BBA 45828

THE SITE OF MANGANESE FUNCTION IN PHOTOSYNTHETIC ELECTRON TRANSPORT SYSTEM

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(Received April 4th, 1969)

SUMMARY

The effects of Mn^{2+} on aerobic photobleaching of carotenoids, on photoreduction of 2,6-dichlorophenolindophenol (DCIP) and on fluorescence above 600 mμ of spinach chloroplasts washed with 0.8 M Tris–HCl buffer were investigated. Carotenoids (mostly carotenes, lutein and violaxanthin) in the Tris-washed chloroplasts were irreversibly bleached by illumination with red light, while carotenoids in normal chloroplasts prepared with a low concentration of Tris–HCl underwent no bleaching upon illumination. The photobleaching of carotenoids observed with Tris-washed chloroplasts was inhibited by Mn^{2+} ($MnCl_2$ or $MnSO_4$) as well as by some inhibitors of the Hill reaction such as dichlorophenyl-1,1-dimethylurea (DCMU), methylthio-4,6-bis-isopropylamino-*s*-triazine and *o*-phenanthroline or by reducing agents such as ascorbate *plus* tetramethyl-*p*-phenylene diamine (TMPD). DCIP photoreduction, which was deactivated by Tris, was reactivated to 50–80% of the rate for normal chloroplasts upon addition of Mn^{2+}. The restored photoreduction of DCIP was inhibited by DCMU and carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The steady-state fluorescence yield of normal chloroplasts measured at room temperature was lowered by Tris treatment, and the decreased yield was restored by adding Mn^{2+} as well as ascorbate *plus* TMPD. CCCP also lowered the yield; the yield was recovered by adding ascorbate *plus* TMPD. Determination of manganese in normal and Tris-washed chloroplasts showed that 30% of the manganese in chloroplast was removed with Tris. It was postulated that Mn^{2+} functions in the electron transport on the oxidizing side of Photosystem II at a site between water and an electron carrier (Y). CCCP as well as Tris inhibits the reduction of Y^+ by Mn^{2+}, and carotenoids are oxidized by Y^+ which is reduced by ascorbate *plus* TMPD.

INTRODUCTION

It was demonstrated previously[1] that carotenes, lutein and violaxanthin are irreversibly bleached when chloroplasts are illuminated by red light in the presence

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine.

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of a Hill reaction inhibitor such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP), hydroxylamine or azide (Type I), while carotenoids in the chloroplasts suspended in a phosphate buffer in the absence of these inhibitors undergo no bleaching upon illumination. The photobleaching of carotenoids was suppressed by another group of Hill reaction inhibitors such as substituted phenylureas, symmetrical triazines and *o*-phenanthroline (Type II) or by reducing agents such as ascorbate *plus* *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD). The bleaching of xanthophylls proceeded more rapidly in air than in N_2 probably because a mechanism in which the photochemical electron transport on the water side (the oxidizing side) of System II (ref. 2 for two photochemical systems of photosynthesis) was blocked by the inhibitor of Type I at a site between water and an electron carrier designated as *Y*. The photobleaching of carotenoids is a result of oxidation by oxidized Y^+ and the inhibitor of Type II, which is considered to block the electron transport between Systems I and II (refs. 2 and 3), suppresses the light-induced formation of Y^+ to inhibit the bleaching.

The photochemical electron transport driven by System II may be elucidated by analyzing the fluorescence emission from chloroplasts, since the emission arises mainly from System II at room temperature[3,4]. DUYSENS AND SWEERS[3] assume that the fluorescence yield depends on the redox state of a primary electron acceptor for System II which was designated as *Q*. For instance the yield may decrease when *Q* is oxidized by the Hill oxidant and may increase when *Q* is reduced in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which inhibits electron transport between *Q* and the Hill oxidant. Thus one may expect the fluorescence yield to be lowered when electrons are not sufficiently supplied to *Q* by blocking of the electron transport between water and system II.

