Plant Physiol. (1967) 42, 997–1007

Studies on the Manganese of the Chloroplast[1]

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Received April 13, 1967.

Summary. Manganese deficiency of green plants is known to affect preferentially the activity of the oxygen evolving system in the photosynthetic apparatus. Our studies showed that the time needed to reactivate photosynthesis in Mn-deficient algae varies with each culture, and is often very short when Mn is added not before illumination but during the light period. The recent finding by Cheniae and Martin that the reactivation requires light, is confirmed. The plain incorporation of ^{54}Mn into deficient algae as distinguished from reactivation was barely affected by light, yet was inhibited by uncouplers of phosphorylation. Higher plants responded to manganese deficiency either by adjusting the number of chloroplasts per cell to the limited Mn supply, or by forming disorganized chloroplasts with low chlorophyll content. These 2 types of responses produced chlorotic plants which had either a few photosynthetically active or many disabled chloroplasts. Photosystem I mediated photophosphorylation turned out to be much more sensitive to manganese deficiency than the system I dependent photoreduction of $NADP^+$.

In 1937 Pirson (20) discovered that the low photosynthetic activity in manganese deficient algae could be restored to normal in less than an hour simply by adding manganese salts to the suspension medium. This easy reversibility underlines the special position of the manganese deficiency among other mineral deficiencies of green plants. About 20 years after Pirson's discovery, Kessler (12) showed that manganese is not required for the photoreduction of CO_2 with hydrogen by adapted green algae. Subsequent studies (5, 6, 13, 22, 27, 28) are consistent with Kessler's interpretation that manganese is needed only for the oxygen evolving system in photosynthesis. This requirement was demonstrated for green algae as well as for blue-green algae and higher plants. The present work was started with the intention of learning more about the properties of chloroplast manganese, the uptake of manganese by deficient algae during reactivation of photosynthesis, and the interrelations between chloroplast structure and photosynthetic activity.

Methods

Manganese deficient *Scenedesmus* D_3 or *Ankistrodesmus* (strain Marburg) cells were grown autotrophically in a mineral nitrate medium (14) or heterotrophically with a supplement of 1% glucose. Autotrophically grown cells were preferred in this study because during heterotrophic growth more cell particles accumulated which adsorbed added manganese unspecifically and thereby interfered strongly with measurements of manganese uptake by living cells. It usually took 10 to 18 days to grow algae with a 50 to 75% deficiency, expressed as

$$100\left(1 - \frac{\text{rate of photosynthesis of deficient algae}}{\text{rate of photosynthesis after reactivation}}\right)$$

at 12,000 ergs sec^{-1} cm^{-2} red light.

The higher plants were grown in a greenhouse on a mineral medium (1, 10). Lack of adequate air conditioning and light control limited the culturing of spinach (*Spinacia oleracea*) to the winter months, and that of the coffeeweed (*Cassia obtrusifolia*) to the summer. Seeds of the tobacco plants were obtained by courtesy of Dr. Heggestad, Beltsville, Maryland.

For growing manganese deficient plants all trace elements were supplied as Specpure reagents (Johnson, Matthey and Co., London, England). Unfortunately, there is no easy way to determine routinely the deficiency of leaves by analyzing them for traces of manganese still present. In order to compare various deficient plants, leaves were selected which seemed to suffer from the same degree of chlorosis. We always made sure that the chlorotic leaves would become healthy looking green leaves upon addition of manganese to the growth medium. This greening started in the developing young leaves and occurred subsequently in a de-

[1] These studies were aided by grant AF-AFOSR-662-65 from the Air Force Office of Scientific Research.

scending fashion in the older leaves below, initially along their veins.

Whole chloroplasts, broken chloroplasts and sonicated chloroplast particles (obtained with a Branson model S-125 sonifier at full output) were prepared according to standard procedures in 0.40 M sucrose containing 0.05 M tris HCl (pH 7.5), 0.01 M NaCl and 0.001 M EDTA (9, 30). The ferricyanide Hill reaction and the photoreduction of $NADP^+$ were measured spectrophotometrically (15, 29). Partially purified ferredoxin (3) was prepared from various plants. Chlorophyll from chloroplasts was determined after extraction into 80% acetone (16), and chlorophyll from leaves and algae was extracted with methanol at 60° and estimated spectrophotometrically in 90% methanol (26).

The incorporation of manganese into algae from growth medium buffered with 15 mM phosphate at pH 6.6 was measured after incubation in the presence of $^{54}Mn^{2+}$. The algae were collected on a Millipore filter (1.2μ) and washed with 20 ml 100 μM EDTA, and, subsequently, with 20 ml water. The use of EDTA assured the removal of unspecifically adsorbed manganese. The same incorporation data were also obtained when autotrophically grown cells were washed with pure water alone, provided corrections were made to take care of an adsorbed small fraction of manganese (intercept with the abcissa of the plot of incorporation vs time). The algae on the Millipore filter were counted in a crystal well detector (Nuclear Chicago) connected to a Nuclear Chicago model 8712 timer and printer. For colorimetric determinations of manganese, the plant material was digested with $HNO_3/HClO_4$ (8).

