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# STUDIES WITH MANGANESE-DEFICIENT SPINACH CHLOROPLASTS

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SUMMARY

1. Chloroplasts were isolated from $Mn^{2+}$-deficient spinach leaves and compared with chloroplasts from control plants grown for 14–19 days in water culture. The $Mn^{2+}$-deficient chloroplasts contained 25% of the $Mn^{2+}$ of control chloroplasts on a chlorophyll basis.

2. $Mn^{2+}$-deficient chloroplasts contained 1 P 700, 1 cytochrome *f*, 2 cytochromes $b_6$ and 2 cytochromes 559 per 400 or so chlorophylls. Thus $Mn^{2+}$ deficiency had not led to any modification of these components of the photosynthetic unit; in addition it caused no difference in carotenoid distribution.

3. The Hill activities of $Mn^{2+}$-deficient and control chloroplasts were saturated at the same light intensities; in the presence of an uncoupler, the light requirement was twice that required in its absence. The $P/e_2$ ratios were the same for both types of chloroplasts. Thus, $Mn^{2+}$ deficiency had not caused an uncoupling of photophosphorylation from electron flow.

4. Electron microscopy and freeze-etching of leaf sections showed that the lamellar structures of deficient and control chloroplasts were indistinguishable.

5. $Mn^{2+}$-deficient chloroplasts contained more chlorophyll *b* relative to chlorophyll *a* than control chloroplasts (chl *a*/chl *b*: control, 2.8; $Mn^{2+}$-deficient, 2.2). As $Mn^{2+}$ deficiency increased, there was a parallel decrease in Hill activity and in the chl *a*/chl *b* ratio. The addition of $Mn^{2+}$ and light to the deficient plants resulted in the restoration of Hill activity of isolated chloroplasts and the chl *a*/chl *b* ratio was restored to normal. It was not possible, however, to get a similar restoration of Hill activity by the addition of $Mn^{2+}$ and light to isolated deficient chloroplasts.

## INTRODUCTION

$Mn^{2+}$ is essential for oxygen evolution by plants and algae[1]. The nature of Photosystem 2, the oxygen-evolving system of photosynthesis, continues to be poorly understood; this is partly due to the extreme lability of this tightly lamellar-bound photosystem and also to the fact that most of its components are as yet hypothetical entities. The absolute requirement of $Mn^{2+}$ for a functional Photosystem 2 is well documented[1–6], though it is not certain whether $Mn^{2+}$ is involved directly as an electron carrier, or is rather exerting some secondary effect on the structure and/or function of this photosystem.

Abbreviations: PMS, phenazine methosulphate; TCIP, 2,3,6-trichlorophenolindophenol.

Recently we examined the fluorescence properties of chloroplasts isolated from $Mn^{2+}$-deficient spinach plants and chloroplasts isolated from non-deficient plants of the same age (referred to as control)[7]. This paper examines the chemical components, photochemical activities and the structure of the $Mn^{2+}$-deficient chloroplasts used in the fluorescence study. These chloroplasts contained only 25% of the $Mn^{2+}$ of control chloroplasts on a chlorophyll basis, yet there were no observable structural changes in their lamellar structure.

## MATERIALS AND METHODS

### *Plant material and preparation of chloroplasts*

Spinach (*Spinacia oleraceae* L.) plants were grown in water culture as described elsewhere[7]; the plants were either grown with the normal nutrient solution (control plants) or with $MnCl_2$-free nutrient solution ($Mn^{2+}$-deficient plants). Leaves, excluding the smaller and younger leaves, were harvested 14–19 days after the seedlings were transplanted into nutrient solutions.

Chloroplasts were prepared by hand-grinding of control or $Mn^{2+}$-deficient leaves in 0.05 M phosphate buffer (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl as described previously[7]. After the second centrifugation, the chloroplasts were resuspended in the above buffer; 0.66 ml buffer per g control leaves and 0.5 ml buffer per g $Mn^{2+}$-deficient leaves to give about 0.5 mg chlorophyll per ml suspension.

### *Trace metal and pigment determinations*

For trace metal determinations, the chloroplasts were prepared in phosphate buffer which had been purified by extraction with 8-hydroxyquinoline in chloroform to remove trace metals[8]. The chloroplasts were washed once in glass-distilled water, prior to lyophilization. The metal components were determined by atomic absorption spectrophotometry according to the procedure described by ANDERSON *et al.*[9].

Chloroplast suspensions were extracted with 80% acetone, the solution clarified by centrifugation and chlorophyll *a* and *b* concentrations were determined spectrophotometrically on the Cary Model 14 R using the simultaneous equations of ARNON[10]. For whole leaf determinations, 1 or 2 g (fresh wt.) were homogenized in 80% acetone in the Servall Omnimixer. The residue obtained on centrifugation was extracted twice with 80% acetone and the combined supernatants made up to 100 ml for chlorophyll determinations. Dry weights were obtained by heating leaf samples at 80° to constant weight. For carotenoid determinations the pigments were transferred from 80% acetone extracts to ether and saponified as described previously[11] and concentrations ($\mu$g/ml) were obtained by dividing the absorbance values at 442 nm by the factor, 0.24. The carotenoids were separated by thin-layer chromatography on Kieselgel G (Merck) using benzene–acetone (75:25, v/v) for development in the dark at 15°, as described elsewhere[11].

Difference spectra of reduced *minus* oxidized cytochromes were determined using chloroplasts without prior extraction of chlorophyll, and acetone-extracted powders of chloroplasts as described previously[11]. These spectra were recorded at 20°C and 77°K with a Cary Model 14R recording spectrophotometer fitted with a Cary Model 1462 scatter transmission attachment containing an RCA Type 6217 photomultiplier. A modified cuvette assembly of BONNER[12] was used; cuvettes had a path length

of 2 mm and a volume of 0.28 ml. Spectra at 77°K were measured in 50% glycerol using the single-freeze procedure[13].