Chloroplasts washed with 0.8 M Tris–HCl buffer exhibit no Hill reaction activity[5,6]. YAMASHITA AND BUTLER[5] ascribed the inhibition to blocking of the electron transport at a site between water and System II from the following facts. (a) Photoreduction of $NADP^+$ observed with Tris-washed chloroplasts in the presence of ascorbate *plus* *p*-phenylene diamine is largely inhibited by DCMU. (b) The increased fluorescence yield induced by superimposed strong illumination in the absence of an electron acceptor for the Hill reaction is less marked in Tris-washed than in normal chloroplasts. (c) The low fluorescence yield is restored by adding ascorbate *plus* *p*-phenylene diamine. These results also indicate that the electron donor system, ascorbate *plus* *p*-phenylene diamine, reduces an electron carrier functioning on the water side of System II.

In the present study, the effects of Mn^{2+} on photobleaching of carotenoids, on photoreduction of DCIP and on fluorescence emission of Tris-washed spinach chloroplasts were investigated in order to elucidate the role of manganese in photosynthesis. Photobleaching of carotenoids occurs in Tris-washed chloroplasts without addition of the Hill reaction inhibitor of Type I, and the bleaching is inhibited by Mn^{2+} as well as by either the inhibitors of Type II or reducing agents. Mn^{2+} inhibition of the bleaching was also accompanied by restoration of 2,6-dichlorophenolindophenol (DCIP) photoreduction once lost after Tris treatment. The fluorescence yield of normal chloroplasts was decreased by the Tris treatment, and the yield was recovered by adding Mn^{2+} or ascorbate *plus* TMPD. It also was demonstrated that manganese is removed from chloroplasts by Tris. After these experiments were completed and this

manuscript was in preparation, HOMANN[7] reported that the fluorescence properties of manganese-depleted tobacco chloroplasts prepared from the leaves heated at 46–50° were greatly affected by adding Mn^{2+}. Thus these results are discussed in reference to his data.

EXPERIMENTAL

Chloroplasts

Chloroplasts were isolated from *Spinacia oleracea* (spinach) by the procedure similar to that described by YAMASHITA AND BUTLER[5]. Green leaves were homogenized with a Waring blender at 0° in 0.05 M Tris–HCl buffer (pH 7.8) containing 0.4 M sucrose and 10 mM NaCl; the homogenate was filtered through cotton cloth, and the filtrate was centrifuged at 300 × *g* for 1 min to remove cell debris. The supernatant was centrifuged at 1200 × *g* for 10 min. Precipitated chloroplasts were divided into two parts; one part referred to as the Tris-washed chloroplast sample was suspended in 0.8 M Tris–HCl buffer (pH 7.8) and the other called normal chloroplasts was suspended in 0.05 M Tris–HCl (pH 7.8) containing 0.4 M sucrose and 10 mM NaCl. After standing for 10 min in the dark at 10 ± 3°, the two suspension batches were centrifuged at 1200 × *g* for 10 min. Chloroplasts obtained as pellet were resuspended in 0.05 M Tris–HCl (pH 7.8) containing 0.4 M sucrose and 10 mM NaCl and were measured.

Photobleaching of carotenoids and photoreduction of DCIP

The light-induced decrease of the total carotenoid content in normal or Tris-washed chloroplasts was followed by measuring the difference spectrum between two chloroplast suspensions in 1.0-cm cells; one was illuminated in air at 20 ± 2° by red actinic light above 680 mμ (170 lux) for a desired period while the other was kept in the dark for the same period. Exact difference spectrum in terms of semi-integral attenuance[8] was measured with a Shimadzu multipurpose recording spectrophotometer model MPS-50 as described previously[1,9]. The rate of DCIP photoreduction in the chloroplasts illuminated by red actinic light above 680 mμ (170 lux) was determined photometrically by assuming the molar extinction coefficient at 610 mμ of DCIP to be 20600 $M^{-1} \cdot cm^{-1}$. Chloroplast suspensions for difference spectrophotometry and for measurements of DCIP photoreduction contained 19 μg chlorophylls per ml. Thin-layer chromatography with microcrystalline cellulose[1,10] was employed for analysis of carotenoids extracted from chloroplasts.