Photophosphorylation was measured as the disappearance of P_i (11) or by using ^{32}P as tracer (2). Photosynthetic oxygen evolution of algae was followed manometrically at 22° in their culture medium supplemented with 15 mM phosphate buffer and equilibrated with a gas phase of 4% CO_2 in air. Leaves or leaf sections were floated on 3 ml 0.1 M bicarbonate-carbonate buffer pH 9.0 (26). CO_2 fixation was measured as incorporation of $^{14}CO_2$ into leaves which were kept in a gas-tight plexiglass chamber (volume 363 cm^3) maintained at 22 to 23°. The leaves or leaf sections were put upright into wet sand, and during a preillumination period of 15 minutes a constant flow of air containing usually 0.5% CO_2 was bubbled through dilute phosphoric acid and then passed through the chamber. Then the gas flow was stopped and the chamber closed. A $Na_2{}^{14}CO_3$ solution of known activity (determined after precipitation of an aliquot as $BaCO_3$) was immediately injected into a vial holding a weighed amount (usually about 8 mg) of $BaCO_3$ as carrier. The carbonates were then decomposed by 20% H_3PO_4 to liberate labeled CO_2. The gas phase was stirred by a magnetically driven propeller. After 8 to 15 minutes the leaves or leaf sections were taken out, coated with a thin film of collodion, dried on planchets and counted in a Nuclear Chicago model 1046 planchet counter. The incorporation of $^{14}CO_2$ in the dark was usually negligible. Red illumination was provided by floodlight or projection lamps and plastic filters which removed all visible light with wavelength shorter than 580 mμ. Light intensities were measured with the remote probe of an Isco Model SR Spectroradiometer between 580 and 700 mμ. In manometric experiments 12,000 ergs sec^{-1} cm^{-2} red light were usually used to illuminate the algae.

The electron micrographs were kindly prepared by Dr. Georg H. Schmid (for methods see ref. 24).

Results

The Reactivation of Photosynthesis in Manganese-deficient Algae. According to Cheniae and Martin (5) light is required to restore normal photosynthetic activity in manganese deficient algae when manganese has been added to the suspension medium. In other words, when algae have been incubated in the presence of manganese but in complete darkness, photosynthesis will start upon illumination at the same rate as is found before Mn has been added. Once the light has been turned on, the reactivation begins and the rate of photosynthesis increases. First indications of a light effect on the reactivation process had already been seen by Pirson et al. 15 years ago (21) but these authors did not become aware of their discovery, and consequently did not design the crucial experiments. Our results lend full support to the findings of Cheniae and Martin (5).

The reactivation of photosynthesis was studied by following the kinetics of the increase of the rate of O_2 evolution after addition of manganese. The addition of 3 μM Mn^{2+} to an algal suspension with about 100 μg chlorophyll per 3 ml was sufficient to restore maximal photosynthesis in the course of

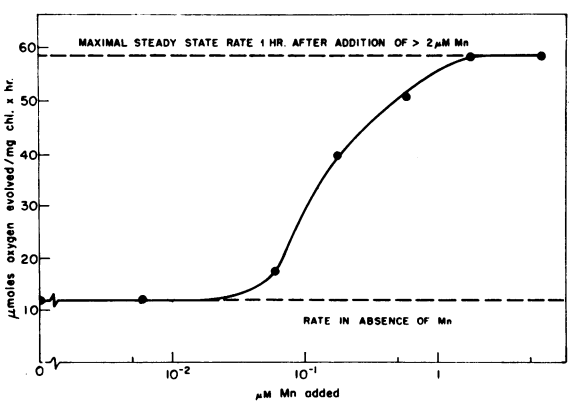

FIG. 1. Dependence of the increased steady state rate of photosynthesis of manganese deficient *Scenedesmus* D_3 (95 μg chl) on the concentration of added Mn^{2+} (10,000 ergs sec^{-1} cm^{-2} red light, 22°).

about an hour (fig 1). Normal algae used as controls attained a steady rate of photosynthesis not later than 10 minutes after the light was switched on. On the other hand, originally Mn-deficient algae which had been incubated with manganese in complete darkness for 50 minutes or more before the onset of illumination, needed about an hour or longer to reach a high constant rate (table I). The initial increase of the rate of photosynthesis consisted of 2 phases (fig 2): immediately after the onset of the light period there was the usual induction period of photosynthesis during which the rate increased rather rapidly for about 5 to 10 minutes. This rise was followed by a slower increase in the samples containing manganese, which reflects the reactivation process proper. The intercept with the ordinate of the slope of the second phase should give us the capacity for oxygen evolution of the algae at the beginning of illumination. Such an extrapolation clearly shows that reactivation had occurred only to a small extent during the dark period. The time needed to reach the manganese induced higher steady rate of photosynthesis will be called the reactivation time. Paradoxically, the reactivation time was often much shorter when manganese was added not before but during the light period (see table I). The dependence on the light intensity is greater for the rate of photosynthesis than for the reactivation process. Consequently, the reactivation time became longer at higher light intensities (fig 2). In general, the time needed to restore normal photosynthesis varied from experiment to experiment, and it was hard to tell whether extremely short reactivation times which were sometimes measured after a dark incubation with manganese, indicated a reactivation process in the dark, or a very rapid reactivation starting at the onset of the light period. It was not possible to obtain a clearcut pattern of response to the addition of manganese when the algae had been starved for 48 hours in the dark before the experiment. The reactivation process may also be influenced by the life cycle of the organism. Our algae were grown in a day-night cycle of 16:8

