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THE FLUORESCENCE PROPERTIES OF MANGANESE-DEFICIENT SPINACH CHLOROPLASTS

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SUMMARY

The fluorescence properties of chloroplasts from Mn-deficient and control spinach plants were studied at 20°C and 77°K, using light of weak intensities for excitation.

1. At 20°C, the quantum yields of fluorescence of the Mn-deficient chloroplasts exceeded those of the control; at 77°K the position was reversed.

2. A comparison of the induction of fluorescence intensity at 20°C showed that the increase from the initial fluorescence to the final steady-state was much lower with the Mn-deficient chloroplasts and they showed a faster induction rate.

3. The fluorescence yields of Mn-deficient chloroplasts in the presence and absence of a Hill oxidant were comparable, whereas with control chloroplasts the fluorescence yield was lower under oxidizing conditions. The fluorescence yields of both types of chloroplasts under reducing conditions were similar.

4. At 77°K, the 693-mμ fluorescence emission band was absent from the Mn-deficient spectrum. Some 80% of the total fluorescence was emitted at 735 mμ for the Mn-deficient chloroplasts as compared to 70% for the control.

5. The effect of DCMU on fluorescence yields was similar to Mn deficiency. At 77°K with DCMU-inhibited chloroplasts, the 683-mμ and 693-mμ emission bands relative to 735 mμ were lower, although not as low as that of Mn-deficient ones; some 74% of the total fluorescence was emitted at 735 mμ.

6. It appears that Mn deficiency causes a block between the primary and secondary reductants of Photosystem 2.

INTRODUCTION

There is a considerable evidence to show that the micronutrient, Mn, is required for photosynthesis in plants and algae[1-6]. With the demonstration of the two light reactions of photosynthesis, it was possible to demonstrate that Mn was essential for Photosystem 2, *i.e.* the oxygen-evolving sequence (for review see KOK AND CHENIAE[7]). However, the biochemical nature of the tightly lamellar-bound Photosystem 2 is proving extremely difficult to elucidate. It has not been demonstrated yet whether the Mn is involved directly in primary events associated with electron transport, or

Abbreviations: chl, chlorophyll; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

is merely exerting some secondary effect on the structure or function of this Photosystem.

Since fluorescence emission arises primarily from Photosystem 2, at least at room temperature[8,9], it may be used to study primary events in this Photosystem. A number of hypothetical intermediates involved in electron transport in Photosystem 2 have been deduced from such fluorescence studies[7]. Therefore, in an attempt to gain some insight into the function of Mn, the fluorescence properties of chloroplasts isolated from Mn-deficient spinach plants and chloroplasts isolated from non-deficient plants of the same age (referred to in this paper as control) were examined at room temperature and that of liquid nitrogen. As expected, the fluorescence properties of the Mn-deficient chloroplasts were considerably modified, and this paper reports their characteristics. The results obtained are consistent with our proposal that manganese deficiency causes a block in electron flow between the primary and secondary reductants of Photosystem 2.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L.) plants were grown in water culture in a glasshouse maintained at 20° by day and 15° by night. Seeds were germinated in vermiculite moistened with glass-distilled water for 10 days and the seedlings were transferred to nutrient solutions of the composition described by TSUI[10]. The solutions which were made with glass-distilled water, had been purified by prior extraction with 8-hydroxyquinoline in chloroform to remove trace metals according to the method of GENTRY AND SHERRINGTON[11]. The plants were either grown with the usual nutrient solution (control plants) or with nutrient solution which contained no $MnCl_2$ (Mn-deficient plants). Each beaker (3 l) held 8 plants and the solutions, which were aerated continuously, were not changed during the growing period; glass-distilled water was added daily to fill the beakers. The leaves were harvested between 14 and 19 days (average length of leaves: control, 12 cm; Mn-deficient, 8 cm).

Chloroplasts from either control or Mn-deficient plants were prepared by gentle grinding of leaves (3–5 g) in a chilled mortar with 9–15 ml of 0.05 M phosphate buffer (pH 7.2), containing 0.01 M KCl and 0.3 M sucrose. The brei was filtered through a double layer of moistened Miracloth (Chicopee Mills Inc., New York) and centrifuged at 1000 × *g* for 10 min. The chloroplasts were then resuspended in 10 ml of buffer and centrifuged for a further 10 min at 1000 × *g* and resuspended in the same buffer; 0.66 ml buffer per g control leaves and 0.5 ml buffer per g Mn-deficient leaves to give about 0.5 mg chlorophyll per ml suspension. Chlorophyll concentrations were determined spectrophotometrically in 80 % acetone using the equations of ARNON[12].

Fluorescence emission and excitation spectra were recorded with a fluorescence spectrophotometer incorporating automatic correction for photomultiplier and monochromatic responses and variation in the output of the light source, as described previously by BOARDMAN, THORNE AND ANDERSON[9] and BOARDMAN AND THORNE[13]. Fluorescence quantum efficiency (ϕ) was determined by an extension of the relative method[14]. Fluorescein in 0.1 M NaOH at 1 μM was used for the standard with an absolute value for quantum efficiency of 0.92 at 20° (ref. 15).