Fluorescence

The fluorescence from either Tris-washed or normal chloroplasts was measured at room temperature (20 ± 2°) with the Type I fluorescence attachment of the Shimadzu multipurpose recording spectrophotometer. A chloroplast suspension (absorbance at 435 mμ = 0.10) in a rectangular transparent cell (1 cm × 1 cm × 4 cm) was illuminated by exciting light at 435 mμ from the monochromator, and the fluorescence emitted above 600 mμ was detected at right angles with a large photocathode of the photomultiplier, R-236, through a red glass filter, Toshiba-VR-60. A xenon short arc lamp (Ushio Electric, Tokyo) used as the light source operated at 15 A.

Manganese

Manganese in either normal or Tris-washed chloroplasts was determined by the permanganate method described earlier[11]. Chloroplasts were heated at 550–600° for 6 h in a crucible, and Mn in ash was oxidized to permanganate by KIO_4 in the presence of $AgNO_3$. The permanganate concentration was determined spectrophotometrically, assuming the molar extinction coefficient at 525 mμ of permanganate to be 2800 $M^{-1} \cdot cm^{-1}$.

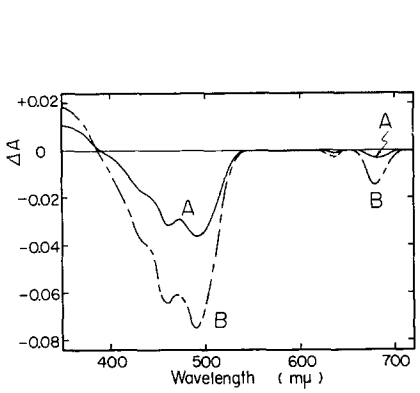
RESULTS

Photobleaching of carotenoids in Tris-washed chloroplasts

Carotenoids in the chloroplasts washed with 0.8 M Tris–HCl buffer were bleached by illumination with red light, while carotenoids in normal chloroplasts prepared at a low concentration of Tris–HCl underwent no bleaching upon illumination. Curves A and B in Fig. 1 are the light-*minus*-dark difference spectra obtained for the Tris-washed chloroplasts illuminated with red light for 5 and 20 min, respectively. These difference spectra clearly show maxima and minima at 458, 470, 490, 630, 650 and 680 mμ and a shoulder at 430 mμ, indicating bleaching of carotenoids as well as of chlorophyll *a*. Chlorophyll *b* was most resistant to the bleaching action of light; the absorbance at 650 mμ was not changed by a prolonged 20-min illumination (Curve B). The absorbance at 490 mμ of a suspension of Tris-washed chloroplasts decreased by 11 % after 20 min of illumination as measured against the chloroplast suspension kept in the dark as the reference. The time-course of the light-induced absorbance change at 490 mμ showed that photobleaching of carotenoids proceeds without an appreciable lag time. In further studies, the rate of photobleaching of carotenoids was estimated from the absorbance drop at 490 mμ during the first 10 min of illumination. The carotenoids in normal chloroplasts were photobleached very slowly, and the rate was at most 5% of the rate (drop of absorbance reading at 490 mμ = 0.046 per 10 min) determined for Tris-washed chloroplasts. The spectra obtained previously in the presence of CCCP or hydroxylamine in phosphate buffer (Fig. 1 of ref. 1) are essentially the same in shape as those obtained for Tris-washed chloroplasts (Fig. 1) without an inhibitor of the Hill reaction. In the case of normal chloroplasts prepared at a low concentration of Tris–HCl, a similar rapid spectral change occurred in the presence of CCCP as found previously in phosphate buffer. The maximal rate (drop of absorbance reading at 490 mμ = 0.060 per 10 min) of photobleaching obtained for normal chloroplasts in the presence of CCCP (10 μM) was 1.3 times greater than the bleaching rate found for Tris-washed chloroplasts. CCCP (10 μM) added to either Tris-washed chloroplasts or normal chloroplasts gave the same rate of photobleaching. Photobleaching of carotenoids in Tris-washed chloroplasts as well as that induced by CCCP were irreversible; the light-induced absorbance decrease at 490 mμ was not reversed by turning off the actinic light.