P 700 was measured in the Cary Model 14R spectrophotometer fitted with the attachments mentioned above and the 0–0.1 slide wire was used. The chloroplast sample in 0.05 M phosphate buffer (pH 7.2) could contain up to 130 $\mu$g chlorophyll per ml. The reference cuvette contained $2 \cdot 10^{-3}$M $K_3Fe(CN)_6$ and the sample cuvette contained $2 \cdot 10^{-3}$M $K_4Fe(CN)_6$. The difference spectrum, measured from 750 to 690 nm had a peak at 702 nm, but the high chlorophyll concentration caused the spectrum to run off scale below 690 nm. A specific extinction coefficient of chlorophyll *a* in 80% acetone (82 $l \cdot g^{-1} \cdot cm^{-1}$) (ref. 14) was used to estimate P 700 at 702 nm This method appears to be satisfactory, since it was checked by using the fractions isolated from digitonin treatment of spinach chloroplasts, and similar results were obtained to those reported by ANDERSON *et al.*[15] where P 700 had been estimated from the light-induced absorbance change at 702 nm.

*Photochemical activities*

Photochemical reduction of $K_3Fe(CN)_6$ and 2,3,6-trichlorophenolindophenol (TCIP) were measured at 20° by recording the absorbance change at 420 nm (ferricyanide) and 610 nm (TCIP). In the standard assays, illumination of 40 000 lux was produced by a 250-W photoflood lamp, with the heat from the lamp being absorbed by a cell filled with water. Varying white-light intensities were obtained from a 250-W photoflood lamp operated at 190 V with the cuvette being positioned at selected distances from the lamp. Illumination intensity was measured with a Weston Meter, Model 603, calibrated in foot candles.

Photophosphorylation assays were performed aerobically at 20°; the illumination provided was about 120 000 lux for 2 min. Reactions were stopped by the addition of 0.3 ml of 20% trichloroacetic acid and aliquots were analysed for $^{32}P$ incorporation by the procedure of AVRON[16]. Bromine-saturated water (0.02 ml) was added to those samples which contained ferrocyanide. Ferrocyanide formation was determined in separate assays by the procedure of AVRON AND SHAVIT[17].

*Electron microscopy*

Pieces of leaf tissue (1 mm × 1 mm) were cut from the central part of one half of the leaf and fixed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature. The tissue was washed in buffer, post-fixed in 2% $OsO_4$ in 0.025 M sodium phosphate buffer (pH 7.2) for 2 h, washed, dehydrated in ethyl alcohol and propylene oxide and embedded in an Epon–Araldite mixture. Silver to gold sections were cut using a Reichart "Om U2" ultramicrotome, stained with uranyl acetate and Fiske's lead citrate and examined with a Philips EM 200 electron microscope.

For freeze-etch electron microscopy, pieces of leaf tissue were floated on a glycerol solution, frozen in Freon and liquid nitrogen and processed using a modified version of the freeze-fracture apparatus of BULLIVANT AND AMES[18]. Details of the modification will be presented elsewhere but it involved essentially the addition of a heating coil which made it possible to sublime ice from the surface exposed by the fracturing process.

## RESULTS

### *Effect of $Mn^{2+}$ deficiency on trace metal and pigment content*

*Manganese.* By atomic absorption analysis, an average value of 1 gatom of manganese per 75 moles of chlorophyll was obtained for control chloroplasts from spinach grown in water culture. This value is similar to data reported in the literature[1] which ranges from 50 to 100 chlorophylls for each manganese. More recently, CHENIAE AND MARTIN[6] using chemical analysis found an average of 1 $Mn^{2+}$ per 53 chlorophylls in greenhouse and locally grown spinach. The $Mn^{2+}$-deficient chloroplasts used in this study contained only 25–30% of the $Mn^{2+}$ of control chloroplasts on a chlorophyll basis; *i.e.* 1 $Mn^{2+}$ per 250–300 chlorophylls. However, the iron and copper contents of the deficient chloroplasts were essentially similar to those of control chloroplasts.

*Chlorophylls.* When the $Mn^{2+}$-deficient chloroplasts were 75% deficient in $Mn^{2+}$ on a chlorophyll basis, the chlorophyll content of deficient leaves was 65–70% of that of control leaves (Table I). As the degree of severity of the $Mn^{2+}$ deficiency is increased by growing the plants for a longer period in $Mn^{2+}$-free nutrient media, the chlorophyll content was more drastically reduced. There was a significant difference in the chl *a* per chl *b* ratio of the two types of chloroplasts; while control chloroplasts had a chl *a* per chl *b* ratio of 2.70–2.83, there was a pronounced enhancement of chlorophyll *b* relative to chlorophyll *a* in the $Mn^{2+}$-deficient ones (chl *a*/chl *b* 2.20–2.40). Using $Mn^{2+}$-deficient tomato plants, SPENCER AND POSSINGHAM[2] noted a very small but statistically significant difference in the chl *a*/chl *b* ratio (control leaves, 3.192; $Mn^{2+}$-deficient leaves, 2.823). With $Mn^{2+}$-deficient Scenedesmus, CHENIAE AND MARTIN[4] also found a slight increase in chlorophyll *b* relative to chlorophyll *a*.

