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ON THE FUNCTIONAL SITE OF MANGANESE IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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SUMMARY

1. Restoration of Hill activity to illuminated Mn-deficient Euglena cultures is complete 4–6 h after Mn addition.

2. The maximum electron transfer rate at infinite light intensity is 2.5 times higher for Mn_R (reactivated) than for —Mn (deficient) chloroplasts, and the quantum efficiency is some 3 times greater. The fluorescence yield and quantum efficiency are likewise 4–5 fold higher in +Mn as against —Mn chloroplasts.

3. At 283°K, +Mn chloroplasts have a characteristic fluorescence maximum at 685 nm, while —Mn chloroplasts have an additional shoulder at 693 nm. Reactivation of —Mn cells increases the fluorescence emission of the isolated chloroplasts uniformly over the entire spectrum.

4. At 77°K, reactivation is accompanied by positive shifts in the fluorescence difference spectrum at 685, 700 and 719 nm. The (+Mn) − (−Mn) spectrum is characterized in addition by a trough at about 730 nm.

5. The initial fluorescence yield induced by a weak measuring beam is 50% higher in either −Mn chloroplasts or partially reactivated chloroplasts compared with +Mn chloroplasts. However, the variable excess fluorescence yield in strong illumination is almost absent in −Mn chloroplasts and is high in Mn_R and +Mn samples.

6. Hydroxylamine can donate electrons to Photosystem II, by-passing the —Mn block in electron flow and restoring high fluorescence yields. In cases of severe Mn deficiency, restoration of electron flow by hydroxylamine is only partial.

7. The results indicate a structural and a functional role for Mn in O_2 evolution; the latter, at least, residing in the span between water oxidation and the System II photoact.

INTRODUCTION

Involvement of bound Mn in the O_2 evolving apparatus of chloroplasts is now a well-established fact[1,2], and it is generally supposed that Mn functions in electron transport between the site of water oxidation and the photoact. Recently an alternative location for the Mn function has been proposed by ANDERSON AND THORNE[3];

Abbreviations: APDC, ammonium pyrrolidine dithiocarbamate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)ethanesulfonic acid; TCPI, *o*-chlorophenolindo-2,6-dichlorophenol; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

namely, on the reducing side of Photosystem II close to the site of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibition. This suggestion was based on studies of the fluorescence properties of normal and Mn-deficient spinach chloroplasts, making the usual assumptions about the mode of fluorescence quenching in Photosystem II[4].

In an investigation of the Cl^- requirement for photosynthetic O_2 evolution[5,6], we examined the fluorescence of normal and Cl^--deficient chloroplasts in order to determine on which side of the photoact the requirement might lie. A decrease in fluorescence yield was observed in the absence of Cl^-, in complete contrast to the high yields seen upon inhibition by DCMU or (according to ANDERSON AND THORNE[3]) by Mn deficiency. We were thus satisfied that Cl^- functions at the oxidizing (water) end of the photosystem.

Electron donor systems have recently been described[7,8] which, by supplying electrons more or less directly into Photosystem II, can by-pass the site of Cl^- requirement. Light-dependent electron flow in Mn-deficient chloroplasts could also be restored by these same donors[9]. This observation pointed to a location of the Cl^-- and Mn-requiring sites on the same (oxidizing) side of Photosystem II.

An investigation of the properties of Mn-deficient chloroplasts was therefore made in an attempt to explain the apparent contradiction between our observations with artificial electron donors and the fluorescence studies of ANDERSON AND THORNE[3].

MATERIALS AND METHODS

Euglena gracilis var. *bacillaris* Strain Z (Pringsheim) was grown in the light on heterotrophic medium[10] at 26°. The 1-l culture flasks were shaken mechanically and illuminated from above with warm white fluorescent lamps providing an incident intensity of 150 ft candles. Cultures intended for inoculation of Mn-free medium were prepared with only 10% of the normal Mn level, to minimize carry-over of Mn. The growth rate (generation time, approx. 10 h) was not affected by this restriction and provided the cells were washed once in Mn-free medium before inoculation, high degrees of Mn deficiency could be obtained without resort to chemicals purified beyond the standard analytical grade. In reactivation experiments, 30 mg $MnSO_4 \cdot H_2O$ were added per l of culture, 2–6 h prior to harvesting. All cultures were harvested after 3 days' growth, washed once and disrupted by a French pressure cell (5000 lb/inch^2) in 0.4 M sucrose, 0.03 M Tricine and 5 mM $MgSO_4$, pH 7.4. The resulting suspension was centrifuged at 500 $\times$ *g* for 5 min to remove cell debris and then at 10000 $\times$ *g* for 10 min to sediment the chloroplasts. These were washed once in the disruption medium before final resuspension. Chlorophyll was determined by the method of ARNON[11].