The photobleaching of carotenoids in Tris-washed chloroplasts was confirmed by chromatographically analyzing the pigments. A dense chloroplast suspension containing 133 μg chlorophylls per ml was illuminated for 10 min by red light above 600 mμ (600 lux), and carotenoids were extracted with diethyl ether after saponification of chlorophylls. Chromatographic analysis of the extract obtained from illuminated chloroplasts in comparison with that from chloroplasts incubated in the

dark revealed that the contents of carotenes, lutein and violaxanthin decreased to 85, 76 and 70% of the original contents, respectively, after illumination. The content of neoxanthin was not altered by the illumination. The same behavior of neoxanthin was found previously[1] in the presence of CCCP.



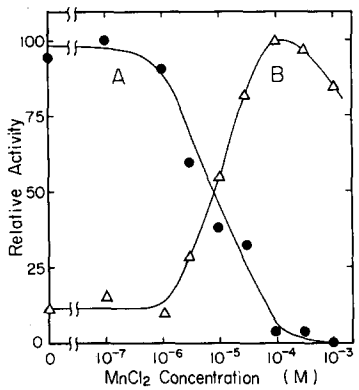


Fig. 1. The light-*minus*-dark difference spectra obtained for the Tris-washed chloroplasts illuminated by red actinic light above 680 mμ (170 lux) for 5 (Curve A) and 20 min (Curve B), respectively. The absorbance of the reference suspension kept in the dark was 0.69 at 490 mμ. The spectra were measured in the dark after illumination.

Fig. 2. The rates for photobleaching of carotenoids (Curve A) and the photoreduction of DCIP (Curve B) in Tris-washed chloroplasts as measured as a function of $MnCl_2$ concentration. For DCIP reduction, 30 μM DCIP was added to the chloroplast suspension. The bleaching rate was estimated from the absorbance drop at 490 mμ of chloroplast suspensions during the first 10 min of red light illumination, and the reduction rate was estimated from the absorbance drop at 610 mμ during the first 15 sec of illumination. The maximal reduction and bleaching rates, which were taken as 100%, were 10.2 μmoles DCIP per mg chlorophylls per h and 0.042 (absorbance drop at 490 mμ) per 10 min, respectively.

The effect of Mn^{2+} on photobleaching of carotenoids and photoreduction of DCIP

The photobleaching of carotenoids in Tris-washed chloroplasts, observed by direct absorption photometry at 490 mμ, was suppressed by $MnCl_2$ or $MnSO_4$. The addition of Mn^{2+} was made in the dark immediately before photobleaching measurements. The concentration of $MnCl_2$ required for 50% suppression of the bleaching rate was determined to be 8.0 μM (Curve A in Fig. 2), and 1 mM $MnCl_2$ completely suppressed bleaching. The degree of suppression was 81% with 0.1 mM $MnSO_4$ and 100% with 1 mM $MnSO_4$.

Tris-washed chloroplasts do not exhibit the Hill reaction activity with ferricyanide or $NADP^+$ as the oxidant[5,6]. Tris inactivated DCIP photoreduction as seen from Curve A in Fig. 3, which is the time-course of the light-induced absorbance change at 610 mμ in the presence of 30 μM DCIP. The DCIP photoreduction rate obtained for normal chloroplasts illuminated with red light (170 lux), estimated from the absorbance drop at 610 mμ during the first 15 sec of illumination, was 20.0 μmoles DCIP/mg chlorophylls per h (Curve B in Fig. 3). The rate obtained for Tris-washed chloroplasts similarly illuminated (Curve A) was 6% of that for normal chloroplasts. The rather low rate for normal chloroplasts was mostly due to the weak intensity of the actinic light. A higher rate of reduction (55.3 μmoles DCIP per mg chlorophylls per h) was obtained when normal chloroplasts were illuminated with strong white

light. DCIP was reoxidized in the dark when the actinic light was turned off (Fig. 3).