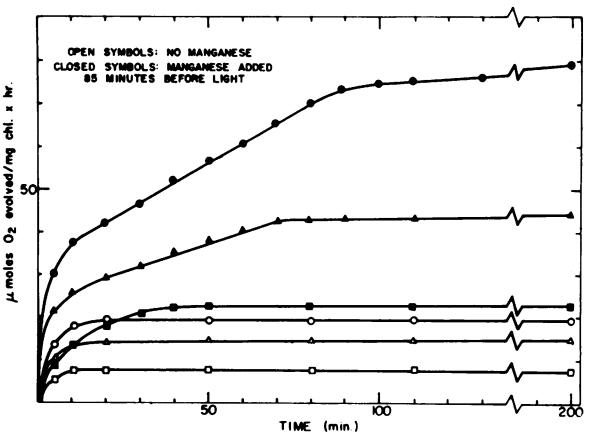

FIG. 2. Reactivation of Mn deficient *Ankistrodesmus* at various light intensities. Rates of O_2 evolution from manometric experiment, 160 μg chl, 22°. 3 μM Mn^{2+} added (closed symbols). 11,500 ergs sec^{-1} cm^{-2} (●, ○); 7000 ergs sec^{-1} cm^{-2} (▲, △); 4000 ergs sec^{-1} cm^{-2} (■, □).

hours. Mature normal algae were not difficult to obtain, but Mn-deficient algae were always found in a stage of incomplete division. Apparently manganese deficiency inhibits cell division, a condition which is also the reason for the production of long, undivided strings of blue-green algae under the stress of manganese deficiency (22). Attempts to use deficient algae at various stages of their life cycle have, therefore, been unsuccessful.

The Incorporation of Manganese into Algae. The uptake of radioactive manganese by deficient cells, apart from the reactivation process itself, was chosen as another approach to the problem of reactivation. The amount of incorporated manganese is here defined as the radioactive manganese which remains associated with the cells after they have been washed with 100 μM EDTA (see Methods). Under our experimental conditions, using 3 μM Mn^{2+}, the amount of manganese taken up irreversibly followed a linear time course for about one-half hour. After this time, a slower uptake

Table I. *Reactivation of Photosynthesis in Manganese Deficient Algae*

Reactivation times were estimated from manometric determinations of O_2 evolution at 22°, but at 35° with *Synechococcus*. The intensity of the red light was 12,000 ergs sec^{-1} cm^{-2}.

Species	Deficiency	Mn^{2+} added	Time of preincubation (min) + Mn	— Mn	Reactivation time
	%	μM	dark	red light	min
Scenedesmus	67	6	...	80	60
		3	...	80	60
Ankistrodesmus	60	3	50	...	40
			...	20	20
Ankistrodesmus	75	3	50	...	70
			...	60	25
Synechococcus	82	3	70	...	140
			10	...	145
			...	70	110

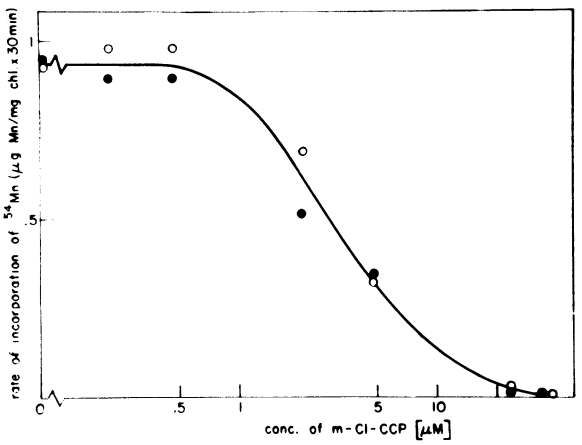

FIG. 3. Inhibition by *m*-Cl-CCP of the incorporation of ^{54}Mn into manganese deficient *Ankistrodesmus* (30 μg chl). Incubation at 25° for 30 minutes with 3 μM labeled Mn^{2+} in 12,000 ergs sec^{-1} cm^{-2} red light (○ - ○) or in the dark (● - ●).

was often measured (see inset, fig 4). The amount of incorporated manganese was proportional to the concentration of algae up to at least 100 μg chlorophyll per ml. During the initial linear time course, the rate of manganese uptake was proportional to the Mn concentration between 0.2 and 3 μM Mn, and increased further by a factor of 1.7 when the Mn concentration was raised from 3 μM to 12 μM. Usually light had only a slight stimulatory effect on the rate of incorporation, at least during the initial linear phase (table II). The incorporation of manganese must be an "active uptake" because it was inhibited by the same low concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) (fig 3) and of 2,4-dinitrophenol (DNP) which inhibit respiratory phosphorylation, and was slightly retarded by anaerobic conditions. The incorporation rate had a strong temperature dependence (only 15 % of the rate at 25° was found at 5°). 3-(*p*-Chlorophenyl)-1,1-dimethylurea (CMU), did not affect the manganese uptake in the light or in the dark. The somewhat faster incorporation in illuminated algae, therefore, was not connected to photosynthesis. The rate of manganese uptake could be inhibited by increasing the pH beyond 7.0, and by addition of bicarbonate at pH 7.5, but not by saturating the medium with 4 % CO_2 at pH 6.6. These effects are probably due to the formation of undissociated manganese hydroxide, phosphate and carbonate.

