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LIGHT-INDUCED SLOW CHANGES IN CHLOROPHYLL *a* FLUORESCENCE IN ISOLATED CHLOROPLASTS: EFFECTS OF MAGNESIUM AND PHENAZINE METHOSULFATE

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SUMMARY

We have investigated the possible relationships between the cation-induced and phenazine methosulfate (PMS)-induced fluorescence changes and their relation to light induced conformational changes of the thylakoid membrane.

1. In isolated chloroplasts, PMS markedly lowers the quantum yield of chlorophyll *a* fluorescence (ϕ_f) when added either in the presence or the absence of dichlorophenyldimethylurea (DCMU). In contrast, Mg^{2+} causes an increase in ϕ_f. However, these effects are absent in isolated chloroplasts fixed with glutaraldehyde that retain (to a large extent) the ability to pump protons, suggesting that structural alteration of the membrane—not the pH changes—is required for the observed changes in ϕ_f. The PMS triggered decrease in ϕ_f is not accompanied by any changes in the emission (spectral) characteristics of the two pigment systems, whereas room temperature emission spectra with Mg^{2+} and Ca^{2+} show that there is a relative increase of System II to System I fluorescence.

2. Washing isolated chloroplasts with 0.75 mM EDTA eliminates (to a large extent) the PMS-induced quenching and Mg^{2+}-induced increase of ϕ_f, and these effects are not recovered by the further addition of dicyclohexyl carbodiimide. It is known that washing with EDTA removes the coupling factor, and thus, it seems that the coupling factor is (indirectly) involved in conformational change of thylakoid membranes leading to fluorescence yield changes.

3. In purified pigment System II particles, neither PMS nor Mg^{2+} causes any change in ϕ_f. Our data, taken together with those of the others, suggest that a structural modification of the thylakoid membranes (not macroscopic volume changes of the chloroplasts) containing both Photosystems I and II is necessary for the PMS-induced quenching and Mg^{2+}-induced increase of ϕ_f. These two effects can be explained with the assumption that the PMS effect is due to an increase in the rate of internal conversion (k_h), whereas the Mg^{2+} effect is due to a decrease in the rate of energy transfer (k_t) between the two photosystems.

4. From the relative ratio of ϕ_f with DCMU and DCMU *plus* Mg^{2+}, we have calculated k_t (the rate constant of energy transfer between Photosystems II and I to be $4.2 \cdot 10^8$ s^{-1}, and ϕ_t (quantum yield of this transfer) to be 0.12.

Abbreviations: PMS, phenazine methosulfate; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; $DCIPH_2$: reduced dichlorophenolindophenol.

INTRODUCTION

In green plant tissues most of the fluorescence and all of the variable fluorescence is associated with the O_2-evolving System II. An approximate competition between chlorophyll *a* fluorescence and O_2 evolution may be seen from (a) the high quantum yield of fluorescence (ϕ_f), when photosynthesis is saturated (low quantum yield of O_2 evolution, ϕ_p)[1–3] (b) from the antiparallel relationship between ϕ_f and ϕ_p during a portion of the fluorescence induction in algae (see a recent review[4]). It has been shown that the short term (sec) changes in the yield of fluorescence reflect the state of the primary Photosystem II electron acceptor Q. An accumulation of reduced Q (Q^-) results in the closure of the traps and causes a high fluorescence yield; conversely, a high rate of electron flow keeps Q in the oxidized state and the fluorescence remains low[5]. The phenomonology of such qualitative competition between the rate of electron flow during photosynthesis and fluorescence linked to the redox state of Q has been amply documented.

Recently, it has been shown[6–8] that slow, long term changes in ϕ_f observed in intact algal cells cannot be easily explained on the basis of the oxidoreduction state of the Photosystem II acceptor as these changes exist even in the presence of dichlorophenyldimethylurea (DCMU), a potent inhibitor of photosynthesis. It has been postulated (see ref. 4) that these changes are controlled by the structural alteration of the pigment bed. In isolated chloroplasts most of the changes in the fluorescence yield have been shown to be related to the oxidation-reduction level of Q. However, Q-independent change in fluorescence yield was first observed by Homann[9] and later by Murata[10] in isolated broken chloroplasts, who have shown that the addition of cations enhances the fluorescence yield even in the presence of DCMU, which suggests that accumulation of reduced Q does not give rise to the maximal yield of fluorescence. Murata[10] proposed that this enhancement in the yield of fluorescence by the addition of cations is due to a suppression of energy transfer from highly fluorescent Photosystem II to weakly fluorescent Photosystem I (see refs 3, 4 and 11). Furthermore, some Photosystem I cofactors [phenazine methosulfate (PMS) and diamino durene] suppress the fluorescence yield, also acting independently of the redox reaction[12–14]. It has been postulated that this type of quenching is associated with the electrochemical state of the thylakoid membranes[14].

The data, reported in this communication, suggest that both cation induced enhancement and cyclic electron transport cofactor induced quenching of fluorescence in isolated broken chloroplasts are correlated with structural alterations of the chloroplast membrane. We have shown that the participation of both Photosystem I and Photosystem II is mandatory for the observed chlorophyll fluorescence changes. Although the molecular mechanism is still unknown, we present data that allow us to speculate on the possible cause of such alterations in the fluorescence yield.

MATERIALS AND METHODS

Oat, maize, lettuce or spinach leaves were either obtained from the market or grown in vermiculite in the laboratory. Broken, Class II chloroplasts from leaves were prepared as described earlier[15] although phosphate buffer, pH 7.8, replaced the Tris–HCl buffer. Tris-washed chloroplasts were made according to the procedure

of Yamashita and Butler[16]. Photosystem II particles were prepared according to the method of Huzisige *et al.*[17].

Removal of the coupling factor by use of 0.75 mM EDTA was done according to McCarty[18] with slight modification (several fold higher chlorophyll concentration than used before). Washing was done at room temperature. Differential centrifugation effectively removed the coupling factor from the chloroplasts, and the latter were then washed several times to remove the EDTA.