A remarkable fact is that the decreased activity of DCIP photoreduction found for Tris-washed chloroplasts is greatly restored by adding $MnCl_2$ or $MnSO_4$. Curve C in Fig. 3 shows the time course of DCIP photoreduction in the Tris-washed chloroplasts illuminated with red light in the presence of 0.1 mM $MnCl_2$. DCIP was rapidly reduced by illumination during the first 15 sec, and the reduction was repeatedly observable. The rate of the photoreduction in the presence of Mn^{2+}, however, decreased markedly after 20 sec of illumination. As estimated from the absorbance drop during the first 15 sec of illumination, the rate in the presence of 0.1 mM $MnCl_2$ (10.2 μmoles DCIP per mg chlorophylls per h) was 51% of that for normal chloroplasts. As estimated from the slope at zero time of illumination, the rate in the presence of 0.1 mM $MnCl_2$ (16.4 μmoles DCIP per mg chlorophylls per h) was as high as 80% of that for normal chloroplasts (20.5 μmoles DCIP per mg of chlorophylls per h). A similar degree of activation was obtained in the presence of 0.1 mM $MnSO_4$. It may be worth noting that the reoxidation of DCIP in the dark is also accelerated by adding Mn^{2+} (Curve C in Fig. 3). Mn^{2+} (0.1 mM $MnCl_2$) without chloroplasts did not, of course, reduce DCIP on illumination with white (200 000 lux) or red light. The photoreduction as well as the dark oxidation of DCIP in normal chloroplasts (Curve B in Fig. 3) was not affected by adding 0.1 mM $MnCl_2$. An experiment with $NADP^+$ in place of DCIP as electron acceptor showed that Tris-washed chloroplasts photoreduce $NADP^+$ rapidly provided that ferredoxin, ferredoxin–NADP reductase and Mn^{2+} are supplied. Addition of 0.1 mM $MnCl_2$ to the Tris-washed chloroplasts in the presence of two other requisites increased the reduction rate by more than 50%. More details on the restoration of $NADP^+$ photoreduction by added Mn^{2+} will be reported elsewhere.

The rate of DCIP photoreduction in Tris-washed chloroplasts increased with increasing Mn^{2+} concentration. Curve B in Fig. 2 presents the dependency of the reduction rate on the $MnCl_2$ concentration. A maximal reduction rate was obtained at 0.1 mM $MnCl_2$, and higher Mn^{2+} concentrations caused a deleterious effect on

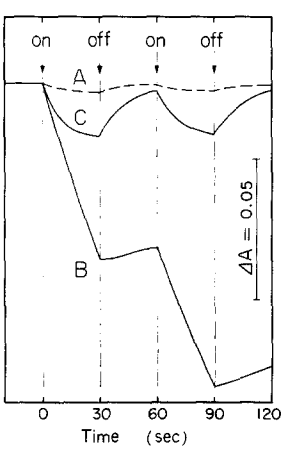


Fig. 3. The time courses of the light-induced absorbance changes at 610 mμ of chloroplasts; Tris-washed (Curve A), normal (Curve B) and Tris-washed *plus* 0.1 mM $MnCl_2$ (Curve C). 30 μM DCIP was added to each sample. Red actinic light was turned on or off as indicated by arrows. The downward change corresponds to photoreduction of DCIP, and the absorbance indicated (0.05) is equivalent to 2.4 μM DCIP.

reduction. The concentration of $MnCl_2$ required to activate the DCIP photoreduction to one half of the maximal rate was 8.0 μM, which approximates the concentration required for 50% suppression of photobleaching of carotenoids (Curve A in Fig. 2).

The activation of DCIP photoreduction by Mn^{2+} may be due to uncoupling of the electron flow from phosphorylation, since the Hill reaction is known to be accelerated by this uncoupling effect[12]; however, DCIP photoreduction in Tris-washed chloroplasts in the absence of Mn^{2+} was not activated by adding 4 mM NH_4Cl as a strong uncoupler.

These observations strongly suggest that the Tris wash has removed manganese from chloroplasts. In fact, repeated determinations of manganese in normal and Tris-washed chloroplasts indicated that 0.8 M Tris–HCl extracts on an average 33% of the chloroplast manganese (Table I). The manganese content in normal chloroplasts was 1.04 gatoms per 50 moles chlorophyll, corroborating that reported previously[13] for spinach chloroplasts. HOMANN[7] reported recently that the manganese content in tobacco chloroplasts decreased by 65–85 % after either the Tris wash or a heat treatment at 50°.

TABLE I

MANGANESE CONTENT IN TRIS-WASHED CHLOROPLASTS AS COMPARED TO THAT IN NORMAL CHLOROPLASTS

The content was determined for five different chloroplast preparations and was expressed in atoms/50 moles chlorophyll.