The increased amount of chlorophyll *b* relative to chlorophyll *a* in $Mn^{2+}$-deficient chloroplasts was clearly visible in an absorption spectrum at 77° K which showed enhancement of the chlorophyll *b* bands *in vivo* at 650 and 470 nm. Fig. 1 shows a difference spectrum of $Mn^{2+}$-deficient chloroplasts *minus* control chloroplasts at 20°, the suspensions being matched to give equal absorbance at their red maxima, 678 nm. The main features of the difference spectrum are a peak at 650 nm, a trough at 686 nm and enhanced absorption at 434 and 475 nm. Although the positions of peaks and troughs do not necessarily correspond to true maxima of individual components, this spectrum can be interpreted as indicating that $Mn^{2+}$-deficient chloroplasts contain more chlorophyll *b* 650 and less chlorophyll *a* 683 than control chloroplasts. It is generally agreed that chlorophyll *a* exists in two major forms, chlorophyll *a* 683 and *a* 673

TABLE I

CHLOROPHYLL CONTENT OF CONTROL AND $Mn^{2+}$-DEFICIENT LEAVES

Chlorophyll from 19-day-old leaves was determined as described in MATERIALS AND METHODS.

| *Expt. No.* | *chl a/chl b* | | *Chlorophyll (μg/g leaf)* | |
|---|---|---|---|---|
| | *Control* | *$Mn^{2+}$-deficient* | *Control* | *$Mn^{2+}$-deficient* |
| 1 | 2.76 | 2.28 | 1057 | 737 |
| 2 | 2.81 | 2.18 | 964 | 649 |
| 3 | 2.83 | 2.30 | 1002 | 672 |

and from studies of action spectra it has been suggested that chlorophyll *a* 683 may provide energy for Photosystem 1 and chlorophyll *a* 673 for Photosystem 2 (ref. 19).

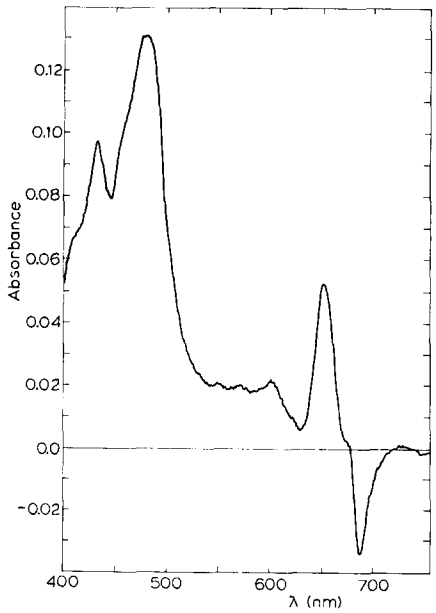


Fig. 1. Difference spectrum at 20° of $Mn^{2+}$-deficient chloroplasts (sample cuvette) *minus* control chloroplasts (reference cuvette). Chloroplasts were resuspended in 0.05 M phosphate buffer (pH 7.2) to give a chlorophyll content of 20 μg/ml. The chloroplasts were matched for equal absorbance at their red maxima, 678 nm.

The increase of absorption throughout the Soret band is probably partly due to increased scattering in $Mn^{2+}$-deficient chloroplasts; it was also seen in this region in the deficient spectrum at 77°K.

*Carotenoids.* A comparison of the chlorophyll and carotenoid content of control and $Mn^{2+}$-deficient leaves showed that the carotenoids were also reduced in $Mn^{2+}$-deficient leaves to about the same extent as the chlorophyll pigments (Table II). Thus, the total chlorophyll/carotenoid ratio was about the same for the two types of leaves as was the dry weight/fresh weight ratio. Although the carotenoid content was reduced in $Mn^{2+}$-deficient leaves, there was no significant difference in the carotenoid composition (Table III); this contrasts with the differences found in the chl *a* /chl *b* ratio.

TABLE II

CHLOROPHYLL AND CAROTENOID CONTENT OF CONTROL AND $Mn^{2+}$-DEFICIENT LEAVES

Pigments from 19-day-old leaves were determined as described in MATERIALS AND METHODS.

| *Leaves* | *Chlorophyll* (*mg/g dry wt.*) | $\frac{chl\ a}{chl\ b}$ | *Carotenoids* (*mg/g dry wt.*) | $\frac{chl\ a + chl\ b}{carotenoids}$ | $\frac{Dry\ wt.\ leaves}{Fresh\ wt.\ leaves}$ (%) |
|---|---|---|---|---|---|
| Control | 11.45 | 2.83 | 1.75 | 6.54 | 8.75 |
| $Mn^{2+}$-deficient | 7.25 | 2.30 | 1.15 | 6.30 | 9.00 |

*Cytochromes.* The cytochrome content of control and $Mn^{2+}$-deficient chloroplasts were calculated from room temperature difference spectra. Total cytochrome *b* (cytochrome $b_6$ and cytochrome 559) was estimated from the dithionite-reduced *minus* ferricyanide-oxidized spectrum (D − F) of chloroplasts after correcting for the absorption of cytochrome *f* (ref. 11). Cytochrome $b_6$ was estimated from the dithionite-reduced *minus* ascorbate-reduced difference spectrum (D − A) of chloroplasts. A difference molar extinction coefficient of $2.0 \cdot 10^4$ was used for cytochrome *b*. Cytochrome *f* was estimated from the ascorbate-reduced *minus* ferricyanide-oxidized spectrum (A − F) of acetone-extracted chloroplasts using a difference molar extinction coefficient of $2.5 \cdot 10^4$.