Glutaraldehyde fixation was done according to Park[19] with minor modifications. For measurements of the PMS-quenching effect, the chloroplasts were finally suspended in 0.05 M phosphate buffer, pH 7.8. In all experiments, all chemical additions were made in dark, and the pH of the additives was adjusted close to neutrality.

Absorption spectra of chloroplast suspensions were measured with a Bausch and Lomb, Inc. (Rochester, New York) recording spectrophotometer (Spectronic 505) equipped with an integrating sphere to reduce the errors due to light scattering. (The half-maximum width of the measuring light was 5 nm.)

Absorbance of chlorophyll solutions was measured in the Cary model 14 spectrophotometer. The chlorophyll concentration was determined according to Bruinsma[20] or Arnon[21].

Fluorescence measurements were carried out with a spectrofluorometer described elsewhere[22,23]. The procedure for measuring the fluorescence transient was essentially the same as that of Munday and Govindjee[24] and Papageorgiou and Govindjee[6]. For measurements of emission at room temperature and at 77 °K, the procedure of Cho *et al.*[25] was followed. The spectra were corrected for the spectral variation of the monochromator and the photomultiplier (EMI 9558B) unless otherwise stated. Other details are given in the legends of the figures and tables.

Light intensity was measured either with a Bi/Ag Eppley Thermopile (No. 6161) and a 150A Keithley microvolt ammeter or a Yellow Springs radiometer (model No. 63).

RESULTS AND DISCUSSION

In the presence of DCMU the fluorescence yield rapidly rises to a maximum[26] (due to the accumulation of reduced Q) and remains constant throughout the period

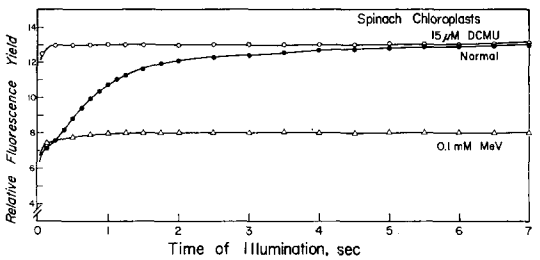

Fig. 1. Time course of chlorophyll *a* fluorescence in broken spinach chloroplasts with and without terminal electron acceptor. Fluorescence was measured at 685 nm (half-band width, 6.6 nm); ●—●, normal chloroplasts without any addition; ○—○, 15 μM DCMU; △—△, 0.1 mM methyl viologen (MeV); 0 level not recorded in these plots. Excitation, broad band blue light (C.S. 4-96 and C.S. 3-73), intensity, $1.4 \cdot 10^4$ ergs·cm^{-2}·s^{-1}; [chlorophyll], 40 μg in 3 ml buffer, 40 mM phosphate *plus* 10 mM KCl, pH 6.8; dark time 10 min before each measurement.

of illumination. However, when an excess of ferricyanide is added to untreated chloroplasts Q is kept in the oxidized state and the fluorescence yield remains low[27]. Fig. 1 illustrates the fluorescence yield changes that typically reflect the redox state of the primary Photosystem II acceptor Q. It shows fluorescence transients with DCMU and methyl viologen (an electron acceptor that is known to accept electrons from X, the primary electron acceptor of Photosystem I) in spinach chloroplasts that are in agreement with the quenching ability of the primary acceptor Q. We present this data to characterize our chloroplast preparations, to show the effect of methyl viologen (not available in the literature, to our knowledge), and to illustrate the changes in ϕ_f that are associated with electron transport as compared to changes that are independent of it (see below).

The cation effect

Table I (columns A and B) illustrates the effect of 5 mM $MgCl_2$ on the fluorescence yield in chloroplasts both in the presence and in the absence of DCMU. The effect of Mg^{2+} in increasing the fluorescence yield in the presence or absence of DCMU is clearly observed. The addition of cations such as Mg^{2+} causes a slow rise in fluorescence yield (approx. 2–3 min to reach the maximal level, figure not shown), as seen in intact cells and requires light. The measurement of the lifetime of fluorescence (τ) (made in collaboration with J. Hammond and H. Merkelo) indicates that the fluorescence intensity increase by Mg^{2+} is accompanied by an equivalent increase in τ. Thus, it is a true increase in the yield (ϕ_f).

TABLE I

RELATIVE FLUORESCENCE YIELD (FINAL STEADY-STATE) LEVEL UPON THE ADDITION OF 5 mM $MgCl_2$ TO NORMAL, DCMU-TREATED, TRIS-WASHED CHLOROPLASTS AND SYSTEM II PARTICLES

Spinach chloroplasts were washed and suspended in low molarity (0.005 M) Tris–HCl buffer. The rate of electron flow in normal chloroplasts was approx. 250 μmoles DCIP reduced per mg chlorophyll per h. Other details as in ref. 30.

Treatment	*A* *Normal chloroplasts*	*B* *Cloroplasts with 10 μM DCMU*	*C* *Tris-washed chloroplasts**	*D* *System II particles***
None	1.00 ± 0.04	1.50 ± 0.05	0.70 ± 0.04	1.00 ± 0.05
5 mM $MgCl_2$	2.25 ± 0.06	2.40 ± 0.06	1.20 ± 0.05	1.02 ± 0.06

* Rates of electron flow (DCIP reduction) ranged from <1 to <8 μmoles/mg chlorophyll per h (with water as donor) and 60 to 100 μmoles/mg chlorophyll per h (with diphenyl carbazide as donor).

** Rates of electron flow (DCIP) reduction ranged from 30 to 50 μmoles/mg chlorophyll per h with diphenyl carbazide as electron donor.

It was then necessary to check if the relative enhancement in fluorescence yield due to cations is not due to an alteration in the base level of fluorescence (F_0). Fig. 2 shows a fluorescence transient in lettuce chloroplasts with and without Mg^{2+}. It is clear that Mg^{2+} does not alter the constant F_0 level, but the steady-state yield increases (about 30–80%) by the addition of Mg^{2+} (*cf.* ref. 9).