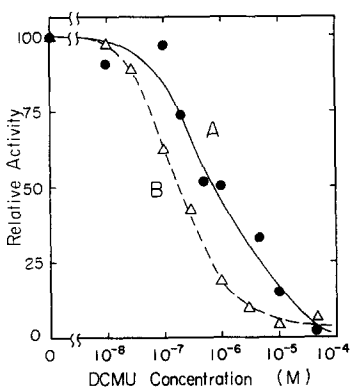
Prep.	*Mn content*		*Difference* (%)
	Normal	*Tris-washed*	
a	1.10	0.77	30
b	1.00	0.62	38
c	1.03	0.71	31
d	0.91	0.64	30
e	1.17	0.78	33
Av.	1.04	0.70	33

The photobleaching of carotenoids observed in Tris-washed chloroplasts without Mn^{2+} and the DCIP photoreduction in the presence of 0.1 mM $MnCl_2$ were inhibited by DCMU as shown by Curves A and B in Fig. 4, respectively. The DCMU concentration for 50% inhibition of the bleaching and that for 50% inhibition of the reduction were 0.80 and 0.17 μM, respectively. DCMU at a concentration of 50 μM was sufficient for complete inhibition of these processes (Fig. 4). The photobleaching of carotenoids was also suppressed by other members of Type II inhibitors of the Hill reaction: 0.1 mM prometryne (2-methylthio-4,6-bis-isopropylamino-*s*-triazine) caused complete inhibition of the bleaching, and 0.1 mM *o*-phenanthroline inhibited 65%. Addition of 1 mM ascorbate *plus* 20 μM *p*-phenylene diamine inhibited the photobleaching in Tris-washed chloroplasts by 62% and 1 mM ascorbate *plus* 20 μM TMPD by 88%. Ascorbate alone was less inhibitory than the coupled systems. These inhibitors and reducing agents also suppressed the photobleaching of carotenoids induced by CCCP as described previously[1]. The photobleaching of carotenoids observed for normal

chloroplasts in the presence of 10 μM CCCP was inhibited 78% upon addition of 0.1 mM $MnCl_2$, and the DCIP photoreduction observed for Tris-washed chloroplasts supplied with 0.1 mM $MnCl_2$ was inhibited 53 and 73% by 10 μM CCCP and by 0.1 mM CCCP, respectively. DCIP photoreduction in normal chloroplasts was inhibited 67% by addition of 10 μM CCCP. However, addition of 1 mM $MnCl_2$ to the CCCP-inhibited normal chloroplasts did not activate photoreduction.

Effects of Tris treatment, Mn^{2+} and CCCP on the fluorescence of chloroplasts

Curves A and B in Fig. 5 are the time-courses of fluorescence obtained for normal and Tris-washed chloroplasts, respectively. The fluorescence yield of these chloroplast samples increased with time and reached a steady-state level after 30–40 sec of illumination. In agreement with previous observations[14,15], the fluorescence rise for normal chloroplasts (Curve A) was biphasic and showed inflection at approx. 1 sec. The data in Fig. 5 revealed that the fluorescence yield for Tris-washed chloroplasts was lower than that for normal chloroplasts: the yield found for Tris-washed chloroplasts was 68% of that for normal chloroplasts when estimated after 40 sec of illumination (Curve B). The steady yield for Tris-washed chloroplasts was recovered to 82% of the yield for normal chloroplasts by addition of 0.1 mM $MnCl_2$ (Curve C in Fig. 5). A similar increase in the fluorescence yield was observed by adding either