For fluorescence measurements, the chloroplasts were diluted to an absorbance of 0.2 at the desired excitation wavelength for measurements at 20°C, and to an

absorbance of 0.1 for measurements at 77° K. Fluorescence yields were determined at room temperature in 0.05 M phosphate (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl, and at 77° K in 0.05 M phosphate buffer (pH 7.2) containing 62 % glycerol. The timecourse of fluorescence intensification was measured at 683 mμ, the maximum of the fluorescence emission spectrum at 20° C. Fluorescence intensity was recorded as a function of time on a recorder (Rikadenki, Japan, Model B-34; full scale response time < 0.5 sec) operated normally at a speed of 80 mm/min.

RESULTS

Fluorescence yields at 20° C

The steady-state quantum yields of fluorescence (ϕ) of control and Mn-deficient spinach chloroplasts excited at a number of wavelengths and at two levels of light intensity are compared in Table I. Regardless of the excitation wavelength used, the Mn-deficient chloroplasts were more fluorescent than the control chloroplasts at the lower light intensity as shown by the high $\phi_{\text{deficient}}/\phi_{\text{control}}$ ratio. The effect of increasing the light intensity resulted in a more marked increase in the fluorescence yield of control chloroplasts as compared to Mn-deficient ones; thus with a 10-fold increase of light intensity, the ratio $\phi_{\text{deficient}}/\phi_{\text{control}}$ was lower, but still greater than unity. The fluorescence yield of the Mn-deficient relative to the control chloroplasts was greatest at 650 mμ (absorption *in vivo* of chl *b*) and least at 670 mμ (absorption *in vivo* of chl *a*). This is due to the enhanced chlorophyll *b* relative to chlorophyll *a* in Mn-deficient (chl *a*/chl *b*, 2.2) as compared to control chloroplasts (chl *a*/chl *b*, 2.8). There was little difference however, between excitation at 436 mμ (absorption *in vivo* for chl *a*) and 470 mμ (absorption *in vivo* for chl *b*). A difference spectrum of Mn-deficient chloroplasts *minus* control chloroplasts showed that the absorption *in vivo* of Mn-deficient chloroplasts, at both 436 and 470 mμ exceeded that of control chloroplasts. Despite the differences in the amounts of extracted chlorophyll *a* and chlorophyll *b* from the two types of chloroplasts, it appears that their pigments systems must be roughly balanced in the region of 400–500 mμ.

TABLE I

STEADY-STATE QUANTUM YIELDS OF FLUORESCENCE OF CONTROL AND Mn-DEFICIENT CHLOROPLASTS AT 20° C

Chloroplasts isolated from control and Mn-deficient spinach by hand grinding, were resuspended in 0.05 M phosphate buffer (pH 7.2) containing 0.3 M sucrose and 0.01 M KCl. The chloroplasts (in vol. 3.5 ml) were diluted to an absorbance of 0.2 at each particular wavelength of excitation. Light intensities (I) were either $I \times 1$ or $I \times 10$, with $I_{436} = 90$ erg·cm^{-2}·sec^{-1} and $I_{470} = 130$ erg·cm^{-2}·sec^{-1}, $I_{650} = 170$ erg·cm^{-2}·sec^{-1} and $I_{670} = 140$ erg·cm^{-2}·sec^{-1}. The bandwidth of excitation at $I \times 1$ was ± 1 mμ and at $I \times 10$, ± 6.0 mμ respectively.

Excitation wavelength (mμ)	*Quantum yield* (ϕ) *at light intensity* $I \times 1$			*Quantum yield* (ϕ) *at light intensity* $I \times 10$		
	Control	*Deficient*	$\frac{\phi_{Deficient}}{\phi_{Control}}$	*Control*	*Deficient*	$\frac{\phi_{Deficient}}{\phi_{Control}}$
436	0.0093	0.0186	2.00	0.0245	0.0280	1.14
470	0.0107	0.0230	2.15	0.0306	0.0306	1.18
650	0.0060	0.0170	2.83	0.0240	0.0350	1.45
670	0.0088	0.0130	1.47	0.0240	0.0300	1.25

The differential effect of light intensity on fluorescence yields of Mn-deficient and control chloroplasts was demonstrated by fluorescence induction studies. Figs. 1 and 2 compare the kinetics of fluorescence increase at 683 mμ by control and Mn-deficient chloroplasts at two light intensities, with an excitation wavelength of 436 mμ. Fluorescence shows changes of intensity, F, with time of illumination. There is a rise from an initial low level observed immediately after illumination, F_0, to a steady-state level, F_∞, as shown in Fig. 1a. MALKIN AND KOK[16] have shown that the shape of the curve depends on the light intensity; at low light intensities, the curve has two

