about 10–20 A per E (ref. 40) and thus the Mn pool is therefore of the same order of magnitude of the A pool. If all the manganese is functional, one must then consider the implication of the similarity of pool size between A pool and Mn. Several conjectures

are possible. Is the bound Mn associated with the reducing-side of System II and thus directly associated with pool A? Or does pool A have a complement on the oxidant side of System II, this complement represented by the pool of Mn? Kinetic data of JOLIOT[34] argues against but does not exclude this latter possibility.

TABLE V

RELATION BETWEEN BOUND MANGANESE AND PHOTOCATALYST *E*

$\frac{Chl}{Mn}$	$\frac{Chl}{E}$	$\frac{Mn}{E}$
50	400	8
250	1330	5.5
1000	8000	8

At present, however, we cannot assess even how much of the manganese is at the specific site in the *E* complex. The true "critical" value may actually be less, since we do not know the active-site manganese content of the presumed Mn protein of Photosystem II. Metalloproteins are known which contain specifically a metal at non-specific sites in addition to the same metal at the active site.

The determination of the true "critical" amount of Mn must await direct measurements of some parameter, possibly oxidation–reduction of the specifically bound manganese within the *E* complex of Photosystem II. Such a measurement awaits a more clearly defined function of Mn in Photosystem II.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Dr. B. KOK for many critical comments and useful discussions throughout the course of this work. This work was supported in part by the Atomic Energy Commission AT(30-1)-3706 and the National Aeronautics and Space Administration (NASW-1592).

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