TABLE III

CAROTENOID COMPOSITION OF CONTROL AND Mn-DEFICIENT CHLOROPLASTS

Carotenoids were separated by thin-layer chromatography. Figures are averaged from 8 experiments.

| *Chloroplasts* | *Percentage composition of carotenoids* | | | |
|---|---|---|---|---|
| | *β-Carotene* | *Lutein* | *Violaxanthin* | *Neoxanthin* |
| Control | 20 | 43 | 20 | 16 |
| $Mn^{2+}$-deficient | 19 | 41 | 24 | 16 |

TABLE IV

MOLAR RATIOS OF CHLOROPHYLL/CYTOCHROME AND CHLOROPHYLL/P 700 FOR CONTROL AND Mn-DEFICIENT CHLOROPLASTS

Cytochrome and P 700 difference spectra were determined as described in MATERIALS AND METHODS. Values are average of four separate determinations.

| *Chloroplasts* | $\frac{chl\ a}{chl\ b}$ | $\frac{chl\ a + chl\ b}{cyt\ b}$ | $\frac{chl\ a + chl\ b}{cyt\ f}$ | $\frac{cyt\ b}{cyt\ f}$ | $\frac{cyt\ b_6}{cyt\ 559}$ | $\frac{chl\ a + chl\ b}{P\ 700}$ |
|---|---|---|---|---|---|---|
| Control | 2.89 | 83 | 350 | 4.2 | 0.87 | 440 |
| $Mn^{2+}$-deficient | 2.52 | 89 | 360 | 4.0 | 0.96 | 460 |

The total cytochrome content of $Mn^{2+}$-deficient leaves was only 65–70% of control leaves, which is comparable to the reduced levels of both total chlorophylls and carotenoids. The molar ratios of chl/cyt *b* and chl/cyt *f* of $Mn^{2+}$-deficient chloroplasts were the same as those of control chloroplasts; similarly, the molar ratios of cyt *b* per cyt *f* and cyt $b_6$/cyt 559 were identical. Previously, we identified in spinach chloroplasts[11], cytochrome *f* and cytochrome $b_6$ with α bands at 554 and 563 nm and cytochrome 559 with an α-band at 559 nm at 20° C and 557 nm at 77°K: these are present in the approximate molar ratio of 1:2:2, respectively. Cytochrome 559 is firmly associated with Photosystem 2 particles[11], so it interesting that $Mn^{2+}$ deficiency has not caused a reduction in this cytochrome. Furthermore, reduced *minus* oxidized difference spectra of both types of chloroplasts at 77°K were similar, confirming that the cytochrome distribution has not been altered by $Mn^{2+}$ deficiency.

*P 700.* $Mn^{2+}$-deficient chloroplasts contained 1 P 700/460 chl which is similar to the ratio found in control chloroplasts (Table IV). Since P 700 is the reaction centre

chlorophyll of Photosystem I it would be excepted that Mn deficiency had not altered the P 700/chl ratio, because Photosystem I activities are unaltered by $Mn^2$ deficiency.

*Photochemical activities*

It is well established that $Mn^{2+}$-deficient chloroplasts have decreased Hill-reaction activities as compared to control chloroplasts, with the reduction in activity

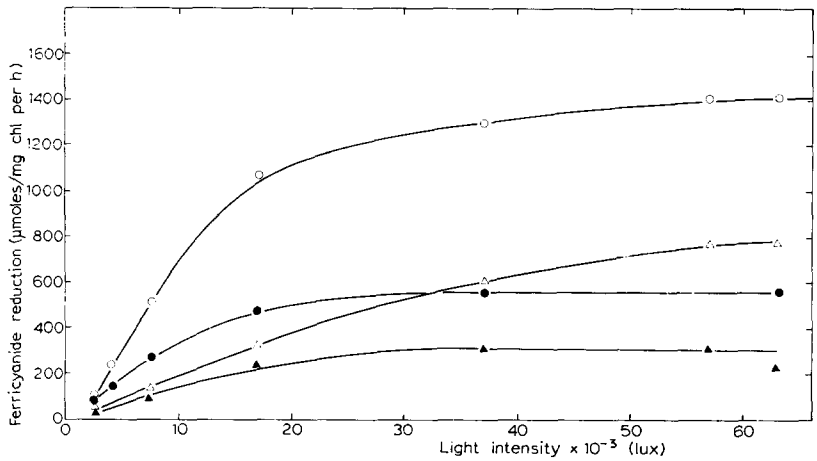


Fig. 2. Effect of light intensity on the rate of reduction of $K_3 Fe(CN)_6$ by control and $Mn^{2+}$-deficient chloroplasts in the absence and presence of methylamine·HCl: ●——●, control without methylamine·HCl; ▲——▲, $Mn^{2+}$-deficient without methylamine·HCl; ○——○, control with methylamine·HCl; △——△, $Mn^{2+}$-deficient with methylamine·HCl. The reaction mixture contained in 3 ml, chloroplasts equivalent to 25 μg chlorophyll and the following in μmoles: Tris (pH 8.0), 40; NaCl, 70; $MgCl_2$, 10; $K_3 Fe(CN)_6$, 1.5, and if added, methylamine·HCl, 100.