Photosystem II electron transfer activity was determined spectrophotometrically at 15° using *o*-chlorophenolindo-2,6-dichlorophenol (TCPI) as electron acceptor. Samples were illuminated with blue light at saturating intensity (140 kergs$\cdot$cm$^{-2}\cdot$sec^{-1}), and the change in absorbance at 620 nm was continuously recorded.

The fluorescence kinetics at 283°K and the fluorescence emission spectrum at 77°K were measured as previously described[6,7]. Emission spectra are plotted as relative quantum flux per unit wave number.

The Mn^{2+} content of chloroplasts was determined using a Perkin–Elmer model 303 atomic absorption spectrophotometer and the following extraction procedure, based on the method of MANSELL[12]. To chloroplasts equivalent to 1 mg of chlorophyll in

a 1.5-ml volume was added 0.5 ml of a mixture of 17 parts HNO_3 and 3 parts 70% $HClO_4$ (v/v). After mixing, the sample was centrifuged for 5 min at 25° and the clear supernatant decanted into a test tube. The pellet was washed once with 2 ml of water. 2 drops of a 0.2% solution of bromophenol blue in 15% ethanol were added to the combined supernatants, followed by 10 M NaOH and dilute NaOH until the color was brownish yellow (pH 3.0–3.5). Ammonium pyrrolidine dithiocarbamate (APDC) was freshly prepared as a 5% aqueous solution and purified by extraction with excess methyl isobutyl ketone and filtration. To the sample, 0.25 ml of APDC was added, and after mixing the complexed Mn was extracted into 4 ml of methyl isobutyl ketone. The supernatant was drawn off into a 75 mm × 12 mm diameter tube, centrifuged briefly, then aspirated directly into the spectrophotometer. The absorbance at 280 nm was linear with respect to Mn up to at least 10 μg Mn per 4 ml. Wet-ashing of the chloroplasts for several hours at 100° did not release Mn in excess of that obtained by the simple acid extraction outlined above.

RESULTS

Table I illustrates the restoration of Hill activity in chloroplasts from cells grown in Mn-deficient medium, at various times after adding $MnSO_4$ to the culture. Complete restoration occurs about 5–6 h after Mn addition, while a level of 80–90% reactivation is obtained after 4 h. There is little change in the first 30 min following Mn restoration, in contrast to the observations with Scenedesmus[2] and Anacystis[13]. The amount of Mn

TABLE I

REACTIVATION OF Mn-DEFICIENT EUGLENA

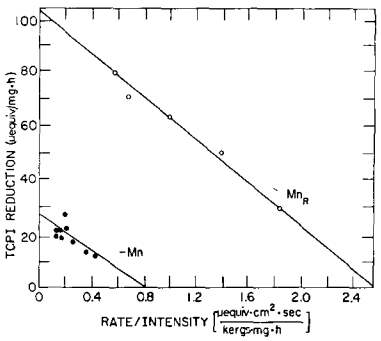
TCPI reduction (μequiv TCPI reduced per h per mg chlorophyll) was assayed in 2.0 ml of 0.1 M sucrose, 0.03 M *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 10 mM NaCl, 5 mM $MgSO_4$, and 25 μM TCPI, pH 7.2, using 20 μg chlorophyll per 2 ml.

Expt. No.	*Moles chlorophyll/atom Mn*			*Reactivation time (h)**	*TCPI reduction*		
	−Mn	Mn_R*	*+Mn***		*−Mn*	Mn_R	*+Mn*
1	327	14	6	3.5	17	101	94
2	220	26	—	4	15	41	—
3	190	15	—	3.5	26	68	—
4	320	76	—	3.5	38	87	—
5	264	25	11	5	9	86	94
6	427	90	—	1.5	2	40	—
6	427	52	—	6	2	70	—

* Reactivated by adding Mn and incubating for given duration.
** Control cells grown in normal (+Mn) medium.

associated with the chloroplasts is also presented in Table I, as moles of chlorophyll per atom of Mn. The minimum ratio appears to be between 6 and 15 moles chlorophyll per atom Mn for normally grown (+Mn) and for reactivated (Mn_R) chloroplasts, rising to between 190 and 427 for Mn-depleted (—Mn) chloroplasts. The ratios vary from preparation to preparation, though the +Mn and Mn_R samples always have a 5–15 times lower value than the deficient cultures.