High concentrations of magnesium in the growth medium suppressed the accumulation of excess manganese in the cell. Thus, during growth of green algae, manganous ions appear to compete with magnesium ions for the same reserve pool. For example, the Mn content of *Scenedesmus* grown in the presence of 10 μM Mn dropped from 40 μg Mn/mg chlorophyll to less than 2 μg Mn/mg chlorophyll, when the Mg concentration was raised from 50 μM to 1 mM. The specific affinity of the photosynthetic system for manganese, however, is so high that this particular competition with magnesium is not sufficient to induce an easily detectable manganese deficiency at the critical sites even with only 1 μM Mn in the medium.

Since the uptake of manganese by algae proceeds rather slowly, attempts were made to interrupt the incorporation process by washing the cells. Their photosynthetic activity ought to be a function of the amount of manganese made available by incorporation during the incubation period up to the time of washing. One experiment is shown in figure 4. It gave the expected relationship but it is not very typical. The majority of such experiments failed for various reasons, such as contaminations with manganese during the filtering process and the subsequent removal from the filter. Moreover, the washing with EDTA had a variety of effects on the photosynthetic activity which have remained unexplained. Because of the many failures in this type of experiments one should possibly not overemphasize that the combined data of reactivation and Mn-incorporation in figure 4 tell us that about one-half μg Mn per 1 mg chlorophyll is needed for complete reactivation. However, the number agrees well with those found by other authors (summarized in ref. 13) and by us (see below) for the manganese content of normal chloroplasts from higher plants or algae.

Attempts to Extract Manganese from Chloroplasts of Higher Plants. After preliminary experiments had shown that a certain bound fraction of

Table II. *Effect of Light and Anaerobiosis on the Incorporation of Manganese into Manganese Deficient Algae*

The amount of labeled Mn incorporated was determined after 30 minute incubation at 20°. Aerobic incorporation in red light is equal to 100.

Alga	Air		Nitrogen	
	Red light*	Dark	Red light*	Dark
Ankistrodesmus (avg from 3 detm)	100	80	...	...
Ankistrodesmus	100	70	50	50
Ankistrodesmus	100**	85**	60	60**
Synechococcus	100	60	...	...

* 12,000 ergs sec^{-1} cm^{-2}.

** Gas phase contained 4 % CO_2.

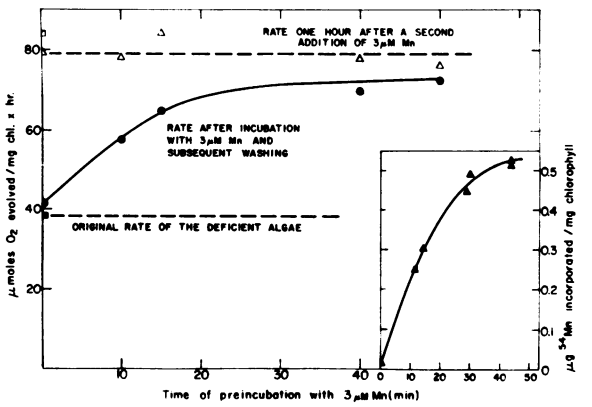

FIG. 4. Steady state photosynthesis of Mn deficient *Ankistrodesmus* after incubation with 3 μM Mn^{2+} in the dark for various lengths of time (22°, 12,000 ergs sec^{-1} cm^{-2} red light) (● – ●). Later another addition of 3 μM Mn^{2+} was made and the new steady state rate recorded (△ – △). The control sample was not incubated with Mn^{2+} but otherwise identically treated (■, □). The insert shows the incorporation of $^{54}Mn^{2+}$ from 3 μM labeled Mn^{2+} as obtained in a parallel experiment.

manganese could not be extracted from active chloroplasts either by agents such as EDTA (5, 28), ethylene glycol *bis*-(β-aminoethyl ether)-N, N'-tetraacetate (EGTA), or by applying an electric field of 100v/cm^2, we tried to isolate a fraction from the chloroplast grana which still contained manganese in its bound form using mechanical and chemical procedures. While this work was in progress Cheniae et al. (5, 13) reported about similar attempts. These authors described the isolation of a manganese containing protein fraction from cell free particles of *Scenedesmus* which they had not yet analyzed because of its instability. Bishop (personal communication) has also succeeded in isolating a manganese containing protein fraction. Without the facilities for large-scale investigations on ^{54}Mn-labeled material, we had mostly to rely on the time-consuming colorimetric assay for Mn. Nevertheless our results on the liberation of manganese from chloroplast fragments or fractions agreed well with those of Cheniae and Martin (5). It was not always easy to separate the active manganese of the chloroplasts from the excess manganese which was often present in chloroplasts of higher plants. Interestingly, this excess manganese could be removed by several washings of the chloroplast preparation with the isolation medium containing 1 mM EDTA, or by sonication and subsequent sedimentation of the sonicated chloroplast particles between 20,000 and 100,000 g. The amount of the manganese which still remained with the chloroplasts or chloroplast particles after these treatments was remarkably constant (table III) and amounted to about 1 atom of manganese per 65 molecules of chlorophyll, a figure which agrees with those reported in the literature (for a summary see 13). When the lipids of the chloroplasts or chloroplast fragments were extracted with acetone or chilled *n*-butanol (18) the manganese remained in the particulate sediment. Although this manganese could not be washed out by water or various buffers, it had now become readily removable by EDTA. The question arises whether EDTA has better access to the lipid free particles than to unextracted chloroplast particles, or whether the binding site has been altered during the extraction procedure. In this context it may be mentioned that Cheniae's (5) protein fraction also contains highly labile manganese. Our results and the data of Cheniae and Martin (5) do not confirm the earlier report