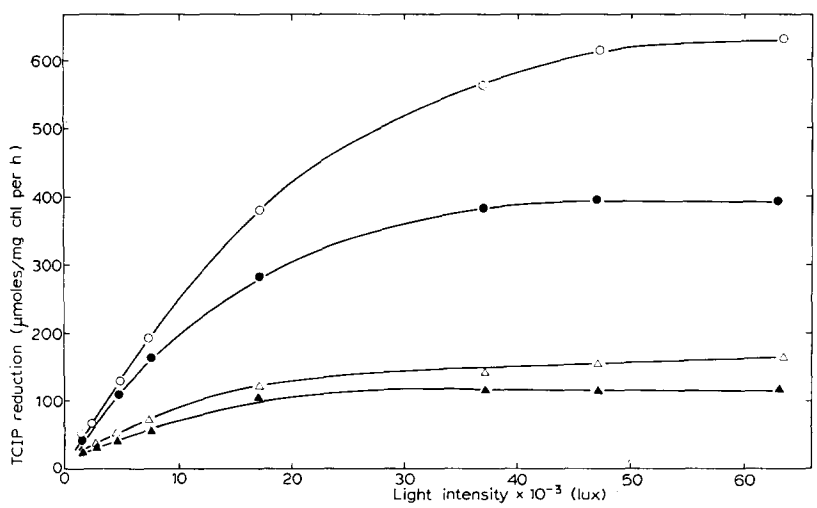


Fig. 3. Effect of light intensity on the rate of TCIP reduction by control and $Mn^{2+}$-deficient chloroplasts in the absence and presence of methylamine·HCl: ●——●, control without methylamine·HCl; ▲——▲, $Mn^{2+}$-deficient without methylamine·HCl; ○——○, control with methylamine·HCl; △——△, $Mn^{2+}$-deficient with methylamine·HCl. The reaction mixture contained in 3 ml, chloroplasts equivalent to 8 μg chlorophyll and the following in μmoles: Tris (pH 7.8), 40; KCl, 80; TCIP, 0.08, and if added, methylamine·HCl, 30.

being correlated with the degree of $Mn^{2+}$ deficiency[2,4,5]. Figs. 2 and 3 show the effect of white light intensity on Hill-reaction activities of control and $Mn^{2+}$-deficient chloroplasts. For ferricyanide reduction, the saturating light intensity for both types of chloroplasts was about 30 000 lux (Fig. 2). At saturation, control chloroplasts reduced ferricyanide at the rate of 540 $\mu$moles/mg chlorophyll per h compared to 358 $\mu$moles/mg chlorophyll per h for $Mn^{2+}$-deficient chloroplasts. In the presence of the uncoupler, methylamine·HCl, the saturation point was again similar for both sorts of chloroplasts, but as would be expected a higher light intensity, about 60 000 lux, was required. $Mn^{2+}$-deficient chloroplasts were only about 50 % as active as control chloroplasts under uncoupled conditions.

As observed with ferricyanide reduction, the saturating light intensities for TCIP reduction were the same for control and $Mn^{2+}$-deficient chloroplasts (Fig. 3). In the absence of the uncoupler, TCIP reductions were saturated at about 37 000 lux while in the presence of the uncoupler, about 70 000 lux was required. $Mn^{2+}$-deficient chloroplasts were only about 30% as active as control ones for TCIP reduction, both in the presence and absence of the uncoupler.

The similar requirements of both types of chloroplasts for saturation of ferricyanide and TCIP reductions in the absence of uncoupler, suggest that the deficient chloroplasts are not significantly more uncoupled than control chloroplasts, since uncoupled systems be expected to require higher light intensities for saturation. Moreover, $Mn^{2+}$-deficient chloroplasts possessed higher rates of Hill activities under uncoup-

TABLE V

PHOTOPHOSPHORYLATION AND FERRICYANIDE REDUCTION BY CONTROL AND $Mn^{2+}$-DEFICIENT CHLOROPLASTS

The reaction mixture contained in 3 ml, chloroplasts equivalent to 50–60 $\mu$g chlorophyll and the following in $\mu$moles: Tris–HCl (pH 7.8, 45; NaCl, 60; $MgCl_2$, 12; ADP, 6; sodium, potassium phosphate, 12; $^{32}P_i$ ($5 \cdot 10^4$–$5 \cdot 10^6$ counts/min) and either PMS, 0.08 or $K_3Fe(CN)_6$, 5. Reaction conditions and assay procedure are described in MATERIALS AND METHODS.

| *Expt. No.* | *Chloroplasts* | *Photophosphorylation ($\mu$moles ATP per mg chl per h)* | | *Ferricyanide reduction ($\mu$moles/mg chl per h)* | $P/e_2$ |
|---|---|---|---|---|---|
| | | *PMS* | $K_3Fe(CN)_6$ | | |
| 1 | Control | | 382 | 828 | 0.92 |
| | $Mn^{2+}$-deficient | | 264 | 526 | 1.00 |
| | $\frac{Mn^{2+}\text{-deficient}}{\text{Control}} \times 100$ | | 69 | 63 | |
| 2 | Control | 441 | 538 | 964 | 1.11 |
| | $Mn^{2+}$-deficient | 406 | 208 | 446 | 0.93 |
| | $\frac{Mn^{2+}\text{-deficient}}{\text{Control}} \times 100$ | 92 | 39 | 46 | |
| 3 | Control | 640 | 342 | 754 | 0.91 |
| | $Mn^{2+}$-deficient | 528 | 123 | 222 | 1.11 |
| | $\frac{Mn^{2+}\text{-deficient}}{\text{Control}} \times 100$ | 83 | 36 | 29 | |

ling conditions, although the increase caused by the addition of the uncoupler was not as much as that exhibited by control chloroplasts: with ferricyanide reduction, the uncoupled rate was 2.5 times greater for control and 2.0 times greater for $Mn^{2+}$-deficient chloroplasts, likewise for TCIP reduction, the uncoupled rate was 1.7 times greater for control and 1.4 times greater for $Mn^{2+}$-deficient chloroplasts. The lower increase caused by uncoupling in the case of TCIP reduction may be due to the uncoupling action of TCIP itself[20]. It should be stressed that Hill activities in the absence of uncoupler are being measured under non-phosphorylating conditions (no ADP or $P_i$ being present) and therefore do not represent coupled electron transport.