It has been shown that ϕ_f and τ are linearly related[28,29]. From such measurements, it was concluded that the common pigment bed is cooperatively shared by

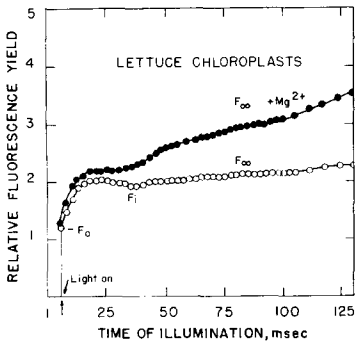

Fig. 2. Oscillograph recording (replotted) of fluorescence yield changes, with and without $MgCl_2$, in lettuce chloroplasts, λ observation, 685 nm. Note the absence of change in F_0 upon the addition of Mg^{2+}; buffer, 1 mM Tris–HCl, pH 7.8; [chlorophyll], 12 μg/ml. All other conditions as in Fig. 1.

several reaction centers of Photosystem II and, also, that the constant level of fluorescence (designated as 0 or F_0) originates from bulk pigments during the process of energy migration to open centers. Cations do not seem to alter the fluorescence yield of F_0 (see above), and thus, they do not appear to change the process of energy migration from the bulk chlorophyll to the photochemical centers.

The independence of Mg^{2+} effect from the net electron transport is already known as this effect can be observed in the presence of DCMU, when all the electron transport is inhibited and all Q is in the reduced state. We checked to see if the Mg^{2+} effect is present when photoreduction of Q is severely inhibited by blocking electron transport on the water side by Tris-washing. During illumination, the fluorescence yield of Tris-washed chloroplasts remains low as a result of an accumulation of both oxidized donor (Z) and acceptor (Q) of Photosystem II[30]. Table I (Column C) shows that under these conditions Mg^{2+} also induces its effect of increasing the fluorescence yield. Thus, whether the primary acceptor is in the reduced state or in the oxidized state, cations induce a similar effect. Thus, the cation effect is independent of the operation of the electron transfer chain.

The Mg^{2+} effect of increasing the ϕ_f can be observed in chloroplasts and in grana fragments, but it is absent in stroma lamellae that contain only Photosystem I[31]. Here we report the complementary experiment: the absence of Mg^{2+} effect on the purified Photosystem II particles from maize. There was no effect of magnesium salts on the fluorescence yield at 685 nm measured at room temperature (Table I, Column D).

These particles have a chlorophyll *a*/*b* ratio of 1.9 and are capable of dichlorophenolindophenol (DCIP) reduction with diphenyl carbazide (0.5 mM) as electron donor (see footnote** in Table I). They, however, do not reduce $NADP^+$ with reduced dichlorophenolindophenol ($DCIPH_2$). Upon the addition of 10 mM $MgCl_2$ in light at room temperature, and cooling to 77 °K, these Photosystem II particles show (Fig. 3) the emission spectra as shown by the drawn line: the ratio of F_{735}/F_{685} is identical (0.42) with and without Mg^{2+}. However, control chloroplasts showed a decrease in the same ratio from 2.75 to 1.70 upon Mg^{2+} addition. These data, along with those of Murata[31] convince us that both Photosystem I and Photo-

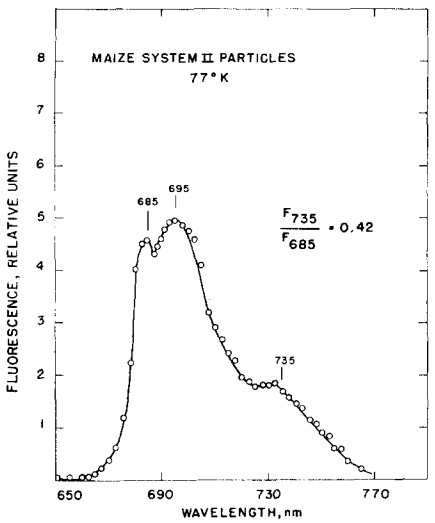

Fig. 3. Emission spectra of chlorophyll *a* fluorescence of photosystem II particles (Huzisige type) from maize chloroplasts at 77 °K. These Photosystem II particles do not show any Mg^{2+} or PMS-induced alteration in the yield; —, with Mg^{2+} or PMS; ○, no additions.

system II must be present on the membrane to show the Mg^{2+} effect. These results indicate that the enhancement of fluorescence yield is not due to change in spacing or orientation of chlorophyll a_{II} (chlorophyll *a* associated with Photosystem II) but due to an interaction with Photosystem I.

Changes in emission spectra at liquid N_2 temperature caused by the addition of cations have been taken as an indication of the mode of action of cations. Although emission characteristics of the two photosystems are clearly distinct at 77 °K, it is necessary to study the similar characteristic changes at room temperature. These are important because of possible errors that may occur with 77 °K spectra (see ref. 32). Fig. 4 shows the room-temperature emission spectra of lettuce chloroplasts with and without 10 mM $CaCl_2$. Here the fluorescence level at 685 nm has been adjusted to give the same value; a decrease in the fluorescence level at 720–740 nm (mainly Photosystem I) bands by the addition of cations is observed. There is a decrease in the ratio of F720/F685, by the addition of Ca^{2+}, suggesting an enhancement of the F685 (primarily Photosystem II) band with Ca^{2+} treatment, Room-temperature spectra of chloroplasts with and without 10 mM $MgCl_2$ showed similar results.

This type of change induced by cations in the emission characteristics of chloroplasts may lead to the suggestion (see ref. 10) that these cations bring about a suppression of energy transfer from Photosystem II to Photosystem I (however, see Concluding Remarks). Assuming that the cations cause a total suppression of energy from Photosystem II to Photosystem I, it is possible to estimate the rate constant (probability per unit time) and the efficiency of energy transfer from Photosystem II to Photosystem I. The rate constant was calculated to be $4.2 \cdot 10^8\ s^{-1}$ and the efficiency of transfer to be 12% (see Appendix).