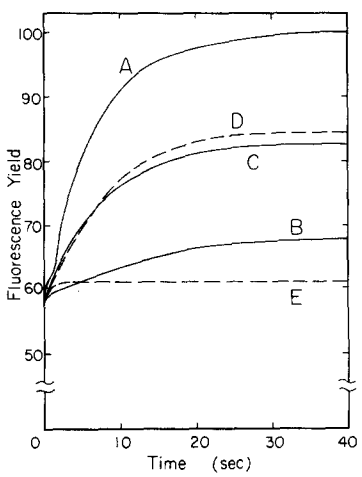


Fig. 4. The rates for photobleaching of carotenoids (Curve A) and the photoreduction of DCIP (Curve B) in Tris-washed chloroplasts measured as a function of DCMU concentration. For DCIP reduction, 30 μM DCIP and 0.1 mM $MnCl_2$ were added to the chloroplast suspension. The bleaching rate was estimated from the absorbance drop at 490 mμ during the first 10 min of red light illumination, and the rate of the reduction was estimated from the drop at 610 mμ during the first 15 sec of illumination. The maximal rates of reduction and bleaching, which were taken as 100%, were 10.2 μmoles DCIP per mg chlorophylls per h and 0.046 (absorbance drop at 490 mμ) per 10 min, respectively.

Fig. 5. The time-courses of the fluorescence above 600 mμ observed for normal chloroplasts (Curve A), Tris-washed chloroplasts (Curve B), Tris-washed chloroplasts in the presence of 0.1 mM $MnCl_2$ (Curve C), Tris-washed chloroplasts in the presence of 1 mM ascorbate *plus* 20 μM TMPD (Curve D) and normal chloroplasts in the presence of 10 μM CCCP (Curve E). The absorbance of the sample chloroplast suspensions was 0.10 at 435 mμ. The fluorescence was excited by 435 mμ light, and the fluorescence yield of normal chloroplasts (Curve A) obtained after 40 sec of illumination was taken as 100%.

1 mM ascorbate *plus* 20 μM TMPD (Curve D in Fig. 5) or 1 mM ascorbate *plus* 20 μM *p*-phenylene diamine. Addition of 0.1 mM $MnCl_2$ to normal chloroplasts had no appreciable effect on the fluorescence. It should be noted that addition of 10 μM CCCP to normal chloroplasts greatly decreased the time-dependent increment of fluorescence, lowering the steady yield to 61% of the original yield (Curve E in Fig. 5). A time-course similar to Curve E was obtained for the Tris-washed chloroplasts in the presence of 10 μM CCCP. The low steady yield found for the CCCP-inhibited normal chloroplasts was recovered to 89% of the steady yield for normal chloroplasts by adding 1 mM ascorbate *plus* 20 μM TMPD. However, addition of 0.1 mM $MnCl_2$ to the CCCP-inhibited chloroplasts only slightly increased the yield, while addition of 10 μM DCMU either to normal or to Tris-washed chloroplasts increased their steady yields by 60–70%.

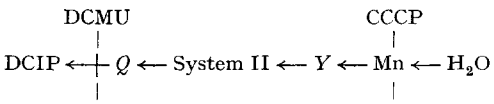
DISCUSSION

Photobleaching of carotenoids occurs in Tris-washed chloroplasts without addition of Type I Hill reaction inhibitors like CCCP, and photobleaching is suppressed by Mn^{2+} as well as by both Type II inhibitors like DCMU and reducing agents like ascorbate *plus* TMPD. The suppression of the bleaching by Mn^{2+} was accompanied by restoration of DCIP photoreduction. On the other hand, the fluorescence yield of chloroplasts was lowered by the Tris-wash treatment, and the decreased yield was recovered by addition of Mn^{2+} or ascorbate *plus* TMPD. These results demonstrate the site of Mn^{2+} function in the photosynthetic electron transport chain.

As discussed previously[1], photobleaching of carotenoids results from oxidation by the oxidized form of an electron carrier (Y) functioning on the water side of System II (see the scheme shown below). Photobleaching of carotenoids occurred in Tris-washed chloroplasts, substantiating the proposal of YAMASHITA AND BUTLER[5] that Tris blocks the electron transport on the water side of System II. According to our interpretation shown in the scheme, Tris blocks the electron transport from water to Y. The Mn^{2+} inhibition of the photobleaching in Tris-washed chloroplasts may be interpreted as an indication that Mn^{2+} participates in the reduction of Y^+ as does ascorbate *plus* TMPD. Ascorbate *plus* *p*-phenylene diamine or ascorbate *plus* TMPD has been considered to donate electrons to Y^+ (refs. 1 and 5). The supposition for the function of manganese is supported by the following facts: (a) Manganese is removed by Tris-wash treatment. (b) DCIP photoreduction is restored to a considerable extent by readdition of Mn^{2+}. (c) The restored photoreduction of DCIP is sensitive to DCMU. Furthermore, the fluorescence yield of chloroplasts is lowered by Tris treatment, and the decreased yield is recovered by addition of either Mn^{2+} or ascorbate *plus* TMPD. The observed lowering of fluorescence yield is due to the block on the water side of System II. This interpretation originates from the previous assumptions[3] that fluorescence yield is higher when a primary electron acceptor Q for System II is more reduced. The block on the water side of System II will decrease the supply of electrons for the reduction of Q and maintains Q more oxidized, thus lowering the yield.