The dependence of the rate of electron transport on light intensity is given in Fig. 1 for reactivated and Mn-deficient chloroplasts. In both cases, the rate of electron transport is linearly dependent on the rate divided by the intensity of light. The maximum rate of electron transport (intercept on the rate axis) is 2.5 times higher for Mn_R than for —Mn chloroplasts. Fig. 1 also shows that the quantum requirement (photons per electron, or inverse intercept of the rate/intensity axis) of —Mn chloroplasts is some 3 times greater than that of the reactivated preparation although the light absorption is very nearly the same. The similar slope of both plots in Fig. 1 indicates that the half-maximal rate of electron flow is reached at the same light intensity with both systems. The much greater relative quantum requirement seen by CHENIAE AND MARTIN[14] for Mn-deficient Scenedesmus chloroplast particles is most probably due to their use of normal (+Mn) rather than reactivated (Mn_R) chloroplasts as controls.



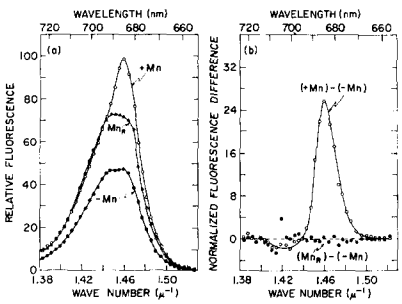


Fig. 1. Dependence of electron flow upon light intensity. Euglena chloroplasts (20 μg chlorophyll per 2 ml) were suspended in 0.1 M sucrose, 0.03 M Tricine, 5 mM $MgSO_4$, 0.01 M NaCl and 25 μM TCPI, pH 7.2, at 15°. Chloroplasts were either Mn deficient (—Mn), having 195 moles chlorophyll per atom Mn; or reactivated for 3.5 h (Mn_R), having 15 moles chlorophyll per atom Mn.

Fig. 2. Fluorescence emission spectrum of Euglena chloroplasts. Chloroplasts (40 μg chlorophyll) were incubated in 2.0 ml of 0.2 M sucrose, 0.03 M Tricine, 5 mM $MgSO_4$, 0.01 M NaCl at pH 7.4. Exciting light intensity, 1 kerg·cm^{-2}·sec^{-1} at 435 nm. Fluorescence given as relative quanta per wave number. +Mn, normally grown; —Mn, Mn deficient; Mn_R, reactivated for 2 h. (a) Emission spectra at 283°K. (b) Difference emission spectra. Values for fluorescence emission from —Mn and Mn_R chloroplasts were normalized at 695 nm to that from the +Mn sample.

In order to locate more closely the site of Mn involvement, an examination of the fluorescence properties of these chloroplasts was undertaken. The fluorescence emission spectra of three classes of Euglena chloroplasts at 283°K are given in Fig. 2a. Normally grown (+Mn) samples have a fluorescence maximum at 685 nm (1.460 μ^{-1}) with a very small shoulder above 693 nm (1.441 μ^{-1}). In contrast the —Mn preparation gives two almost equal peaks at 685 and 693 nm, and these same peaks are also indicated in the Mn_R chloroplasts. The normal and Mn_R chloroplasts are significantly more highly fluorescent over the entire emission band, though the normal (+Mn) spectrum alone shows the characteristic high 685-nm peak.

In Fig. 2b, which is a fluorescence difference spectrum obtained from the data of Fig. 2a by normalization at 695 nm, there is no difference between the fluorescence of —Mn and Mn_R chloroplasts. However, in +Mn chloroplasts the 685-nm peak is strikingly displayed and there is evidence for an associated broad trough at about 703 nm. This suggests that growth in the presence of Mn for long periods must produce

changes in Photosystem II over and above those accomplished in the 5 or 6 h needed for complete restoration of electron flow rates by the reactivation procedure.

Here, then, is a clear instance in which the fluorescence of normal chloroplasts would provide a poor control in attempting to elucidate the strictly functional effects of Mn deficiency; from which it follows that comparison between the fluorescence characteristics of normal (+Mn) and Mn-deficient cultures[3], without reference to the effects of reactivation, should be eschewed.

The fluorescence emission spectrum of Euglena chloroplasts at 77°K has a large broad peak around 720 nm which obscures details in the 680–700-nm range[15]. However, a difference spectrum can clearly show small changes in this region. Fig. 3A shows that at the 721-nm (1.385 μ^{-1}) fluorescence emission peak, the —Mn chloroplasts are less fluorescent than the Mn_R chloroplasts, whereas the peak for +Mn chloroplasts is equal in intensity to the peak for —Mn chloroplasts but shifted towards a larger wave number by 0.008 μ^{-1} (approx. 2 nm). At the minor 685-nm (1.460 μ^{-1}) peak the emission from the Mn_R chloroplasts lies between the lower intensity of —Mn and the higher of +Mn.