Table III. *Manganese Content of Chloroplast Preparations*

All chloroplast treatments were done at 0 to 5°; the EDTA was present in 0.4 M sucrose containing 0.05 M tris buffer pH 7.5 and 0.01 M NaCl. Sonication was carried out for 8 minutes at full output and the particles were collected between 20,000 and 100,000 g.

Species	Preparation	Treatment	Method of Mn determin.	μatoms Mn/ mmoles chl
Medicago lupulina	Broken chlpl	Passed through Chelex column	colorim.	11
Cassia obtrusifolia	Whole chlpl	Washed with 5 mM EDTA	colorim.	19
Cassia obtrusifolia	Broken chlpl	Sonication	colorim.	17
Cassia obtrusifolia	Broken chlpl	None	colorim.	91
	Same	Sonication	colorim.	17
Medicago polymorpha	Whole chlpl	None	colorim.	22
	Same	Sonication	colorim.	17
Phytolacca americana	Whole chlpl	Washed with 1 mM EDTA	colorim.	14
	Same	Sonication	colorim.	11
Nicotiana tabacum JWB*	Broken chlpl	Washed with 1 mM EDTA	^{54}Mn	14
	Whole leaves	None	^{54}Mn	35
*Spinacia oleracea***	Whole chlpl	Washed with 1 mM EDTA	^{54}Mn	14
	Broken chlpl	Washed with 1 mM EDTA	^{54}Mn	14

* Mn deficient plants supplied with 1.5 μM Mn (in 3 liters) 40 days before harvest.
** Mn deficient plants supplied with 0.6 μM Mn (in 3 liters) 20 days before harvest.

by Boichenko and Udelnova (4) that manganese is associated with the lipid fraction of the chloroplasts. It seems superfluous to enumerate more of our findings, since they agree with those published recently by Cheniae and Martin (5). One important exception has been our experience with the release of Mn from chloroplasts in slightly acid media. In 1 mM phosphate buffer (pH 4.8) with 15 mM NaCl only a little Mn was lost during an overnight incubation at 0°. Cheniae and Martin used 0.2 M phosphate buffer as suspension medium, and found a 60 % loss of Mn at the same pH. Chloroplasts become rapidly inactivated in pure phosphate buffer but not in dilute saline. Therefore, the release of manganese in 0.2 M phosphate buffer may have been a consequence of damage to the chloroplast structure.

Attempts to remove the bound manganese from chloroplast particles by various lipases and phosphatases were not successful, but the proteolytic enzyme pronase was effective in releasing free manganese into the suspension medium.

The Effect of Manganese Deficiency on the Chloroplasts of Higher Plants. The activity of chloroplasts isolated from manganese deficient higher plants has been determined already by other investigators (6, 27, 28). Because of the care they need, and because of their slow growth, water culture grown, manganese deficient higher plants are more difficult to study than manganese deficient algae. Most important, however, is that manganese deficiency affects the metabolism of the differentiated organism of a higher plant in more general ways than that of a simple green alga. The plant first chosen for our studies, the coffeeweed (*Cassia obtrusifolia*), responded to manganese deficiency in an unexpected way. Under the stress of manganese deficiency, the newly developing leaflets became progressively chlorotic. In earlier stages of the deficiency they retained the normal green coloration at least along the veins. Later, when the deficiency became more severe, they remained small, looked uniformly yellowish (8 to 14 μg chl/cm^2 vs about 40 μg chl/cm^2 in normal leaves) and became brittle and easily detachable. Surprisingly, the photosynthetic activity of many of these apparently strongly deficient leaves was not as low as could be expected from the description in the literature. On the contrary, when the activity was calculated on the basis of the little chlorophyll present, it often came out the same as for normal leaves, and sometimes even higher.

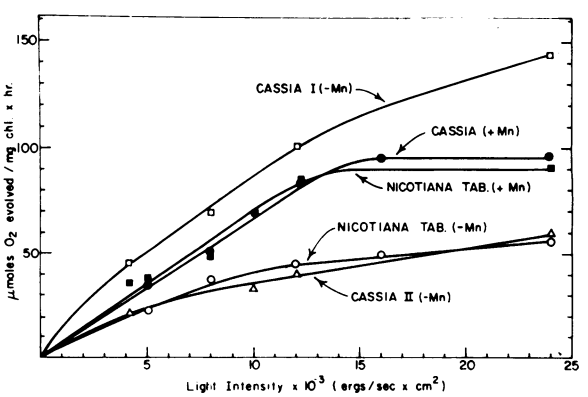

FIG. 5. Light intensity curves for photosynthetic O_2 evolution of Mn deficient leaves, determined manometrically and corrected for respiration: 25°; T+: *Nicotiana tabacum* John Williams Broadleaf, control (45 μg chl/cm^2); T—: same, Mn deficient (8 μg chl/cm^2); C+: *Cassia obtrusifolia*, control (38 μg chl/cm^2; C— I and II: same, 2 Mn deficient leaves (9 and 11 μg chl/cm^2 resp).