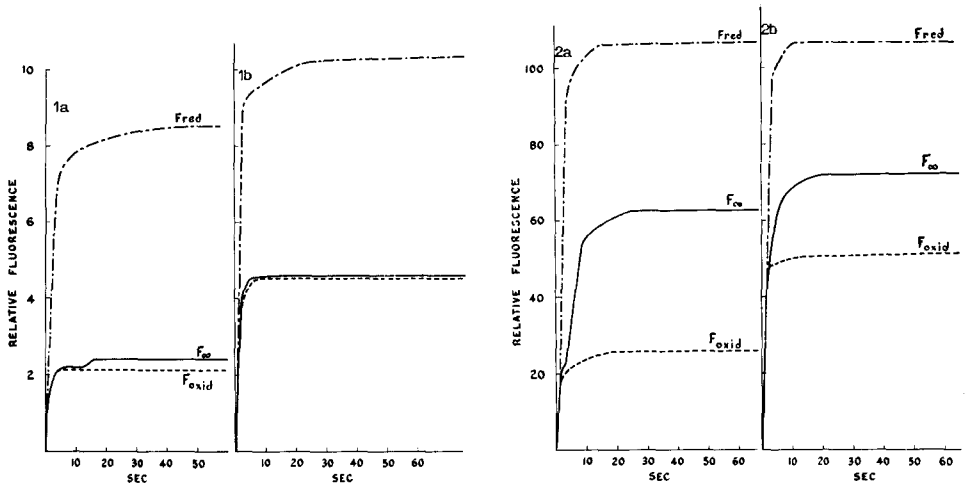

Fig. 1. Time-course of increase in fluorescence intensity at 683 mμ of (a) control and (b) Mn-deficient chloroplasts at 20°C. Excitation wavelength 436 mμ with a low intensity of 90 ergs·cm^{-2}·sec^{-1}. Concentration of chloroplasts, 0.2 absorbance unit; ———, no additions; – – – –, + ferricyanide; –·–·–, + dithionite.

Fig. 2. Time-course of increase in fluorescence intensity at 683 mμ of (a) control and (b) Mn-deficient chloroplasts at 20°C. Conditions as for Fig. 1, except that a higher light intensity at 436 mμ of 900 ergs·cm^{-2}·sec^{-1} was used. ———, no additions; – – – –, + ferricyanide; –·–·–, + dithionite.

phases of increase with an inflection point between them which tends to be less apparent as the light intensity increases. In agreement with previous workers[16–18], the control chloroplasts showed similar biphasic kinetics (Figs. 1a and 2a). At both light intensities the increase in fluorescence intensity upon illumination ($F_\infty - F_0$) was greater at 436 and 470 mμ than at 650 and 670 mμ. A 10-fold increase in light intensity produced a F_∞/F_0 value of 3–4 which was about twice the value obtained at the lower light intensity (Table II).

A comparison of the fluorescence time-rise curves for control and Mn-deficient chloroplasts and the corresponding values of F_∞/F_0 (Table II) shows that at both light intensities the increases in fluorescence intensity upon illumination were lower for the Mn-deficient chloroplasts. Indeed at the lower light intensity, F_∞ was almost equal to F_0 when the excitation wavelengths used to illuminate the Mn-deficient chloroplasts were 650 and 670 mμ. At the higher light intensity, F_∞ exceeded F_0, but the increase was less than 2-fold. Moreover, the Mn-deficient chloroplasts showed a faster rate of induction than did the control chloroplasts (Figs. 1 and 2).

TABLE II

RATIO OF STEADY-STATE INTENSITY (F_∞) TO INITIAL FLUORESCENCE INTENSITY (F_0)

The fluorescence intensity was recorded at 683 mμ; other conditions similar to Table I. F_0 = initial fluorescence intensity at onset of illumination and F_∞ = steady-state fluorescence intensity.

Excitation wavelength (mμ)	*F_∞/F_0 ratio*			
	Light intensity I × 1		*Light intensity I × 10*	
	Control	*Deficient*	*Control*	*Deficient*
436	1.85	1.40	3.70	1.70
470	2.00	1.30	4.30	1.79
650	1.20	1.05	2.68	1.25
670	1.39	1.06	2.73	1.21

TABLE III

RATIO OF STEADY-STATE QUANTUM YIELDS OF FLUORESCENCE OF Mn-DEFICIENT AND CONTROL CHLOROPLASTS WITH FERRICYANIDE

All conditions similar to Table I. $K_3Fe(CN)_6$ was added to each sample to give a final concn. of $5 \cdot 10^{-5}$ M.

Exitation wavelength (mμ)	$\phi_{oxidized,\ deficient} / \phi_{oxidized,\ control}$	
	Light intensity I × 1	*Light intensity I × 10*
436	2.0	2.0
470	2.3	1.6
650	1.7	2.2
670	1.5	2.0

The addition of a relatively high concentration of a Hill oxidant, potassium ferricyanide, to control chloroplasts in the dark caused a decrease in the level of the final steady-state fluorescence intensity, $F_{oxidized}$, upon subsequent illumination (Figs. 1 and 2). With control chloroplasts, $F_{oxidized}$ lies between the original F_0 and F_∞ values obtained with chloroplasts to which no oxidant had been added. This is not the case with the Mn-deficient chloroplasts, since $F_{oxidized}$ is about the same as F_∞ and only slightly greater than F_0 at the lower light intensities. With a 10-fold increase in light intensity, $F_{oxidized}$ was lower than F_∞, but the decrease was again less pronounced than with control chloroplasts. Fluorescence quantum yields under oxidizing conditions are compared at 4 excitation wavelengths and two light intensities in Table III. Regardless of the excitation wavelength or light intensity, the $F_{oxidized}$ values of Mn-deficient chloroplasts always exceeded those of the control.