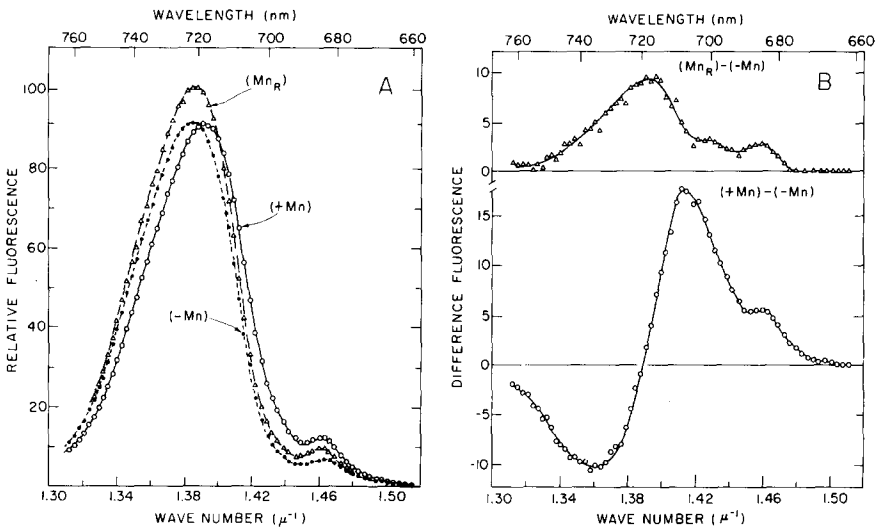


Fig. 3. Fluorescence emission spectrum of Euglena chloroplasts at 77°K. Chloroplasts (40 μg chlorophyll) were suspended in 0.4 M sucrose, 0.03 M Tricine, 5 mM $MgSO_4$, 0.01 M NaCl, pH 7.4 and frozen in liquid N_2. Chloroplasts were either Mn deficient (—Mn), having 327 moles chlorophyll per atom Mn; or reactivated for 3.5 h (Mn_R), having 14 moles chlorophyll per atom Mn; or normally grown (+Mn), having 6 moles chlorophyll per atom Mn. (A) Emission spectra at 77°K. (B) Difference emission spectra. Fluorescence given as relative quanta per wave number.

In Fig. 3B the difference curves of the fluorescence emission quite markedly show the effect of Mn reactivation. During the course of reactivation the first change in emission is the production of a band at 719 nm and two small bands at 700 nm and 685 nm (Fig. 3B, uppermost curve). Chloroplasts from cells grown in the presence of Mn give an emission difference spectrum (Fig. 3B, lower curve) which can be interpreted as follows: the aforementioned bands at 685, 700 and 719 nm have further increased but the contribution of a component emitting around 730 nm has decreased. The

emission maxima at 685 and 700 nm have been linked with excitation of Photosystem II (ref. 16).

Although CHENIAE AND MARTIN[2] did not present data for reactivated chloroplasts, their results from low temperature fluorescence studies are in essential agreement. Indeed, there is a striking resemblance between their spectrum of normal cells and our reactivated *minus* deficient difference spectrum (Fig. 3B), especially with regard to the minor peak at about 685 nm.

TABLE II

NORMALIZED YIELDS OF FLUORESCENCE INDUCED BY LOW-INTENSITY EXCITING LIGHT

Samples in 2nd column, in plots of fluorescence yield *vs.* exciting light intensity, gave nonzero intercepts with the intensity axis at 50 ergs·cm^{-2}·sec^{-1}. Chloroplasts were incubated as in MATERIALS AND METHODS with DCMU (10 μM) or $Na_2S_2O_4$ (a few crystals). Mn_{R2}, Mn_{R4}: chloroplasts from cells reactivated with Mn for 2 and 4 h, respectively. Fluorescence yield measured as in Fig. 5 and text, at 685 nm.

Experiment	*Additions*		
	None	*DCMU*	*$Na_2S_2O_4$*
1. +Mn	132	126	154
−Mn	65	80	100
2. Mn_{R4}	102	105	127
−Mn	59	86	100
3. Mn_{R2}	68	83	101
−Mn	55	77	100

The effect of DCMU or dithionite addition on the fluorescence yield at 12° is shown in Table II. The yield induced by light is seen to be about double in +Mn and fully reactivated samples compared with those deficient in Mn. Adding DCMU does not affect the +Mn and 4-h reactivated (Mn_{R4}) yields because Photosystem I activity is in any case mostly blocked by loss of the soluble cytochrome *f* during chloroplast isolation[17]. Some increase in yield is seen upon adding DCMU to Mn-deficient preparations, in which the fluorescence yield is otherwise low.

Dithionite reduction gives 10–20% more fluorescence than addition of DCMU, as first reported by HOMANN[9]. Although the deficient and +Mn fluorescence yields tend to converge upon addition of dithionite, a significant difference usually remains. We believe that this difference is due to structural changes induced by the deficiency which somehow render the chloroplasts less fluorescent. In chloroplasts reactivated for only 2 h (Expt. 3) there is little difference in the dithionite level relative to the −Mn sample, while after 4 h of reactivation (Expt. 2) an intermediate situation is observed. Presumably, reversal of structural derangements occurs only later during the time-course of reactivation. These findings illustrate again the importance of measurements on both reactivated and normally grown samples.