As shown in Table V, the rates of cyclic photophosphorylation exhibited by the two types of chloroplasts were similar in agreement with previous studies[3]. In contrast to cyclic photophosphorylation, non-cyclic photophosphorylation with ferricyanide as the electron acceptor was significantly reduced by $Mn^{2+}$-deficiency (Table V). Similarly SPENCER AND POSSINGHAM[3] showed that $Mn^{2+}$ deficiency in spinach caused a reduction in FMN-mediated photophosphorylation.

The rates of ferricyanide reduction and of ferricyanide-mediated photophosphorylation exhibited by $Mn^{2+}$-deficient chloroplasts were inhibited to the same extent by $Mn^{2+}$ deficiency (Table V). The $P/e_2$ ratios varied from 0.8 to 1.1 (11 experiments), but in all cases there was no significant difference between the ratios obtained from control and $Mn^{2+}$-deficient chloroplasts, regardless of the degree of $Mn^{2+}$ deficiency as shown by the reduction of Hill activity.

*Chloroplast structure*

There were no apparent differences in the size, shape and distribution of the chloroplasts in the mesophyll cells of control and $Mn^{2+}$-deficient leaves selected for this study. Examination by phase contrast and fluorescence light microscopy showed that both types of isolated chloroplasts were similar. Representative electron micrographs of control and $Mn^{2+}$-deficient chloroplasts are shown in Fig. 4. The lamellar structure of $Mn^{2+}$-deficient chloroplasts did not appear to be disorganized in comparison with control chloroplasts. A statistical analysis similar to that reported by GOODCHILD *et al.*[21] did not indicate any highly significant differences in either the number of grana per chloroplast or lamellae per granum for the two types of chloroplasts. No differences were seen in the shape, distribution and presence of starch grains (the starch being dependent on the time of harvesting the leaves in both cases). Osmiophillic granules, ribosomes and fibril-containing areas were also present in both chloroplasts. Non-membrane bound crystals with an average lattice spacing of 110 Å, similar to those described by PERNER[22], were occasionally seen in both cases.

Freeze-etch electron microscopy visualized the 175 and 110 Å subunits reported by BRANTON AND PARK[23] and showed no differences between the two types of chloroplast.

The effect of $Mn^{2+}$ deficiency on chloroplast structure has given conflicting results; as pointed out by CHENIAE AND MARTIN[6] a direct comparison between different plant materials is impossible unless made on some comparable basis such as the ratio of bound $Mn^{2+}$/chl. POSSINGHAM *et al.*[24] showed a progressive increase in chloroplast disorganization from the oldest leaf to the youngest leaf of 26-day-old $Mn^{2+}$-deficient spinach; at this stage all chloroplasts were showing some signs of structural

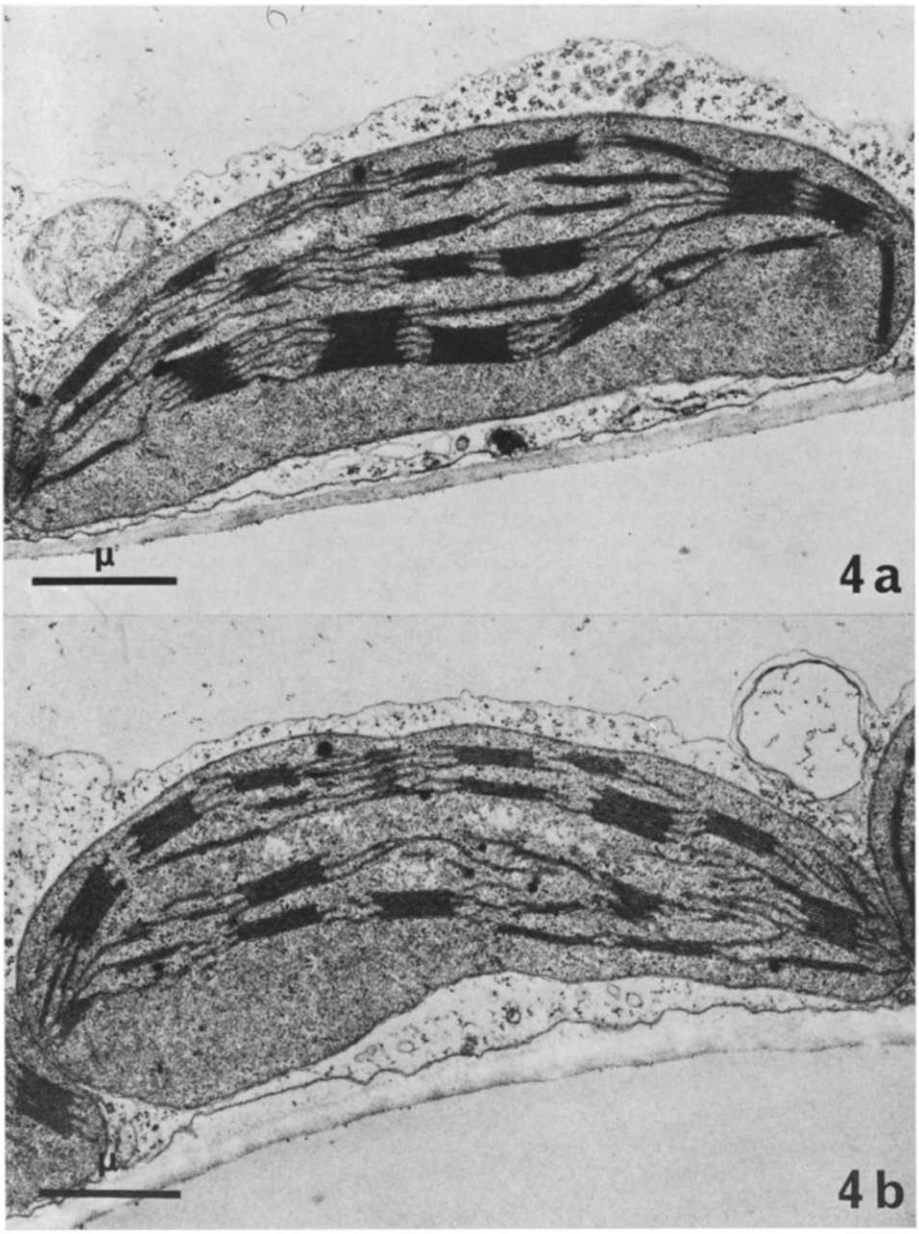


Fig. 4. Electron micrographs of control (a) and $Mn^{2+}$-deficient (b) chloroplasts.