The addition of a reducing agent, sodium dithionite, caused a marked increase in fluorescence to a final steady-state level, $F_{reduced}$, well in excess of the original F_∞ value (Figs. 1 and 2). It should be noted that the light intensities used in our work were relatively low. If the light intensity is high, approx. $2 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}, as in the MALKIN AND KOK[16] study, then the fluorescence under reducing conditions is the same or only slightly in excess of F_∞. Under our conditions for maximum fluorescence, there was little difference between the $F_{reduced}$ values of Mn-deficient and control

chloroplasts. The comparison of the quantum yields, $\phi_{reduced}/\phi_{oxidized}$ is a useful index which indicates the span of fluorescence arising from the change of reducing to oxidizing conditions. Table IV illustrates a striking difference for $\phi_{reduced}/\phi_{oxidized}$ for the two types of chloroplasts; this ratio is 3–4 for control chloroplasts and only about 2 for Mn-deficient chloroplasts. The lower values of the latter, resulted from their higher $F_{oxidized}$ values.

TABLE IV

RATIOS OF STEADY-STATE QUANTUM YIELDS OF FLUORESCENCE IN DITHIONITE AND FERRICYANIDE

All conditions similar to Table I. Sodium dithionite (1–2 mg) was used for reducing conditions and a final concn. of $5 \cdot 10^{-5}$ M $K_3Fe(CN)_6$ for oxidizing conditions with identical samples of control and Mn-deficient chloroplasts.

Excitation wavelength (mμ)	$\phi_{reduced}/\phi_{oxidized}$			
	Light intensity I × 1		*Light intensity I × 10*	
	Control	*Deficient*	*Control*	*Deficient*
436	4.0	2.4	4.1	2.1
470	4.1	2.1	3.3	2.1
650	3.0	2.2	3.0	1.6
670	3.1	2.1	2.8	1.6

TABLE V

QUANTUM YIELDS OF FLUORESCENCE AT 77° K

Chloroplasts were resuspended in 0.05 M phosphate buffer (pH 7.2) containing 62% glycerol to give an absorbance of 0.1. Excitation wavelength was 436 mμ with a light intensity of 90 ergs·cm^{-2}·sec^{-1}.

	$\phi_{(683+693 m\mu)}$	$\phi_{735\ m\mu}$	ϕ_{Total}	$\frac{\phi_{735}}{\phi_{Total}}$
Control	0.056	0.140	0.196	0.71
Mn-deficient	0.029	0.114	0.143	0.80

Fluorescence yields at 77° K

It is known that lowering the temperature from 20° C to 77° K results in a marked increase of fluorescence of chloroplasts and that the shape of the spectrum is also altered considerably; the most intense band occurs at 735 mμ and a new band at 693 mμ is visible[9,19]. The quantum yields of fluorescence at 77° K are given in Table V; the control chloroplasts were more fluorescent than the Mn-deficient chloroplasts. This is the reverse of the situation found with similar excitation conditions at room temperature. It was observed on lowering the temperature from 20° C to 77° K, that the quantum yield of fluorescence had increased some 20-fold for control chloroplasts (*i.e.* 0.0093–0.196) but less than 8-fold for the Mn-deficient chloroplasts (*i.e.* 0.0186–0.143).

The fluorescence emission spectra at 77° K of the two types of chloroplasts are illustrated in Fig. 3. The band at 693 mμ is clearly visible in the control spectrum, but

is absent from the Mn-deficient spectrum, which does not even have a shoulder at 693 mμ on the 683-mμ band. The effect of Mn deficiency on the emission spectrum is more clearly shown by a comparison of the relative band heights (Table VI). The fluorescence emission at 735 mμ as compared to that at both 683 and 693 mμ is greater for the Mn-deficient chloroplasts than it is for the control ones, with the difference being intensified at 693 mμ. Since in our experience, the height of the 693-mμ band relative to that of the 683-mμ band is somewhat variable, especially with digitonin-treated chloroplast fractions, we recorded emission spectra of chloroplasts from many Mn-deficient plants of different ages to ensure that the absence of 693-mμ emission was a consistent fact; this fluorescence emission was absent in all cases. The addition of $MnCl_2$ (10^{-5} M and 10^{-3} M) to the Mn-deficient chloroplasts prior to freezing did not alter the spectrum. However, chloroplasts isolated from plants transferred from Mn-deficient media to Mn-containing media 2 days prior to harvest gave the 693-mμ emission band and the F_{735}/F_{683} and F_{735}/F_{693} ratios were similar to the control. Chloroplasts isolated from plants which had been grown since germination in excess Mn also gave ratios similar to the control. Both of these

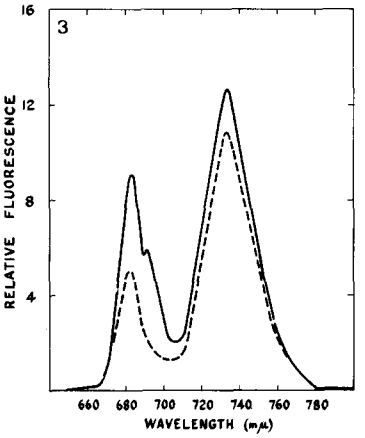

Fig. 3. Fluorescence emission spectra of control (———) and Mn-deficient (– – – –) chloroplasts at 77° K. Excitation wavelength, 436 mμ; light intensity 90 ergs·cm^{-2}·sec^{-1}. Concentration of chloroplasts, 0.1 absorbance unit at 436 mμ.