A final comment about Table II (data not presented) is that a plot of fluorescence *vs.* intensity of exciting light gave straight line relationships intercepting at zero intensity with all samples in the presence of DCMU or dithionite, but not so in the absence of additions. Nonzero intercepts were observed by MURATA *et al.*[18] and are probably due, in this case, to removal of electrons from the quencher by Photosystem I.

The fluorescence yield induced by strong actinic illumination is an indicator of the ability for the quencher (Q) to become reduced by Photosystem II activity[4]. Fig. 4a shows that +Mn chloroplasts undergo a fluorescence increase of nearly 200 % with actinic illumination, whereas only a slight change is observed with a Mn-deficient preparation (Fig. 4B). In contrast, the control level of fluorescence induced by the

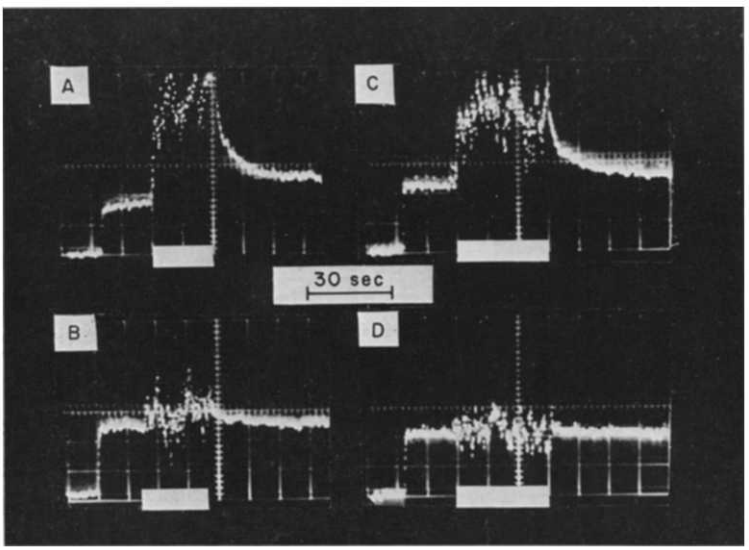


Fig. 4. Comparison of the fluorescence yields at 693 nm for normal and Mn-deficient chloroplasts at high light intensity. Chloroplasts were suspended in 0.1 M sucrose, 0.03 M TES, 5 mM $MgSO_4$, pH 7.2, 12°. Intensity of measuring light, 20 ergs·cm^{-2}·sec^{-1} and of actinic light 5 kergs·cm^{-2}·sec^{-1}. The actinic light was turned on during the periods indicated by solid white bars. A and B are the yield kinetics in one experiment, for +Mn and −Mn chloroplasts respectively. C and D are the kinetics in a second experiment, for Mn_R (reactivated for 5 h) and −Mn chloroplasts.

weak measuring beam alone (ϕ_0) is nearly 50 % higher for the −Mn compared with the +Mn chloroplasts. It must be stressed, however, that there is no known relationship between the ϕ_0 component of fluorescence and the redox condition of Q or any other electron carrier. Fig. 4C shows that in a partially reactivated system, the fluorescence yield induced by the actinic beam increased considerably before any effect on the ϕ_0 component is recognizable (compare Fig. 4D). Perhaps the ϕ_0 component, like the fluorescence level in the presence of dithionite, is indicative of structural features within the O_2 evolving mechanism which modify the environment of the fluorescent chlorophyll.

The fluorescence yield is dependent on the intensity of actinic illumination in a Michaelis–Menten fashion[6]. Table III gives the yield parameters found by plotting fluorescence against the intensity of actinic light as described in the legend. ϕ_0 (the fluorescence due to the measuring beam) is higher for Mn-deficient chloroplasts at both 685 and 693 nm, whereas ϕ_{max}, ϕ_{DT} and Φ_e are lower. Partially reactivated samples have intermediate values for the various parameters. Φ_e (the quantum efficiency of fluorescence) seems to be the most sensitive indicator of Mn deficiency as well as for Cl^- deficiency[6]. Under all conditions, the fluorescence level in the presence of added dithionite is greater than that produced by saturating illumination.