Slow changes in the yield of fluorescence have been correlated with changes in the structural alterations of the thylakoid membranes (see review[4]). Some in-

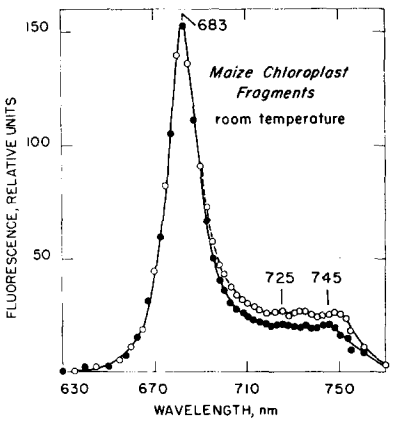

Fig. 4. Room-temperature emission spectra of chlorophyll *a* fluorescence of maize chloroplast fragments in the presence and the absence of $CaCl_2$; the fluorescence yield at 683 nm has been adjusted to give the same value; ○—○, no addition; ●—●, with 10 mM $CaCl_2$; λ excitation, 435 nm with 5-nm slits; λ observation, variable; 1.5-nm slits. Corning C.S. 2-61 before the photomultiplier; [chlorophyll], 15 μg/ml; buffer, 50 mM Tris-HCl, pH 7.4.

vestigators have suggested that the membrane-bound ATPase exerts a regulatory effect on the membrane conformation of chloroplasts[33-35]. We wanted to ascertain whether the conformational change attributed to the coupling factor is somehow linked to the cation-induced increase in Photosystem II fluorescence. As shown in Table II, no stimulation of 685 nm fluorescence yield by Mg^{2+} can be seen. Further addition of dicyclohexyl carbodiimide, that is known to restore pH changes[33], also did not cause any significant change in the 685-nm fluorescence yield. Thus, these

TABLE II

EFFECT OF EDTA-WASHING ON THE FLUORESCENCE YIELD CHANGES DUE TO Mg^{2+} AND PMS IN THE PRESENCE OF DCMU

3 ml of sample containing 15 μg chlorophyll/ml in 0.05 M phosphate buffer, pH 7.8, and 10 μM DCMU. Additions were made in the dark and 5 min were allowed for incubation. The rate of electron flow (DCIP reduction) in EDTA-washed chloroplasts (without DCMU) ranged from 70 to 100 μmoles/mg chlorophyll per h. Experimental procedure as indicated in the legend of Fig. 1.

Additions	*Relative fluorescence yield at 685 nm*
I. None	57.5
5 mM $MgCl_2$	55.5
10 mM $MgCl_2$	55.5
0.1 mM dicychlohexyl carbodiimide	51.0
0.1 mM dicyclohexyl carbodiimide +5 mM $MgCl_2$	48.5
II. None	57.3
30 μM PMS	52.7
0.1 mM dicyclohexyl carbodiimide	45.0
0.1 mM dicyclohexyl carbodiimide + 30 μM PMS	45.0

data suggest that the structural role of the coupling factor may be important for showing the effect of divalent cations on chloroplast membranes. Furthermore, proton transfer is probably not linked with fluorescence yield changes. We do not know the cause of the slight decrease in ϕ_f obtained upon the addition of dicoclohexyl carbodiimide but it is clear that the effect of Mg^{2+} in enhancing the yield of fluorescence is suppressed when the coupling factor is removed by EDTA extraction.

Fixation of membranes with glutaraldehyde immobilizes most of the structural changes of the chloroplasts[36,37]. Chloroplasts fixed with glutaraldehyde were then washed several times to deplete the contents of the ions. No significant increase in fluorescence yield, as compared with unfixed chloroplasts, was observed upon the addition of Mg^{2+}. Table III shows that the ratio of the long-wavelength fluorescence band to the short-wavelength fluorescence band (F735/F685) of chloroplasts, fixed with glutaraldehyde, with and without Mg^{2+} remains unchanged. Unlike unfixed chloroplasts, no shift in the emission characteristics was observed. This result suggests that ions induce a structural movement which in turn alters the fluorescence yield and emission characteristics. Loss of ability of structural alteration brings about a loss of enhancement of ϕ_f by cations.

TABLE III

COMPARISON OF THE RATIO OF THE LONG-WAVELENGTH (F730) EMISSION BAND TO THE SHORT-WAVELENGTH (F685) EMISSION BAND AT LIQUID-N_2 TEMPERATURE

Approximately 0.2 ml of chloroplasts, containing 3 μg chlorophyll/ml, were illuminated before cooling quickly to 77 °K. Emission spectra were measured with an excitation at 435 nm (half-band width, 6.6 nm).

Chloroplasts	*Additions*	*F730/F685*
I. Normal	None	2.4
	5 mM $MgCl_2$	1.8
	15 μM PMS	2.2
II. Fixed with glutaraldehyde*	None	2.3
	5 mM $MgCl_2$	2.3
	15 μM PMS	2.2

* The rate of electron flow (DCIP reduction) ranged from 50 to 70 μmoles/mg chlorophyll per/h.