TABLE VI

COMPARISON OF THE RELATIVE FLUORESCENCE BAND HEIGHTS OF CHLOROPLASTS AT 77° K

All conditions are similar to Table V. Chloroplasts were isolated from 19-day-old leaves from control plants (Control); from Mn-deficient plants (Deficient); from Mn-deficient plants grown for 17 days followed by 48 h with additional Mn (Deficient + 48 h Mn), and from plants grown from germination with excess Mn (Mn excess).

Chloroplasts	$\frac{F_{735}}{F_{683}}$	$\frac{F_{735}}{F_{693}}$	$\frac{\phi_{735}}{\phi_{Total}}$ (%)
Control	1.4	2.3	71
Deficient	2.2	6.2	80
Deficient + 48 h Mn	1.7	2.3	72
Mn excess	1.7	2.3	71

latter growth conditions gave chloroplasts with complete Photosystem 2 activity. Thus we are confident that Mn-deficient chloroplasts do not possess an emission band at 693 mμ.

There is a significant difference between the fraction of light emitted at 735 mμ compared to the total fluorescence emission for the two types of chloroplasts (Table VI). While some 70% of the total fluoresence is emitted at 735 mμ with control chloroplasts, it is always at least 80% with the Mn-deficient ones. CHENIAE AND MARTIN[20] observed a similar difference in the relative fluorescence emission at 77° K with normal and Mn-deficient Scenedesmus; the fluorescence band at 730 mμ was much larger than the bands at 685 mμ and 698 mμ with the Mn-deficient cells.

Effect of DCMU

The addition of DCMU, an inhibitor for Photosystem 2, to chloroplasts or algae causes a rapid induction of fluorescence and the steady-state yield is high; furthermore, Photosystem 1 light is unable to decrease this fluorescence[7,8,16,18]. The effect of DCMU on the induction of fluorescence at 683 mμ of control and Mn-deficient chloroplasts is shown in Fig. 4. At room temperature (Fig. 4a), the fluorescence yields of both control and Mn-deficient chloroplasts in the presence of 10^{-5} M DCMU (F_{DCMU}) exceeded F_∞ and were the same as $F_{reduced}$ (*cf.* Figs. 1a and 1b). The Hill reaction activity of both types of chloroplasts was completely inhibited by 10^{-5} M DCMU, and the fluorescence yield is therefore maximal. With lower concentrations of DCMU, when some electron flow occurred, the fluorescence yields were progressively decreased, but the decline in the final steady-state fluorescence yield was much less with Mn-deficient than with the control chloroplasts, as would be expected.

For measurements at 77° K, DCMU was added to control chloroplasts prior to their appropriate dilution in 62% glycerol buffer. The higher fluorescence yield of

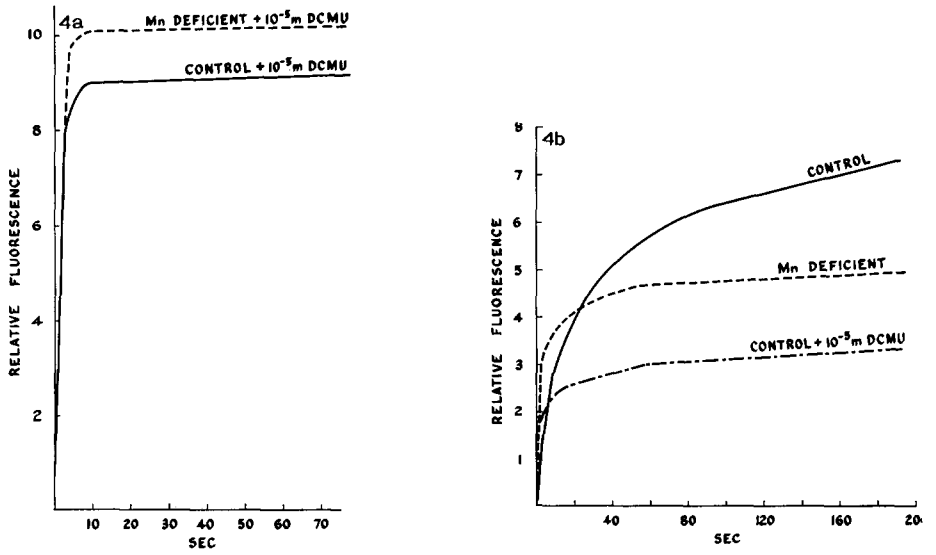

Fig. 4. Time-course of increase in fluorescence intensity at 683 mμ of chloroplasts at (a) 20°C and (b) 77° K. Excitation wavelength, 436 mμ with an intensity of 90 ergs·cm^{-2}·sec^{-1}. Concentration of chloroplasts in absorbance units, 0.2 at 20°C and 0.1 at 77° K.

DCMU-inhibited chloroplasts at 20° C relative to the control chloroplasts, was reversed at 77° K; this reversal was obtained also with Mn-deficient chloroplasts. The fluorescence induction time at 77° K is rather slow with control chloroplasts, it is faster with the DCMU-inhibited chloroplasts and the fastest rise-time was obtained with Mn-deficient chloroplasts (Fig. 4b). While the fluorescence emission at 693 mμ was still visible in the DCMU-inhibited chloroplast spectrum, both the 683- and 693-mμ bands relative to the 735-mμ band were lower ($F_{735}/F_{683} = 1.8$ and $F_{735}/F_{693} = 2.9$). These values are thus intermediate between the control and Mn-deficient ones (Table VI). The fraction of light emitted at 735 mμ relative to the total fluorescence had increased from 70 % (control) to 74 % and was thus approaching the Mn-deficient value of 80 %.