Figure 5 compares the light intensity curves of Mn deficient leaves of tobacco and *Cassia* with those of their non-deficient controls. The curves for the tobacco leaves are typical for a diminished photosynthetic activity due to manganese deficiency (5, 20, 21). In contrast to tobacco, the upper curve for a deficient *Cassia* leaf (*Cassia* I) shows that the photosynthetic activity on the basis of chlorophyll is much higher than that of the control leaf. Another curve (*Cassia* II) shows a leaf with the low photosynthetic activity normally associated with Mn deficiency. Because such leaf material is unavoidably heterogenous, many intermediate stages between these 2 curves were observed, some rising even higher than the 1 shown in figure 5. On 2 occasions we found with very chlorotic *Cassia* leaves an oxygen evolution of 220 μmoles O_2 per mg chlorophyll per hour. This was about twice the maximal rate of a normal control leaf under these conditions, which gave only 125 μmoles O_2 per mg chlorophyll per hour.

Manometric measurements of photosynthesis with leaves of low chlorophyll content often require a large correction for their high respiration rate. To eliminate any doubt that the high rates of photosynthesis were real, the fixation of $^{14}CO_2$ was also measured. The saturation rates for carbon dioxide fixation by manganese deficient and normal leaves from various plants have been summarized in figure 6. The bars represent the average of at least 10 determinations. The saturation rates in this type of experiment exceed those measured as evolution of oxygen, because photosynthesis of *Cassia* leaves becomes saturated at 0.5 % CO_2 in air. The gas phase in the Warburg vessels, however, contained only about 0.35 % CO_2. Moreover, the heat exchange of a leaf surrounded by stirred gas may have been less efficient than in the Warburg vessel where the leaf floated on a bicarbonate solution. Apart from this difference, the $^{14}CO_2$ experiments confirmed that the chlorophyll of the manganese deficient *Cassia* leaves had a normal photosynthetic activity.

Because manganese deficiency ought to affect the chloroplast Hill reaction in the same manner as photosynthesis, the Hill activities of various chloroplast preparations were compared. In gen-

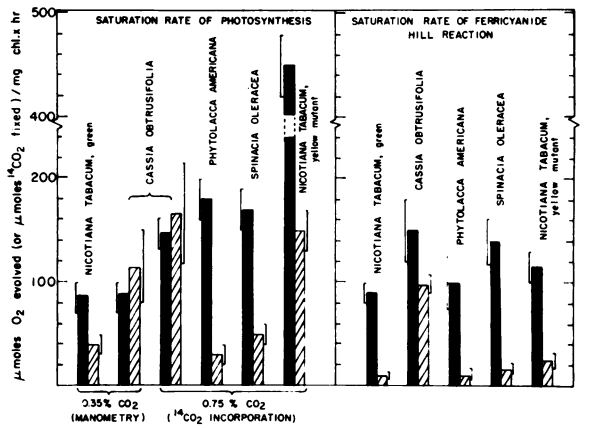

FIG. 6. Activity of leaves or chloroplasts in photoreactions involving photosystems I and II. Black bars: controls; shaded bars: manganese deficient leaf material with about 25 % of the normal chlorophyll content per area (for information about the yellow tobacco mutant "Su/su" see 23, 24, 25). Left side: light saturation rates for photosynthesis of various leaves determined by manometry or $^{14}CO_2$ incorporation. Right side: light saturation rates for ferricyanide Hill reaction of various chloroplast preparations in the presence of 15 mM methylamine (for reasons of comparison recalculated as μmoles O_2 evolved/mg chlorophyll hr).

eral, the deficient chloroplasts were much less active than those of the control plant, with the exception of *Cassia*. It is unlikely, therefore, that factors other than the activity of the chloroplasts (e.g., the stomatal aperture) contributed significantly to the differences in the photosynthetic activity of the normal and deficient leaves.

After testing photosynthesis and Hill reaction it was interesting to see how those reactions which involve photosystem I only would fare in such a comparison. Theoretically the $NADP^+$ photoreduction with the ascorbate 2,6-dichlorophenol-indophenol couple as electron donor as well as the PMS mediated cyclic photophosphorylation should remain unaffected by lack of manganese. Figure 7 sums up the results. The photoreduction of $NADP^+$ was indeed nearly unaffected by manganese deficiency. The photophosphorylating activity, on the other hand, was rather low in the deficient chloroplasts, with the exception of 1 preparation from spinach. No data on the $NADP^+$ photoreduction and phosphorylation of deficient *Cassia* chloroplasts are available at the present time, but one might expect them to be just as active in these reactions as they were found to be in CO_2 fixation or Hill reaction.