High concentrations of hydroxylamine inhibit the oxidation of water and donate

TABLE III

FLUORESCENCE YIELD AND QUANTUM EFFICIENCY AS AFFECTED BY Mn DEFICIENCY AND DITHIONITE ADDITION

Euglena chloroplasts (40 μg chlorophyll/2 ml) were suspended in sucrose (0.2 M), Tricine (0.03 M), $MgSO_4$ (5 mM), NaCl (0.01 M), pH 7.4 at 12°. R2 = reactivated for 2 h following Mn addition. ϕ_0 is the fluorescence yield due to the measuring beam. The maximum change in fluorescence yield due to actinic illumination (ϕ_{max}) and the fluorescence quantum efficiency (Φ_e) are obtained from the relation

$$\phi - \phi_0 = \frac{\phi_{max}\, I}{I + (1/\Phi_e)}$$

where I = light intensity and ϕ = observed fluorescence. ϕ_{DT} = fluorescence yield in the presence of $Na_2S_2O_4$.

$\lambda(nm)$	*Mn*	*Relative yield*			Φ_e ($cm^2 \cdot sec \cdot kerg^{-1}$)
		ϕ_0	ϕ_{max}	ϕ_{DT}	
685	+	1.0	1.0	2.3	2.8
	−	1.1	0.5	1.1	0.8
693	+	1.0	2.7	2.8	5.4
	+(R2)	1.2	0.4	2.4	2.7
	−	1.3	0.5	1.4	1.1

electrons to Photosystem II between the photoact and the Cl^--requiring site[8]. The depression in fluorescence yield due to Cl^- deficiency can accordingly be overcome by addition of hydroxylamine[8]. The same effect is observed with Mn-deficient chloroplasts, as shown in Fig. 5. For convenience, the curves in Fig. 5 are divided into three phases as illustrated and as previously defined[6]. It is apparent that hydroxylamine increases

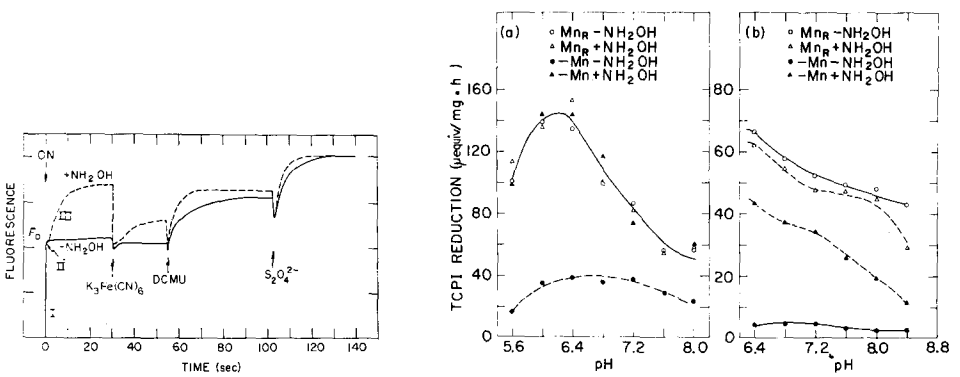


Fig. 5. Restoration of high fluorescence yield by hydroxylamine. Mn-depleted chloroplasts (40 μg chlorophyll per 2 ml) were suspended in 0.1 M sucrose, 0.03 M TES, 5 mM $MgSO_4$, 0.01 M NaCl, pH 7.2, 12°; *plus* the following, where indicated: DCMU, 10 μM; $Na_2S_2O_4$, a few grains; $K_3Fe(CN)_6$, 20 μM; $(NH_2OH)_2 \cdot H_2SO_4$ (pH 7.2), or, instead, Na_2SO_4, 25 mM. Exciting beam intensity, 260 ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1}.

Fig. 6. Restoration of electron transport rates by hydroxylamine in Mn-depleted chloroplasts. Euglena chloroplasts (20 μg chlorophyll per 2 ml) were suspended in 0.1 M sucrose, 0.03 M buffer, 5 mM $MgSO_4$, 25 μM TCPI and either 25 mM Na_2SO_4 or 25 mM $(NH_2OH)_2 \cdot H_2SO_4$ (pH 7.2) at 15°. Buffers used: pH 5.6–6.8, 2-(*N*-morpholino)ethanesulfonic acid (MES); pH 7.2–8.0, TES; pH 8.4, Tricine. (a) −Mn, 320 and Mn_R, 76 moles chlorophyll per atom Mn. (b) −Mn, 540 and Mn_R, 15 moles chlorophyll per atom Mn. Reactivation time, 3.5 h throughout.

both the rate of fluorescence rise in Phase III and the final steady state level, but has little effect on the initial (F_0) fluorescence jump or the final level in the presence of dithionite. Ferricyanide lowers the high steady state fluorescence level produced by addition of hydroxylamine, indicating that reduction of Q by this donor is essentially reversible and physiological. The reduction of Q by Photosystem II in the presence of DCMU is not quite complete, so that addition of the donor yields a significant increase in fluorescence (Fig. 5), an effect which has also been observed to a lesser extent with Cl^--deficient chloroplasts[8].