Changes in membrane conformation are believed to be linked to electron transport *via* ion transport. Some authors consider membranes of chloroplasts[38,39] and mitochondria[40] to possess fixed anion groups performing many functions. Electron transport induced ion transport would alter the charge distribution and hence, the conformation of the membrane. Deionization of chloroplasts would lead to the removal of loosely bound cations and would induce repulsion between like charges on the membrane and hence, change in the conformation of the membrane. According to this picture, alkaline pH would enhance repulsive interaction and acidic pH would reduce it. The associated changes in membrane structure would

also change the chlorophyll *a* emission characteristics of chloroplasts. Fig. 5 shows changes in emission spectra measured at 77 °K of oat chloroplasts suspended in neutral, acidic and alkaline medium before freezing. We observed that in acidic pH (Curve I) the main Photosystem II fluorescence band is enhanced and the long wavelength band is suppressed extensively over the control at neutral pH (Curve II). This emission spectrum (Curve I) looks similar to the emission spectra of chloroplasts treated with Mg^{2+} and other cations. On increasing the pH of the medium, by adding NaOH, the emission at 685 nm is progressively lowered, and the long-wavelength fluorescence band (735 nm band) is enhanced (Curve III). Unlike Mg^{2+} treatment, acidification did not cause a large increase in yield of fluorescence at room temperature. This may be due to some loss of chlorophyll from the membrane at low pH. However, our results indicate that the spectra are reversible and therefore, any specific loss of any particular form of chlorophyll *a* is perhaps not the cause for the change in fluorescence emission characteristics. The shift in emission characteristics may be due to specific alteration in the membrane structure. It seems that ionic forces play an equally important role in hydrophobic interaction in the organization of the membrane and associated changes of energy transfer between the two photosystems.

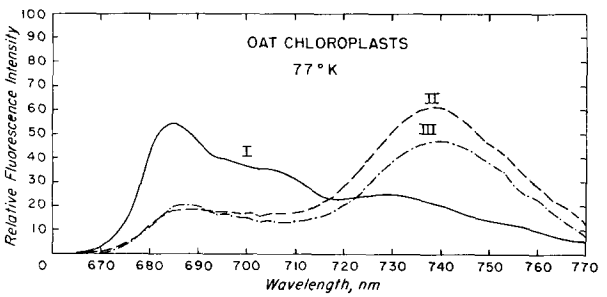

Fig. 5. Emission spectra of chlorophyll *a* fluorescence in oat chloroplasts suspended in acidic, alkaline and neutral medium. – –, suspended in 5 mM phosphate buffer, pH 7.0; —, sample was acidified to pH 3.8; —.—, same sample was titrated to pH 9.5 by adding 0.2 M NaOH.

PMS-induced changes in fluorescence yield

Another type of slow change in fluorescence yield not linked with the redox state of Q is the lowering in the yield of fluorescence with cofactors of cyclic electron transport such as PMS or diaminodurene[12–14]. Wraight and Crofts[14] showed that diaminodurene-induced fluorescence quenching in the presence of DCMU is reversed by a variety of uncouplers and ionophorous antibiotics, suggesting that this quenching is linked with energy conversion processes. Furthermore, Cohen and Sherman[41] have shown a direct correlation between proton translocation and diaminodurene-induced quenching of chlorophyll *a* fluorescence. It is also known that PMS induces structural changes in chloroplasts[42,43]. Our data, reported below, indicate that structural alterations are also associated with fluorescence lowering by PMS (probably also by diaminodurene). Fig. 6 confirms the effect of PMS in causing a decline in the chlorophyll fluorescence yield. This decline is slow, with a $t_{\frac{1}{2}}$ of approximately 10–15 s. The lowering of fluorescence yield by PMS occurs only in the light; the fluorescence yield recovers in the dark to its original level which requires a 30–40 s dark period. The extent of quenching decreases on sucessive illumination.

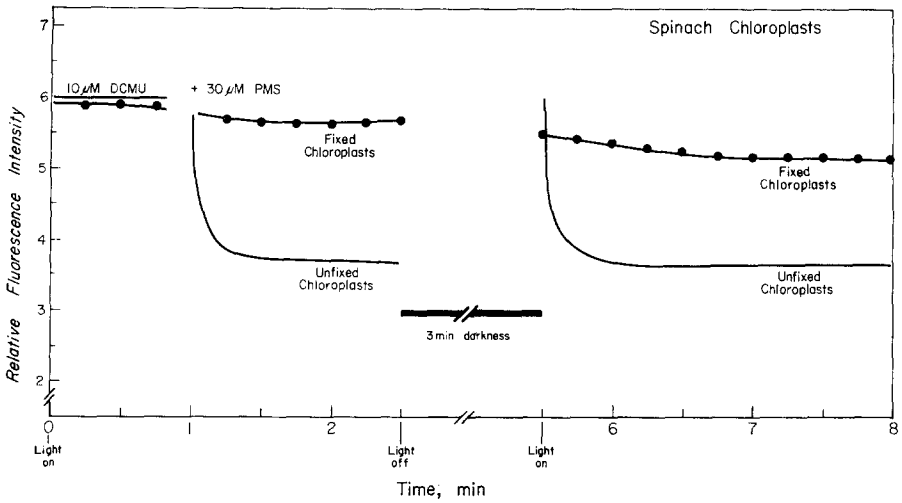

Fig. 6. PMS-induced quenching of chlorophyll *a* fluorescence in spinach chloroplasts in the presence of DCMU in unfixed and glutaraldehyde fixed chloroplasts. (Left) First 10 μM DCMU was added and then chloroplasts were illuminated and fluorescence yield was measured; then 30 μM PMS was added and fluorescence recorded again until new steady state fluorescence was reached. (Right) Recovery of (light induced) PMS quenched fluorescence after 3 min dark time. [Chlorophyll], approx. 14 μg/ml, 3 ml total volume; buffer, 50 mM Tris–HCl, pH 7.8. Excitation, blue light, $1.8 \cdot 10^6$ ergs·cm^{-2}·s^{-1}. Note the absence of PMS-induced quenching of ϕ_f when chloroplasts were fixed with glutaraldehyde.

We measured the effect of PMS, $PMSH_2$ $DCIPH_2$ on fluorescence yield of DCMU-treated oat chloroplasts. Reduced PMS and oxidized PMS caused about the same amount of quenching (Fig. 7). $DCIPH_2$ did not cause any appreciable quenching of ϕ_f. Ascorbate alone (not shown) also did not alter the yield. Increasing the concentration of PMS to 100 μM suppresses the initial fluorescence level, but the extent of slow lowering in the yield remains fairly constant, both in the presence and in the absence of DCMU, if the exciting light is sufficiently bright.