DISCUSSION

Before commenting on the results, it must be stressed that the chloroplasts obtained from Mn-deficient spinach almost certainly comprise a heterogeneous population, therefore only comparative information can be gained. In this regard, studies of deficient chloroplasts are more equivocal than those with mutants which, by contrast, may be completely lacking in a specific component of the photosynthetic apparatus. The Mn-deficient chloroplasts used in this work still contained some Mn, although it was only about one quarter of the amount per unit chlorophyll present in the control chloroplasts. They were only about 50 % as effective in oxygen evolution and contained more chlorophyll *b* relative to chlorophyll *a* than the control chloroplasts (these and other properties will be reported in a following paper). Although the Mn-deficient plants were still growing at the time of chloroplast isolation, the leaves were smaller and much paler green and such material cannot be classed as optimal.

As expected, the quantum yields of fluorescence of the Mn-deficient chloroplasts were greater than those of the control at room temperature, consistent with the loss of efficiency of trapping of energy by a partly non-functional Photosystem 2. Current theory[8] states that the chlorophylls of Photosystem 2 are capable of relatively strong fluorescence which is dependent on redox conditions, while the fluorescence arising from Photosystem 1 is relatively weak. DUYSENS AND SWEERS[8] have suggested that fluorescence is dependent on the state of reduction of a primary quencher, Q. The fluorescence will be high when Q is in the reduced (*i.e.* non-quenching) state, which results when light is absorbed by Photosystem 2. Exposure to far-red light or to darkness, causes the oxidation of Q^- and a lower fluorescence yield. Similarly, a Hill oxidant depresses the fluorescence yield by oxidizing Q, either directly or *via* Photosystem 2.

This concept of DUYSENS AND SWEERS[8] has been enlarged, and a number of hypothetical intermediates are now postulated to be involved in the changes of fluorescence yield. The primary catalyst of Photosystem 2, has been estimated from the yield of oxygen obtained with short, intense light flashes in the classic experiments of EMERSON AND ARNOLD[21] and more recently by JOLIOT[22] to be 1 O_2 per 2200 chlorophylls, *i.e.* 1 equiv per 500 chlorophylls. Fluorescence studies with DCMU-inhibited chloroplasts, which show a very fast rise in fluorescence also indicate that there is a small pool of primary quencher of 1 equiv per 500 chlorophylls[7,16,18,23–25]. A larger pool of internal chloroplast oxidants has been deduced from the detailed kinetic analyses of the changes in fluorescence yields of chloroplasts by MALKIN AND

KOK[16,17] and MURATA, NISHIMURA AND TAKAMIYA[18,23,24] and from the studies of JOLIOT *et al.*[22,26,27] on the kinetics of fluorescence and O_2 evolution. It appears to be composed of at least two components called Q and P by MALKIN AND KOK[16,17], X_2 and X_3 by MURATA, NISHIMURA AND TAKAMIYA[18,23,24], and A_1 and A_2 by JOLIOT[26,27] and this total pool was estimated as approx. 1 equiv per 30–50 chlorophylls.

There are differences in opinion on the actual position of these hypothetical intermediates in the electron transport chain; some authors[16,28] assume that the small primary pool is located on the oxidizing side of Photosystem 2, while others[7,8,23,25] prefer it to be located on the reducing side and thus lie between the two pigment systems. The photochemistry of Photosystem 2 is extremely complex[7]; certainly all the observations from fluorescence studies cannot be easily encompassed into one simple scheme as has been clearly pointed out by KOK *et al.*[25] and KOK AND CHENIAE[7]. At the present time it seems simpler to assume that both of these pools are located on the reducing side of Photosystem 2.

It is proposed that the results of the room-temperature fluorescence study can be explained by Mn deficiency causing some sort of block towards electron flow from the primary quencher (*i.e.* primary reductant after illumination), which we denote as Q after DUYSENS[29], to the larger secondary pool of photoreductants, which we denote as A after JOLIOT[22]. Mn-deficient chloroplasts have a faster induction of fluorescence to a higher fluorescence yield than that observed with control chloroplasts. The biphasic nature of the fluorescence induction curve of control chloroplasts is assumed to arise because after Q has been photoreduced it is reoxidized in the dark by the secondary pool, A; hence the total rise in fluorescence reflects the reduction of both Q and A[25]. The faster induction of fluorescence by Mn-deficient chloroplasts would then be explained by the fact that A can no longer be efficiently reduced due to the block in electron flow between Q and A. The actual fluorescence yield is greater with Mn-deficient chloroplasts because there is less efficiency of electron trapping due to a diminished ability for electron flow and this means Q remains more in the reduced state than normal and the fluorescence yield is greater. This is analogous to the situation with DCMU; as the percentage of DCMU inhibition increases, so does the fluorescence yield until, with no electron flow, the fluorescence yield is maximal, and the same as that obtained under reducing conditions. This means that Mn-deficient chloroplasts exhibit the same fluorescence properties as partially DCMU-inhibited chloroplasts.