Effect of Manganese Deficiency on the Chloroplast Structure. Mercer et al. (17) have shown that the normal arrangement of the grana in spinach chloroplasts becomes severely disorganized as the result of manganese deficiency. We found the same disorganized structure in chloroplasts of deficient pokeweed (*Phytolacca americana*) and tobacco plants. The chlorophyll content of these chloroplasts from chlorotic leaves is certainly less than normal, because the number of chloroplasts per cell is about the same as in the control plant. The reverse is true for leaves from manganese deficient *Cassia* plants. They have much fewer chloroplasts per cell, but these chloroplasts look healthy, and on the average the orderly arranged grana have even more lamellar layers than the normal ones (fig 8). It appears as if *Cassia* plants try to adapt themselves to the lack of manganese by packing whatever traces of manganese are available into higher stacked grana of a few chloroplasts. This was verified by analyzing chloroplasts from clearly deficient *Cassia* plants colorimetrically for their manganese content. About one-half μg Mn per mg chlorophyll (10 μ atoms Mn per mmole chl) could be estimated as content of the little chloroplast material available for this determination. Of course, the chlorotic manganese deficient leaves never reach the same photosynthetic capacity per leaf area as the healthy leaves. This can be calculated easily from the data of figure 5.

Discussion

The results presented in this paper can be summarized as follows. First, they support the generally accepted idea that a special manganese fraction in the chloroplasts of green plants is essential for a fully functional photosystem II. Second, they show that there is no such thing as a normal pattern of deficiency symptoms in higher plants cultivated in absence of manganese. And third, they make clear that we are far from under-

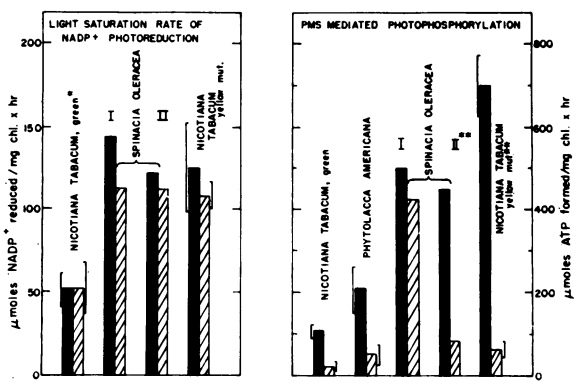

FIG. 7. Activity of chloroplasts in photoreactions involving photosystem I only. Black bars: controls; shaded bars: chloroplasts from manganese deficient plants. The same chloroplast preparations were used in both reactions shown in the graph. Left side: light saturation rates for photoreduction of $NADP^+$ in the presence of saturating (or, in one case(*), non-saturating) amounts of ferredoxin. Right side: Phenazine methosulfate mediated photophosphorylation determined either according to (11) at 40,000 lux, or according to (2) at 80,000 lux(**).

standing the steps involved in the reactivation of photosynthesis in manganese deficient algae.

Extensive studies on the role of manganese in green algae have recently been published by Cheniae and Martin (5) and by Kok and Cheniae (13) while this work was in progress. These authors report that light is necessary to reactivate photosynthesis in deficient algae. Our studies confirm this light requirement.

The time course of reactivation, however, was different in our experiments. Upon addition of manganese in the light, the rate of photosynthetic O_2 evolution increased steadily over a period of 3 hours in the deficient algae of Cheniae and Martin, whereas in this study the reactivation process was found to take only about 1 hour. Any attempts to explain these discrepancies can be only speculations. One can deduce from the data of Cheniae and Martin that these authors used saturating light intensities for the determination of the photosynthetic activity of their algae, but they give no information about the light intensity used during reactivation. In this study photosynthetic O_2 evolution during the reactivation process was followed continuously using a light intensity well below saturation. Therefore, the light intensity may be partly responsible for the differences mentioned above (see also fig 2). Other important factors may be the degree of deficiency of the algae (see, for example, 21), the temperature, and the nature of the suspension medium.

The mere uptake of manganese by algae, in contrast to the reactivation process, did not require light (table I). Hence, if the manganese which could not be removed from the algae with EDTA is not just bound in the cell wall, the light requiring step in the reactivation process must occur close to or within the chloroplast structure. It may then be related to changes of the lamellar structure in the chloroplast which occur upon transition from complete darkness to light (19). This can be assumed to influence the access of manganese to its binding site as well as the binding process itself. Indeed, the reactivation time was generally much shorter when manganese was added one hour after the light was switched on. It would be advantageous to study the reactivation process with cell free preparations of chloroplasts but so far normal oxygen evolution could not be restored in isolated manganese-deficient chloroplasts. Chloroplasts of algae are probably a better choice for in vitro reactivation experiments because manganese deficiency of higher plants induces such an unpredictable variety of changes in chloroplast structure and activity. An additional problem arises from the general lability of the oxygen evolving system in isolated chloroplasts or their fragments. Just about any manipulation of this material results in some loss of system II activity which, as a rule, does not go hand in hand with a release of manganese. In fact, at the present time there is no evidence that the usual deterioration of the activity of photosystem II in isolated chloroplasts results from a loss of a soluble cofactor. The more probable reason is the sensitivity of the lamellar structure. Schmid and Gaffron (23, 25) have presented evidence for the suggestion (31) that green plant photosynthesis can only occur either in grana or where at least 2 lamellae are closely packed. We have now some support for the contention that it is specifically system II which has this structural requirement (7). Such a strong dependence of photosynthetic O_2 evolution on a special lamellar structure ought to be contrasted with 1 of the least structure dependent photoreactions in photosynthesis, namely the ferredoxin mediated photoreduction of $NADP^+$ by photosystem I. This process occurred with high rates in manganese deficient chloroplasts even when the structural integrity was strongly disturbed (fig 7). On the other hand, the activity for cyclic photophosphorylation was nearly lost in the same manganese deficient chloroplasts, with the exception of 1 preparation of spinach chloroplasts which possibly suffered only from a very slight disorganization of their lamellar system. It may be, however, that the phosphorylating cyclic electron flow involves cofactors of photosystem II to a much greater extent than currently believed. This is also indicated by the observation that low concentrations of DCMU are not without effect on the photophosphorylation dependent glucose uptake by algae in far red light (32).