The level of fluorescence in the presence of dithionite is in all cases higher than the level in the presence of sufficient DCMU to block all electron flow between the photosystems (Fig. 5). The occurrence of cyclic electron flow around the second photoact, could lead to incomplete reduction of the quencher, and this flow would be susceptible to inhibition by strong reductants such as dithionite. Although the increase in fluorescence induced by dithionite is slow (5–10 sec) even at saturating light intensity, the reduction of cytochrome oxidase by dithionite is also reported to be slow[19]. Thus the kinetic evidence does not discount the reduction of endogenous electron carriers by dithionite and consequent elicitation of high fluorescence yields.

Table IV documents the effects on Mn-deficient chloroplasts of hydroxylamine, TCPI and dithionite. Hydroxylamine increases the rate of the Phase III fluorescence rise and the final steady state value. It will be noticed that for a given experiment, the steady state fluorescence in the presence of TCPI is unaffected by hydroxylamine. The TCPI-induced steady state value is nearly that of F_0 (or ϕ_0)[4,6]; hence, hydroxylamine most probably does not greatly change F_0.

Figs. 6a and 6b illustrate, respectively, the effect of hydroxylamine and pH on electron transport in chloroplasts from moderately Mn-deficient Euglena (chl/Mn = 320) and from a severely deficient culture (chl/Mn = 540). In the absence of hydroxylamine, the maximum activity for Mn_R chloroplasts is reached at about pH 6.2. The deficient chloroplasts show only slight dependence of reduction rate on pH, with the maximum value at pH 7.0, and have much lower activity at all pH values. pH optima

TABLE IV

FLUORESCENCE RISE RATE AND STEADY STATE LEVEL WITH AND WITHOUT ADDED HYDROXYLAMINE

Euglena chloroplasts (40 μg chlorophyll per 2 ml) were suspended in 0.1 M sucrose, 0.03 M TES, 5 mM $MgSO_4$ and 0.01 M NaCl, pH 7.2 at 10°. Where indicated: 25 mM $(NH_2OH)_2 \cdot H_2SO_4$ (pH 7.2); or instead, 25 mM Na_2SO_4; 2 μM TCPI, 10 μM DCMU and $Na_2S_2O_4$, a few grains. F_{SS} = steady state (Phase III) fluorescence level at 684 nm relative to $-Mn$; $-NH_2OH$; $+Na_2S_2O_4$ value set at 100%. Exciting light intensity, 0.26 kerg·cm^{-2}·sec^{-1}.

Growth conditions	*Hydroxylamine*	*Control*		*+TCPI*	*+DCMU*	*+$Na_2S_2O_4$*
		*sec^{-1}**	*F_{SS}*	*F_{SS}*	*F_{SS}*	*F_{SS}*
1. −Mn	−	0.2	57	50	73	100
	+	3.8	82	50	80	100
+Mn	−	14	127	50	141	180
2. −Mn	−	0.4	47	36	81	100
	+	9.8	88	38	84	98

* Rate of Phase III fluorescence rise, in relative fluorescence units/sec.

below 7.0 have already been described for indophenol dye reduction by Euglena chloroplast fragments[17] and bean chloroplasts damaged by phospholipase[20]. Addition of serum albumin to Euglena chloroplasts during isolation and storage, to bind fatty acids possibly released by phospholipase activity, resulted neither in more active chloroplasts nor in a more alkaline pH optimum. It is likely, therefore, that the low pH optimum is a constitutional phenomenon related to cytoplasmic factors which differ from those in the higher plant cell.

Hydroxylamine has little or no effect on the pH–activity profile of Mn-reactivated samples (Fig. 6a). The electron flow rate of Mn-deficient chloroplasts is, however, increased by the donor to the control level and acquires the same dependence on pH. If the chloroplasts are initially more deficient in Mn (Fig. 6b), hydroxylamine is only partially able to overcome the corresponding inhibition in electron flow.The apparent failure of hydroxylamine to be a fully competent donor under these circumstances may be due to inhibition by structural defects in the O_2-evolving system, resultant on excessive Mn depletion, which are reversible only by prolonged reactivation with Mn.

DISCUSSION

Under conditions of Mn deficiency the structural organization of chloroplasts is known to be altered to an extent dependent on the severity of the deficiency, resulting in disappearance of intergranal lamellae (in higher plants) or swelling and disruption of the thylakoids[21]. Clearly, Mn is involved in the maintenance of lamellar structures. However, as CHENIAE AND MARTIN[2] have demonstrated, Mn deficiency as expressed in electron transfer rates can be reversed by added Mn under conditions, and in times, which would permit minimal structural regeneration. Thus, to simplify the ensuing discussion, it will be convenient to introduce the terms "functional Mn effect" and "structural Mn effect". In so doing, we wish to imply neither that the chloroplasts contain more than one locus of Mn function, nor that structural and functional properties are totally independent.