The following electron transport mechanism can be postulated. CCCP may inactivate manganese, substantiated by the fact that CCCP lowers the fluorescence yield of normal chloroplasts and that the lowered yield is recovered by ascorbate *plus*

TMPD. In contrast to Type II inhibitors of the Hill reaction, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone[16] (an analogue of CCCP) or hydroxylamine[17] does not increase the fluorescence yield of algal cells even when the O_2 evolution is being completely inhibited by these agents. DCMU does not inhibit the electron transport on the water side of System II, judging from the fact[1] that DCMU cannot induce the photobleaching of carotenoids and only inhibits the bleaching.



Participation of manganese in the photosynthetic O_2 evolution has been proposed from other approaches (for review, see ref. 2). Cultures of algae and of higher plants deficient in manganese show a decreased activity of O_2 evolution; the activity is restored upon addition of manganese salts to the growth medium. According to CHENIAE AND MARTIN[18], this restoration observed with Scenedesmus cells is independent of *de novo* protein or chlorophyll synthesis but dependent on light. GERHARDT AND WIESSNER[19] determined the action spectrum for the restoration in Anacystis, which showed the participation of System II in the reaction. Furthermore, the yield of O_2 obtained with a brief saturating flash of light decreased when observed for manganese-deficient Scenedesmus cells, and these cells showed no marked changes in chloroplast lamellar structure and respiratory activity[13]. CHENIAE AND MARTIN[18] (also cited in ref. 2) isolated chloroplasts from normal Scenedesmus and removed manganese completely from the chloroplasts by a heat treatment at 50°. They demonstrated that System I activity such as $NADP^+$ photoreduction in the presence of ascorbate *plus* DCIP or photooxidation of reduced cytochrome *c* is not affected by completely removing manganese, while the Hill reaction with $NADP^+$ or ferricyanide is abolished. By fragmenting chloroplasts with digitonin, ANDERSON AND BOARDMAN[20] obtained two particle fractions responsible for System I and System II reactions, respectively. Determination of manganese contents in the two particle fractions[21] showed that the relative manganese content per Mg is higher in the System II particle.

KENTEN AND MANN[22] showed that Mn^{2+} is oxidized to Mn^{3+} by illumination when chloroplasts are added to a medium containing $MnSO_4$ and pyrophosphate. The chloroplast-mediated photooxidation of Mn^{2+} was inhibited by DCMU[23]. On the other hand, chloroplast-mediated photooxidation of 2,3-diketogulonate is stimulated by addition of $MnCl_2$ (ref. 24) and is inhibited by DCMU[23,25]. HABERMANN *et al.*[25] showed that diketogulonate is oxidized in the dark without chloroplasts provided that mangani pyrophosphate (Mn^{3+}–pyrophosphate complex) is present in the medium and that the quantum requirement for the chloroplast-mediated photooxidation of diketogulonate increases above 700 mμ where System I activity predominates. Recently HOMANN[7] reported that the fluorescence yield of manganese-depleted tobacco chloroplasts, which were prepared from leaves heated for 5 min at 46–50°, is lower than that of normal chloroplasts without heat treatment. They also demonstrated that the yield is restored upon addition of $MnCl_2$ and that the yield recovered by Mn^{2+} is again decreased by adding ferricyanide. It may be concluded that manganese is acting as the electron donor for Y^+ in the photosynthetic electron transport.

ACKNOWLEDGMENT

This work was supported by a grant in aid for Special Project Recearch on Biophysics given by the Japanese Ministry of Education.

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