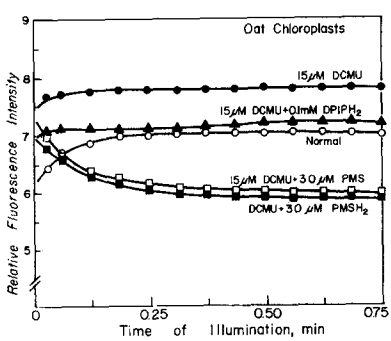

Fig. 7. Time course of chlorophyll *a* fluorescence in DCMU-poisoned oat chloroplasts in the presence of cofactors of Photosystem I reaction. 12.5 μg chlorophyll/ml; 50 mM Tricine buffer, pH 7.8. Additions as shown; PMS and DCIP were reduced with 0.02 M neutralized ascorbate. Excitation, blue light; intensity, $2.0 \cdot 10^6$ ergs·cm^{-2}·s^{-1}.

Murakami and Packer[42] have shown that PMS-catalyzed cyclic electron flow decreases the thickness and spacing of grana stacks. It is also known that cations strengthen the stacking of grana in broken chloroplasts. Table IV shows that addition of Mg^{2+} does not alter the chlorophyll *a* fluorescence quenching by PMS; the extent of quenching by 30 μM PMS remains approximately the same in the presence or in the absence of 5 mM $MgCl_2$ in normal or Tris-washed chloroplasts. The observed small decrease in the extent of quenching in the $MgCl_2$-treated chloroplasts is too negligible to account for any appreciable effect of cations in the presence of PMS. Thus, cations do not override the effect of PMS on lowering the fluorescence yield.

TABLE IV

EFFECT OF Mg^{2+} ON THE PMS-INDUCED LOWERING OF STEADY-STATE FLUORESCENCE YIELD IN NORMAL AND TRIS-WASHED CHLOROPLASTS IN THE PRESENCE OF 10 μM DCMU

3 ml of samples, containing 18.5 μg chlorophyll/ml, were suspended in 0.005 M Tris–HCl buffer, pH 7.8 containing 10 μM DCMU. PMS was added, after the initial fluorescence measurement, to give a final concentration of 30 μM. Fluorescence was again measured after 3 min of equilibrium with PMS. Fluorescence was excited by blue light (C.S. 4–72 and C.S. 3–73); intensity, 10 kergs·cm^{-2}·s^{-1}. λ observation, 685 nm (half-band width, 6.6 nm). Corning C.S. 2–61 filter was used to guard the entry of stray excitation light into the photomultiplier.

Treatment	*Initial intensity of fluorescence*	*Final intensity of fluorescence after addition of 30 μM PMS*	*Percent Quenched*
I. Normal chloroplasts	1.00	0.59	41.0
+ 5 mM $MgCl_2$	1.20	0.76	36.6
II. Tris-washed chloroplasts	0.70	0.45	34.1
+ 5 mM $MgCl_2$	1.02	0.75	26.4

Table III shows the ratio of F735/F685 at 77 °K in normal, $MgCl_2$- and PMS-treated samples. No significant change in this ratio was obtained when PMS was used, whereas Mg^{2+} showed its usual effect in these preparations. Results of Tables III and IV indicate that the quenching in fluorescence intensity by PMS is not associated with an observable shift in the distribution of quanta between the two photosystems. This is fully confirmed as shown by the complete emission spectra at 77 °K (Fig. 8). Apparent absence of changes in the spillover of energy between Photosystem I and Photosystem II is also shown when chloroplasts are washed with 0.8 M Tris, pH 8.0. The fact there is no observable shift in emission characteristics does not indicate that there is no change in spillover from Systems II to I, as it is possible that PMS induced structural change may in fact favor increased energy transfer from Photosystems II to I, but the energy thus transferred may be quenched there so efficiently that no change in yield or emission occurs.

PMS-induced (cyclic electron transport supported) change in the thickness and spacing of the membrane is markedly pronounced if the chloroplasts are incubated with weak acids[42]. Fig. 9 shows the effect of sodium acetate and NaCl on the

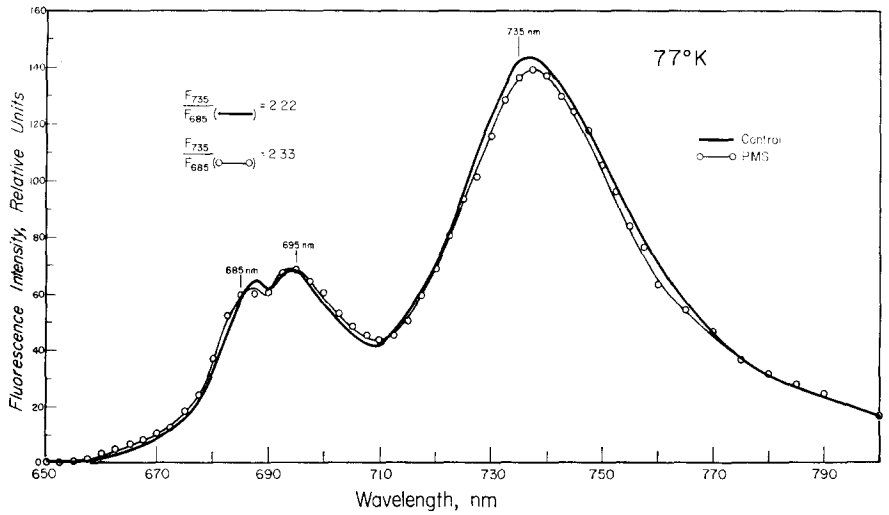

Fig. 8. Emission spectra of chlorophyll *a* fluorescence in spinach chloroplasts at 77 °K, with and without 30 μM PMS. 5 μg chlorophyll per ml; 10 μM DCMU; 0.2 ml total volume, preilluminated, at room temperature for 3 min and then cooled. λ excitation, 435 nm (10 nm slit width), λ observation, variable (3.3 nm slit width). C.S. 2–58 and 2–62 filters before the photomultiplier.

chlorophyll *a* fluorescence lowering by PMS. As with $MgCl_2$, NaCl does not cause any alteration in quenching, whereas in the presence of sodium acetate the fluorescence declines to a low level but again slowly increases to some intermediate level. We consider this small rise in the fluorescence level to be due to some microscopic alteration in the arrangement of the two photosystems. Although a general visible shrinkage of chloroplast volume may be achieved in a variety of ways, it does not necessarily result in the same type of alteration of chlorophyll *a* fluorescence. It is probably the specific molecular organization or environment that governs the interconversion of "states" between the two photosystems.