Under reducing conditions, the fluorescence yield of Mn-deficient chloroplasts was the same as that of control ones. This shows that the primary reductant can still be effectively reduced. It also suggests that the primary reductant is present in the same concentration as in control chloroplasts. However, the results under oxidizing conditions were quite different with Mn-deficient chloroplasts; in distinct contrast to normal chloroplasts, potassium ferricyanide had little effect in lowering the fluorescence yield. With control chloroplasts, a Hill oxidant such as potassium ferricyanide causes a decrease in the final steady-state fluorescence yield. This is due to the increased electron flow in the presence of an external electron acceptor which keeps Q in the oxidized state. The fact that potassium ferricyanide is unable to reduce the fluorescence yield, however, with chloroplasts which have been completely inhibited by DCMU, means that potassium ferricyanide cannot oxidize Q directly but must accept electrons from it, *via* A. Thus, the proposed block between the primary and

secondary reductant is consistent with the observed lack of effect of potassium ferricyanide at low light intensity on Mn-deficient chloroplasts.

The fluorescence properties of Mn-deficient chloroplasts are very similar to those of DCMU-inhibited chloroplasts. DCMU acts at a site very close to Photosystem 2, and the return to a low fluorescence yield by far-red illumination or darkness is inhibited[7,8,16,25]. Moreover, upon addition of DCMU to dark-adapted chloroplasts, the rise-time curve of fluorescence induction is very fast and indicates that only the small pool of primary reductant is being reduced. This fact has been held to signify that DCMU acts at a site between the primary reductant and the larger pool of internal chloroplast oxidants[7], which is the same conclusion we have reached to explain Mn deficiency. This does not mean that DCMU is directly inhibiting or binding the functional Mn, but rather that the "site", in the gross sense, is the same for Mn function as for DCMU inhibition.

Indeed, it has been observed by many workers[5–7] that the described effect of Mn deficiency upon oxygen evolution resemble very closely the inhibitory effects of DCMU or hydroxylamine. Moreover, CHENIAE AND MARTIN[30] showed that the restoration of photosynthesis in manganese-deficient cells of the blue-green alga, Anacystis was dependent on Mn^{2+} and showed an absolute requirement for light. Significantly this photorestoration was inhibited by DCMU to the same extent as was the oxygen evolution.

These results of this paper also suggest that the site of inhibition of Mn deficiency cannot be located prior to the primary photoreductant, *i.e.* between water oxidation and the primary photoreductant. If this were the case, upon illumination Q would rapidly become reduced, but there would be no electrons forthcoming from water to allow electron flow to Q and this would mean that Photosystem 1 would ultimately oxidize Q and the fluorescence yield would be low. Since this was not so, it suggests that the site of deficiency cannot be located between water and the primary photoreductant. However, it should be pointed out that this interpretation is dependent on the assumption that the small primary pool is located on the reducing side of Photosystem 2.

It is interesting to compare the fluorescence properties reported by BOARDMAN AND THORNE[13] of the barley mutant of HIGHKIN AND FRENKEL[31] which is completely lacking in chlorophyll *b*, with the Mn-deficient chloroplasts. The barley mutant chloroplasts had a much lower yield of fluorescence than the normal, in contrast to the Mn-deficient ones. They showed very little induction of fluorescence and the span of fluorescence from oxidizing to reducing conditions, $\phi_{reduced}/\phi_{oxidized}$, was only half of that obtained with normal barley, but this lower value resulted from a correspondingly lower $\phi_{reduced}$. In the case of the Mn-deficient chloroplasts, which also had a lower $\phi_{reduced}/\phi_{oxidized}$ value, it was caused by a higher $\phi_{oxidized}$. The low-temperature fluorescence emission spectrum of barley mutant chloroplasts was identical with that of the normal, although the yields were lower. These results with the barley mutant were ascribed to a lower amount of chlorophyll in Photosystem 2. With the Mn-deficient chloroplasts, it does not seem likely that there is a significant imbalance in the amounts of pigments in the Photosystems, at least in the 400–500-mμ region.

One of the most definitive characteristics of the Mn-deficient chloroplasts is the absence of the low-temperature fluorescence emission at 693 mμ. From our

previous studies[9], which were confirmed by KOK AND RURAINSKI[32] and CEDERSTRAND AND GOVINDJEE[33], it was suggested that the fluorescence emitted at 683 and 693 mμ arises from Photosystem 2 and that at 735 mμ mainly, though not only from Photosystem 1. Excitation studies[19,34–36], particularly with non-green algae where there is a clearer separation of the two photosystems thus allowing more selective activation, have also shown that the 693-mμ fluorescence is associated with Photosystem 2. KREY AND GOVINDJEE[37] suggested that the 693-mμ fluorescence in Porphyridium may be due to the "trap" of Photosystem 2 becoming light-saturated. However, in a subsequent study with blue-green algae, GOVINDJEE, MUNDAY AND PAPAGEORGIOU[38] showed the 693-mμ band existed even at low light intensity and they point out that the nature of this band is not clear. At the present time, it is only possible to state that the 693-mμ band is associated with Photosystem 2, and that chloroplasts from manganese-deficient spinach do not possess this band. In view of the similarity in fluorescence properties at room temperature of manganese-deficient and DCMU-inhibited chloroplasts, it is interesting that the low-temperature fluorescence emission spectra are also alike. Inhibition of control chloroplasts by 10^{-5} M DCMU resulted in a decrease in fluorescence emission at both 683 and 693 mμ relative to the emission at 735 mμ. This decrease was not as pronounced as that obtained with Mn-deficient chloroplasts, and the 693-mμ band was still visible. At 77° K, the quantum yields of fluorescence of both DCMU-inhibited and Mn chloroplasts were lower than that of control chloroplasts which is a reversal from the room-temperature measurements where the fluorescence yield of both types of chloroplasts exceeded those of the control. The significance of this result can only be ascertained when the actual nature of low-temperature fluorescence is understood.