disorganization (no Mn/chl ratios were reported). HOMANN[5] states that $Mn^{2+}$-deficient tobacco and pokeweed chloroplasts showed lamellar disorder but chloroplasts from $Mn^{2+}$-deficient Cassia were, if anything, more ordered than those of the control. CHENIAE AND MARTIN[6] showed no difference in the chloroplast structure of moderately $Mn^{2+}$-deficient Scenedesmus.

*Effect of the addition of $Mn^{2+}$ to spinach plants and chloroplasts*

Plants were grown with varying amounts of $Mn^{2+}$ included in the nutrient media applied at transplanting of seedlings, and the leaves were harvested at the same age as those routinely used in this study. Chloroplasts, isolated from plants grown in nutrient media containing 15, 30, 45, 60, 75 and 90% of the $Mn^{2+}$ used for control plants, showed no signs of $Mn^{2+}$ deficiency: both Hill activities and chl *a*/chl *b* ratios were the same as those of control chloroplasts. However, an effect was observed in the range of 0–10% of the normal $Mn^{2+}$ concentration (Table VI). Chloroplasts isolated from leaves harvested at Day 17 showed a range of chl *a*/chl *b* ratios from 2.91 to 2.30. After 2 additional days of growth, the chl *a*/chl *b* ratios had declined further. Similarly, the Hill activities of the isolated chloroplasts decreased both with limiting amounts of manganese and with age. Thus, there exists a parallel relationship between chl *a*/chl *b* ratios and Hill activities.

TABLE VI

Chl *a*/Chl *b* RATIOS AND HILL ACTIVITIES OF CHLOROPLASTS WITH VARYING DEGREES OF $Mn^{2+}$-DEFICIENCY

Chloroplasts were isolated from plants grown for 17 and 19 days after transplanting into nutrient media containing varying $Mn^{2+}$ concentrations: Control, 100% $Mn^{2+}$; 10% $Mn^{2+}$; 3.3% $Mn^{2+}$; $Mn^{2+}$-deficient; 0% $Mn^{2+}$. Hill activities were assayed at saturating light intensity as described in Figs. 2 and 3.

| *Chloroplasts* | *Chl a/Chl b* | | *Ferricyanide reduction (% of control)* | | *TCIP reduction (% of control)* | |
|---|---|---|---|---|---|---|
| | *17 days* | *19 days* | *17 days* | *19 days* | *17 days* | *19 days* |
| Control | 2.91 | 2.78 | 100 | 100 | 100 | 100 |
| 10% $Mn^{2+}$ | 2.70 | 2.58 | 88 | 57 | 90 | 52 |
| 3.3% $Mn^{2+}$ | 2.55 | 2.40 | 70 | 37 | 70 | 43 |
| $Mn^{2+}$-deficient | 2.30 | 2.19 | 60 | 32 | 53 | 34 |

The effect of the addition of excess $Mn^{2+}$ to the nutrient medium of 17-day-old deficient plants was investigated also. Chloroplasts isolated from these plants 1 day after the addition of $Mn^{2+}$, had Hill activities about 65% those of control and the chl *a* per chl *b* ratio had increased from 2.21 to 2.56. After 2 days with $Mn^{2+}$-containing media, the chloroplasts had the normal chl *a*/chl *b* ratio and the Hill activity was restored to 100%. Light was necessary for this recovery: if the $Mn^{2+}$-deficient plants given additional $Mn^{2+}$ were kept in the dark they remained deficient and no changes were observed in chl *a*/chl *b* ratios or Hill activities. CHENIAE AND MARTIN[25] obtained a very fast recovery (few minutes) of photosynthesis with $Mn^{2+}$-deficient cells of Anacystis which was dependent on $Mn^{2+}$ and light.

Attempts to restore the activity of isolated $Mn^{2+}$-deficient chloroplasts by the addition of $Mn^{2+}$ and light failed. Chloroplasts were isolated by methods which lead to preparations of chloroplasts with intact membranes (Class I chloroplasts as defined by SPENCER AND UNT[26]); these included the procedure of JENSEN AND BASSHAM[27] and SPENCER AND WILDMAN[28]. The addition of bovine serum albumin to various media was also tried[29]. It appears likely that reactivation *in vitro* will be difficult to accomplish with deficient chloroplasts.

## DISCUSSION

$Mn^{2+}$-deficiency studies with higher plants involve long-term growth experiments under sub-optimal conditions which may result in secondary effects such as structural disorganization of the chloroplasts and the lack or modification of components of the photosynthetic unit other than the primary loss of $Mn^{2+}$. Even with photosynthetic mutants, which in themselves are less equivocal in that there may be 100% loss of a component, there also may be secondary effects. Algae offer an advantage over higher plants in that highly deficient algae (*e.g.* Scenedesmus) may be obtained relatively quickly (48 h) after inoculation of normal algae into a $Mn^{2+}$-free medium[6].