Similar to cation induced increase in the fluorescence yield at 685 nm, the PMS-induced fluorescence decline represents a true alteration in fluorescence yield

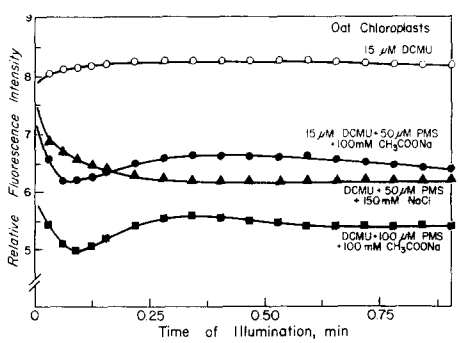

Fig. 9. Time course of chlorophyll *a* fluorescence in oat chloroplasts. ○—○, 15 μM DCMU; ●—●, 15 μM DCMU+50 μM PMS+100 mM sodium acetate; ▲—▲, 15 μM DCMU+50 μM PMS+150 mM NaCl; ■—■, 15 μM DCMU+100 μM PMS+100 mM sodium acetate. Other conditions as in Fig. 7.

(ϕ_f), as the lifetime of chlorophyll *a* fluorescence (τ) declines (data obtained in collaboration with J. Hammond and H. Merkelo). Thus, the decrease in yield by PMS, in the presence of DCMU, may result from an increase in the rate constant (probability per unit time) of radiationless loss. We suggest that this acceleration in loss arises from a structural modification of the membrane.

If the PMS effect is not due to observable changes in the spillover of excitation energy in favor of Photosystem I, then the question was whether it is still related to structural changes of another kind. Thus, we fixed chloroplasts with glutaraldehyde. Such a fixation abolishes all PMS induced quenching (Fig. 6). This is rather an interesting finding because such chloroplasts, although they have lost the ability to phosphorylate, retain about 40–50% of their ability to pump protons[37]. In collaboration with Stemler and Arntzen (unpublished observations, using the method of Dilley[39]) we confirmed that indeed our fixed chloroplasts (that showed no PMS-induced quenching of fluorescence) have pH changes as reported earlier[37]. For example, it was found that the rate of H^+ pumping (μmoles/mg chlorophyll per h) was reduced from 226 only to 80, and the extent (μmoles/mg chlorophyll) from 0.45 to 0.17; $3.3 \cdot 10^{-5}$ M carbonyl cyanide *m*-chlorophenylhydrazone, however, abolished these changes. These data clearly show that there is no simple correlation between pH changes and chlorophyll *a* fluorescence yield changes. This conclusion is in disagreement with that obtained recently by Cohen and Sherman[41] who observed a direct correlation between pH and diaminodurene-induced fluorescence quenching. Even though there was no quenching of chlorophyll *a* fluorescence by PMS in fixed chloroplasts, yet we checked the effect of PMS on the 77 °K fluorescence spectra of fixed chloroplasts. No significant effect was found (Table III).

Extraction of the coupling factor by EDTA (ref. 33) also causes a loss of ability of PMS to quench fluorescence, as shown in Table II. Addition of dicyclohexyl carbodiimide does not restore the PMS effect. (However, see ref. 41.)

If the structural changes abolished by glutaraldehyde fixation or EDTA washing* involve the movement of chlorophyll molecules closer or farther from each other in one and the same pigment bed, we should be able to detect quenching of chlorophyll *a* fluorescence by PMS in Photosystem II particles. No alteration of fluorescence yield by PMS was observed in Photosystem II particles; thus, the above hypothesis may be incorrect. This indicates that Photosystem I plays an important role in the PMS effect, and the absence of PMS induced quenching of ϕ_f in glutaraldehyde-fixed and DCMU-treated chloroplasts shows that the cyclic flow of electrons in Photosystem I alone is not sufficient to show the PMS effect.

However, cyclic electron flow and the energy conservation process therein related does seem to be, at least, indirectly related to the PMS-induced quenching effect, as uncouplers (carbonyl cyanide *m*-chlorophenylhydrazone, and salicylaniliden S_{13}) and an ionophorous antibiotic (gramicidin) reverse the effect of PMS (Table V) (also see refs 13 and 14). We imagine that cyclic electron flow in Photosystem I provides energy for the movement of Photosystem I and Photosystem II membranes closer to each other which results in the spillover of energy from strongly fluorescent

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* We cannot visualize how the coupling factor would directly control chlorophyll *a* fluorescence. But, it could probably undergo light-induced conformational alteration which may alter the orientation of the two pigment systems.

TABLE V

REVERSAL OF QUENCHING OF FLUORESCENCE INDUCED BY 20 μM PMS BY UNCOUPLERS OF PHOSPHORYLATION

3 ml of oat chloroplasts, containing 20 μg chlorophyll/ml, were suspended in 0.05 M phosphate buffer, pH 8.0 with 10 μM DCMU. Fluorescence was measured at 685 nm as excited by blue light (intensity, 10 kergs·cm^{-2}·s^{-1}). The uncouplers alone, at the concentrations used, had no appreciable effects on the steady-state fluorescence yield.

Addition	*Relative steady-state fluorescence intensity*	*Fraction of control*
None	115.5	1.000
PMS	83.5	0.722
+PMS+5 μM carbonyl cyanide *m*-chlorophenyl-hydrazone	105.2	0.919
+PMS+1 μM salicylanilide S_{13}	111.8	0.968
+PMS+4 μM gramicidin	98.7	0.855

Photosystem II to weakly fluorescent Photosystem I, but the excess energy is lost by radiationless decay and not as fluorescence.