Acknowledgments

I am greatly indebted to Dr. H. Gaffron for many encouraging discussions during this work. I also thank Mrs. Diane M. Fass, Mrs. Susan S. Manley, and Mrs. Mary C. Fray for their excellent technical assistance during different stages of this work, and Mr. E. Walter Herold for building the chamber for the $^{14}CO_2$ incorporation studies.

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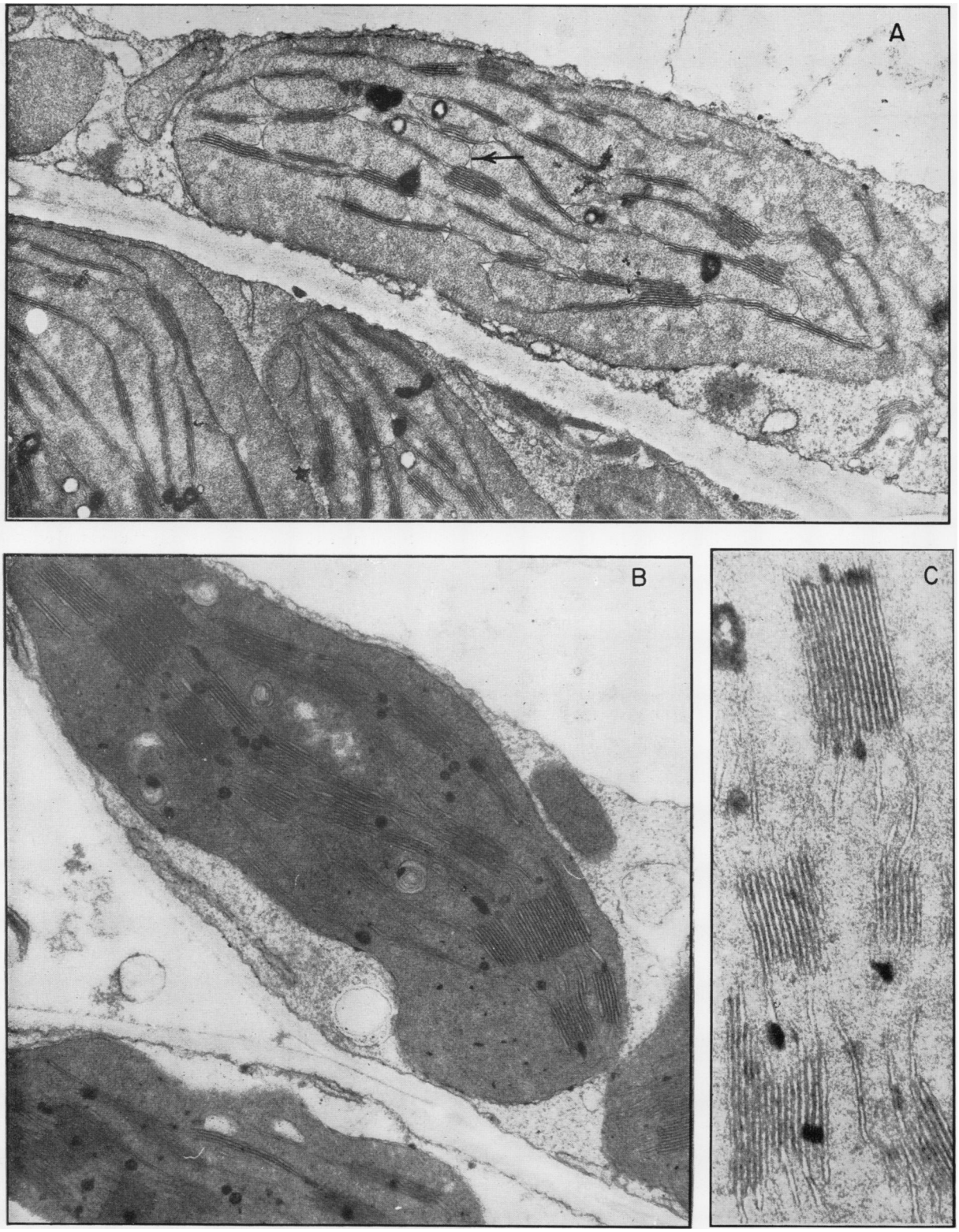

FIG. 8. Chloroplasts from *Cassia obtusifolia*: A) chloroplasts from a normal leaf with 37 μg chl/cm^2 ($\times$ 22,500) (the arrow indicates a hitherto undescribed type of secondary thylakoid multiplication). B) Chloroplasts from a Mn-deficient leaf with 13 μg chl/cm^2 and a photosynthetic saturation rate of 200 μmoles O_2 evolved/mg chl $\times$ hr ($\times$ 20,500). C) Part of another manganese deficient chloroplast from the same leaf at higher magnification ($\times$ 53,000).

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