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REFERENCES

1 A. PIRSON, *Z. Botan.*, 31 (1937) 193.
2 C. EYSTER, T. E. BROWN, H. A. TANNER AND S. L. HOOD, *Plant Physiol.*, 33 (1958) 235.
3 E. KESSLER, *Arch. Biochem. Biophys.*, 59 (1955) 527.
4 E. KESSLER, W. ARTHUR AND J. E. BRUGGER, *Arch. Biochem. Biophys.*, 71 (1957) 326.
5 D. SPENCER AND J. V. POSSINGHAM, *Australian J. Biol. Sci.*, 13 (1960) 441.
6 D. SPENCER AND J. V. POSSINGHAM, *Biochim. Biophys. Acta*, 52 (1961) 379.
7 B. KOK AND G. M. CHENIAE, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 1, Academic Press, New York and London, 1966, p. 1.
8 L. M. N. DUYSENS AND H. E. SWEERS, in S. MIYACHI, *Studies on Microalgae and Photosynthetic Bacteria*, Japanese Soc. Plant Physiologists, Tokyo, 1963, p. 353.
9 N. K. BOARDMAN, S. W. THORNE AND J. M. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 586.
10 C. TSUI, *Am. J. Botany*, 35 (1948) 172.
11 C. H. R. GENTRY AND L. G. SHERRINGTON, *Analyst*, 75 (1950) 17.
12 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
13 N. K. BOARDMAN AND S. W. THORNE, *Biochim. Biophys. Acta*, 153 (1968) 448.
14 C. A. PARKER AND W. H. REES, *Analyst*, 85 (1960) 587.
15 G. WEBER AND F. W. J. TEALE, *Trans. Faraday Soc.*, 53 (1957) 646.
16 S. MALKIN AND B. KOK, *Biochim. Biophys. Acta*, 126 (1966) 413.
17 S. MALKIN, *Biochim. Biophys. Acta*, 126 (1966) 433.
18 N. MURATA, M. NISHIMURA AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 120 (1966) 23.

19 J. C. GOEDHEER, *Biochim. Biophys. Acta*, 88 (1964) 301.
20 G. M. CHENIAE AND I. F. MARTIN, *Brookhaven Symp. Biol.*, 19 (1966) 406.
21 R. EMERSON AND W. ARNOLD, *J. Gen. Physiol.*, 16 (1932) 191.
22 P. JOLIOT, *Biochim. Biophys. Acta*, 102 (1965) 116.
23 N. MURATA, M. NISHIMURA AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 112 (1966) 213.
24 N. MURATA, M. NISHIMURA AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 126 (1966) 234.
25 B. KOK, S. MALKIN, O. OWENS AND B. FORBUSH, *Brookhaven Symp. Biol.*, 19 (1966) 446.
26 P. JOLIOT, *Biochim. Biophys. Acta*, 102 (1965) 135.
27 P. JOLIOT, R. DELSOME AND A. JOLIOT, in J. B. THOMAS AND J. C. GOEDHEER, *Currents in Photosynthesis*, Donker, Rotterdam, 1966, p. 359.
28 M. AVRON, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 2, Academic Press, New York, 1967, p. 1.
29 L. M. N. DUYSENS, *Progr. Biophys. Mol. Biol.*, 14 (1967) 1.
30 G. M. CHENIAE AND I. F. MARTIN, *Biochem. Biophys. Res. Commun.*, 28 (1967) 89.
31 H. R. HIGHKIN AND A. W. FRENKEL, *Plant Physiol.*, 37 (1962) 814.
32 B. KOK AND H. J. RURAINSKI, *Biochim. Biophys. Acta*, 126 (1966) 587.
33 C. N. CEDERSTRAND AND GOVINDJEE, *Biochim. Biophys. Acta*, 120 (1966) 177.
34 J. A. BERGERON, in B. KOK AND A. T. JAGENDORF, *Photosynthetic Mechanisms of Green Plants*, Natl. Acad. Sci., Natl. Res. Council, Publ. 1145, Washington, 1963, p. 527.
35 B. KOK, in B. KOK AND A. T. JAGENDORF, *Photosynthetic Mechanisms of Green Plants*, Natl. Acad. Sci., Natl. Res. Council, Publ. 1145, Washington, 1963, p. 45.
36 A. KREY AND GOVINDJEE, *Biochim. Biophys. Acta*, 120 (1966) 1.
37 A. KREY AND GOVINDJEE, *Proc. Natl. Acad. Sci. U.S.*, 56 (1964) 1568.
38 GOVINDJEE, J. C. MUNDAY AND G. PAPAGEORGIOU, *Brookhaven Symp. Biol.*, 19 (1966) 434.