Recently other ways of obtaining the release of $Mn^{2+}$ from chloroplasts have been found:

(i) 10-min incubation of chloroplasts in 0.8 M Tris–HCl (pH 8.0) [30] and (ii) heat treatment of leaves for 5–10 min at 40–50°, prior to the isolation of chloroplasts[31]. Both of these methods speedily deplete chloroplasts of at least 70% of their $Mn^{2+}$ (ref. 31) and thus minimize the possible secondary effects which may be elicited by long-term growth experiments. However, with these latter two methods, manganese may not be the only component released from chloroplasts; proteins might easily be lost or denatured and there may be some structural deformation. Thus, $Mn^{2+}$-removal procedures are not necessarily better than growth methods for inducing deficiency, even though the time factor has been greatly reduced.

Our $Mn^{2+}$-deficient chloroplasts possess the normal components of the photosynthetic unit. The photosynthetic unit is defined here as the minimum number of chlorophyll molecules necessary for the transport of a single electron from $OH^-$ to $NADP^+$, rather than the original definition of EMERSON AND ARNOLD[32] which was based on the minimum number of chlorophyll molecules required for the evolution of 1 molecule of $O_2$ or the fixation of 1 molecule of $CO_2$. KOK AND HOCH[33] found 1 P 700 for every 400 or so chlorophyll molecules. Spinach chloroplasts possess 1 cytochrome *f* per 400 chlorophylls confirming that the photosynthetic unit contains about 400 chlorophylls; in addition it contains 2 moles each of cytochrome $b_6$ and cytochrome 559 (ref. 11). Since the photosynthetic units of $Mn^{2+}$-deficient chloroplasts contain 1 P 700, 1 cytochrome *f*, 2 cytochrome $b_6$ and 2 cytochrome 559 for 400 or so chlorophylls, there is no indication that lack of $Mn^{2+}$ has modified these components of the photosynthetic unit. Moreover, the moles of carotenoids per photosynthetic unit are the same for $Mn^{2+}$-deficient chloroplasts.

It is concluded that $Mn^{2+}$-deficient chloroplasts are no more uncoupled than control chloroplasts, since the Hill-reaction activities of both types of chloroplasts were saturated at the same light intensities, and twice the light intensity was required for saturation in the presence of an uncoupler as compared to its absence. This conclusion is strengthened since both control and $Mn^{2+}$-deficient chloroplasts had the same $P/e_2$ ratios. This is an interesting observation, because it implies that manganese is acting at a site in the electron transport chain of Photosystem 2 which differs from the site which is directly concerned with the actual energy transfer reactions leading to photophosphorylation.

Our study has shown that it is possible to obtain $Mn^{2+}$-deficient spinach chloroplasts which are 70–75% deficient in $Mn^{2+}$-on a chlorophyll basis, and yet which show no observable differences in lamellar structure. It is concluded therefore that $Mn^{2+}$

does not exert a primary effect on structure *per se*. A similar conclusion was reached by CHENIAE AND MARTIN[6] with $Mn^{2+}$-deficient Scenedesmus. However, prolonged growth in the absence of $Mn^{2+}$ will result in severe alterations of spinach chloroplast structure[24].

It would seem reasonable to postulate that the loss in $Mn^{2+}$-deficient leaves of some 30% of chlorophylls, carotenoids and cytochromes of control leaves on a fresh weight basis might result either in altered lamellar structures or in a decrease the actual number of chloroplasts per leaf of $Mn^{2+}$-deficient plants. In grana-containing chloroplasts, the pigments appear to be mainly confined to the granal areas and their loss might preferentially influence the structure of the grana. Since the $Mn^{2+}$-deficient chloroplasts did not possess an altered lamellar structure it is suggested that the number of chloroplasts per leaf may be lowered. This may result from $Mn^{2+}$ deficiency inhibiting chloroplast formation. $Mn^{2+}$-deficient spinach plants showing mild chlorosis contained a reduced number of chloroplasts per leaf ($Mn^{2+}$/chl ratio was not reported) [34].

$Mn^{2+}$-deficient chloroplasts showed one abnormal feature; they contained more chlorophyll *b* relative to chlorophyll *a*. As $Mn^{2+}$ deficiency increased, there was a parallel decline in Hill activities and chl *a*/chl *b* ratios; similarly, the addition of $Mn^{2+}$ to deficient plants resulted in an increase in Hill activity and chl *a*/chl *b* ratio. Thus the chl *a*/chl *b* ratio is an index of $Mn^{2+}$ deficiency. The question may be asked: why has $Mn^{2+}$ deficiency caused an alteration in the chl *a*/chl *b* ratio? From studies on the aerobic photobleaching of chlorophylls in spinach quantosomes[35] and Aspidistra chloroplast[36] if has been shown that chlorophyll *a* 683 is more susceptible to photobleaching than chlorophyll *a* 673, while chlorophyll *b* 650 is relatively resistant. The addition of $K_3Fe(CN)_6$ to spinach chloroplasts caused the main absorption band of chlorophyll *a* *in vivo* at 678 nm to be shifted to shorter wavelengths, whereas there was little alteration in chlorophyll *b* absorption at 650 nm (ref. 37). Therefore chlorophyll *a* *in vivo* is more susceptible to aerobic photobleaching and to oxidation than chlorophyll *b* *in vivo*. It is suggested that there has been preferential destruction of chlorophyll *a* under conditions of $Mn^{2+}$ deficiency, and that this alteration in the chlorophyll distribution is a direct consequence of the $Mn^{2+}$ deficiency. It is interesting to note that we have found that spinach chloroplasts which were deprived of some 70% of their $Mn^{2+}$ by the two methods listed earlier[30,31], also possess lower chl *a*/chl *b* ratios.

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