The apparent dual role of Mn has been confirmed in the present study, being seen in the dissimilar properties of chloroplasts from cells grown in the presence of Mn (+Mn) and from cells grown without Mn but reactivated for several hours prior to use (Mn_R). The fluorescence spectra in Fig. 2, for example, show that +Mn and Mn_R chloroplasts are considerably more fluorescent than their —Mn counterpart, in disagreement with previous reports[3,9]. The normalized difference spectra, however, show that while 2 h of reactivation restores a high fluorescence yield over the entire spectrum, it does not allow regeneration of the 685-nm peak of +Mn chloroplasts. We are therefore inclined to regard this 685-nm contribution as reflecting a structural effect of Mn, due perhaps to changes in pigment orientation near the Photosystem II trapping center.

In like manner, one may surmise from Fig. 3B that the large increase in fluorescence at 719 nm upon reactivation is a functional Mn effect, whereas the minor peaks in the (Mn_R)—(—Mn) difference spectrum, at 685 nm and 700 nm, are structural in origin. The relatively low fluorescence at 730 nm in chloroplasts from +Mn cultures may be due to a larger proportion of young cells, which are known to have low fluorescence in this region[15].

Table II again illustrates differences between Mn_R and +Mn chloroplasts: in this case, the relatively higher maximum fluorescence yield observed upon dithionite

reduction of +Mn samples. At low exciting light intensity and without DCMU or dithionite, however, the fluorescence induced by the measuring beam, ϕ_0, is slightly greater in the absence of Mn than in the +Mn or Mn_R situation (Table III and Fig. 4); however, ϕ_0 may vary slightly with wavelength. Thus, at 685 nm for all cases of Mn content, ϕ_0 is nearly the same (Table III), but at 693 nm, ϕ_0 for —Mn is consistently higher than that for +Mn (Fig. 4 and Table III).

ANDERSON AND THORNE[3] reported that the initial fluorescence of Mn-deficient spinach chloroplasts was much higher than that of a +Mn control, but that the final steady state fluorescence of the —Mn chloroplasts with added DCMU was much lower than that of +Mn chloroplasts. This correlates well with what we have seen for +Mn and—Mn Euglena chloroplasts, in which the structural Mn effect is observed. However, it is clearly not justifiable to conclude[3] that the high initial fluorescence of the —Mn samples signifies inhibition at the DCMU-sensitive (reducing) side of the photoact. The variable fluorescence which is the true measure of the quencher redox state, is minimal in the absence of Mn; but is close to maximal in the presence of DCMU and maximal when the quencher is reduced by dithionite (Table III, Fig. 4). Unfortunately Mn reactivation experiments are not feasible with spinach plants. In Euglena, by selective restoration of the functional Mn effects, the confusion resulting from the increase of ϕ_0 in —Mn chloroplasts is avoided (Fig. 4).

But there are other grounds for locating the Mn-dependent site between water and the photoact. In Table IV we see that Mn deficiency decreases the Phase III fluorescence rise rate: DCMU and the electron donors hydroxylamine and dithionite increase it. If Mn were involved as a component of the quencher itself, these results would not be obtained. Instead the fluorescence of —Mn chloroplasts would rise rapidly to the dithionite level and DCMU or hydroxylamine would be ineffective, as is observed in System II mutants[9,21]. In fact DCMU, hydroxylamine and +Mn effects show good additivity. Fig. 6 shows that these same conclusions are also reached by measurement of electron flow rates. How could high hydroxylamine concentrations possibly by-pass a Mn-requiring site on the reducing side of the photoact to give light-dependent reduction of TCPI at control (Mn_R) rates and with the control pH profile?

The functional Mn effect is very similar to the effect of Cl^- on electron transport[6,8] in that Cl^- deficiency does not lead to gross fluorescence spectral changes and is readily reversed by hydroxylamine. Cl^-, however, can be restored rapidly to isolated chloroplasts whereas Mn incorporation requires several hours (in Euglena; less in Scenedesmus[2]) and the machinery of the intact cell.

The structural Mn requirement is an engrossing subject which has been actively investigated[14,22]. An interesting facet of the structural effect may be seen in Fig. 6b, where extreme deficiency has resulted in loss of ability to recover the control (Mn_R) reduction rate upon adding hydroxylamine. The term "structural" is clearly to be interpreted loosely in this context. It should be mentioned that other cellular processes require Mn, though at lower concentration and with less specificity than does photosynthesis[23]. Metabolic processes ultimately responsible for maintenance of the thylakoid structure could be so affected by extreme Mn deficiency as to cause lesions at points in the electron transport chain other than those intimately concerned with O_2 evolution.

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