CONCLUDING REMARKS

The slow fluorescence yield changes in isolated chloroplasts stimulated either by cations or by cofactors of Photosystem I-mediated electron transport appear to be analogous to the long term, slow changes in yield observed in intact cells. Both these changes are independent of Q-linked changes in ϕ_f. The data reported here clearly show that both photosystems are required to observe cation stimulated increase and PMS-induced decrease in the yield of fluorescence (Figs 3 and 4); an alteration of the structural organization between the two interacting photosystems is necessary for the change in the fluorescence yield. If this structural alteration is arrested by fixation or suppressed by the removal of the coupling factor, which seems to play a role in this type of conformational alteration, all fluorescence yield changes by either cations or PMS are inhibited or suppressed (Tables II and III; Fig. 6). We have clearly shown that Photosystem I plays the key role in (indirectly) altering the fluorescence yield, in addition to its effect on the redox state of Q. Although a macroscopic thylakoid shrinkage and change in grana stacking[12] are observed upon addition of cations or PMS, the macroscopic chloroplast changes do not regulate the fluorescence yield. This is obvious as in one case the ϕ_f increases, and in the other case it decreases. Also, as shown in Fig. 9, addition of sodium acetate, which causes a shrinkage of chloroplasts[43], results in a fluorescence yield decline and then a rise to an intermediate level when added together with PMS. Hence, it seems that the microscopic molecular organization and orientation of the two photosystems, of which we lack detailed knowledge at this time, regulate the fluorescence yield.

It is conceivable that ionic strength and concentration of specific ions govern the maximum yield of fluorescence by modifying one or all of the rate constants (Eqn 1 in Appendix) that regulate the decay of exited singlet chlorophyll. It can be

easily seen that a decrease in k_H (k_r+k_t) alone can account for all the observed cation induced effects. From the change in emission spectra by cations, Murata[10] concluded that cations suppress the rate of spillover from Photosystem II to Photosystem I. On the other hand, Sun and Sauer[44] believe that cation induced shrinkage of chloroplasts increases the rate of "back spillover" from Photosystem I to Photosystem II, although such a back transfer has not been experimentally shown. One can extend this argument to suggest that cation induced shrinkage of thylakoids and increased stacking of grana induce spillover of energy from Photosystem I to Photosystem II (as stated above), while PMS-stimulated macroscopic chloroplast structural changes favor energy transfer from Photosystem II to weakly fluorescent Photosystem I. However, it is difficult to visualize this model because closeness of Photosystem I to Photosystem II in both cases should increase energy transfer in both directions. As mentioned earlier, PMS does not cause any alteration in emission characteristics of Photosystem I compared to untreated samples even at liquid N_2 temperature where Photosystem I becomes strongly fluorescent. This difficulty does not, however, entirely rule out the above suggestion, as it is possible that continual electron transport is necessary to maintain the two photosystems in a specific organization or environmental conditions to assure enhanced spillover from Photosystem II to Photosystem I by PMS. Upon freezing, the Photosystem I-mediated PMS-catalyzed reaction stops, and we do not observe the effect of PMS. This suggestion derives support from the sensitivity of PMS-induced quenching to uncouplers and the requirement of continual cyclic electron transport. On the other hand, cations exert their increasing effect on the ϕ_f without the requirement of a sustained electron flow.

If cations and cyclic cofactors in fact do exert an antagonistic effect, as one would assume from the above proposition, one would expect a marked suppression of PMS-stimulated quenching by Mg^{2+} which is in direct contradiction with our experimental data. However, again this observation, although admittedly weakening the case, does not entirely rule out the possibility that PMS promotes a spillover from Photosystem II to Photosystem I and cations promote a reverse transfer. One can argue that PMS induced organization of the membrane, being associated with energy conservation, overrides the effect of cations. But the greater difficulty lies in the fact that experimental support for the back transfer from Photosystem I to Photosystem II by Mg^{2+} or other cations is not yet available.

In contrast, if we assume, as suggested by Murata[10], that cations suppress the spillover of quanta from Photosystem II to Photosystem I and PMS favors such a transfer it is equally difficult to visualize how a close positioning of the two photosystems that seem to be induced by Mg^{2+} and PMS will suppress spillover in one case and promote it in the other case. Admittedly, a meaningful correlation between structural states and fluorescence yield and emission characteristics cannot be made at this time because of the lack of information regarding the changes in molecular organization of the two photosystems. It seems, however, clear that PMS-induced slow change in fluorescence yield may be associated with some specific charged state of the membrane but not the H^+ concentration across the membrane. It is also clear that Photosystem I, besides its role in oxidizing QH to Q (Fig. 1), also regulates the yield and emission of Photosystem II by modifying the rate and extent of energy transfer, and that some specific alteration of membrane structure controls the mode of energy distribution between the two photosystems.

APPENDIX

Recently, Mar *et al.*[32] made an evaluation of the three modes of decay of the excited chlorophyll *a* singlet in algae from the measurement of τ with and without DCMU. However, the rate constant for dissipation into heat calculated from τ measurements included the energy transfer from fluorescent Photosystem II to non-fluorescent Photosystem I.

The fluorescence yield and the four possible modes of dissipation of the excited chlorophyll *a* in chloroplasts or algae are related by the following formulation:

$$\phi_f = \frac{k_f}{k_f + k_r + k_t + k_p[T]} \tag{1}$$

where k_f, k_r, k_t, k_p are first-order rate constants of fluorescence, radiationless loss by heat, loss by energy transfer to Photosystem I, and photochemistry, respectively; $[T]$ is the concentration of the open traps (see Weber[45]). If all Q (the primary acceptor of electrons of Photosystem II) is in the oxidized state, all the traps of Photosystem II are open, then ϕ_f given in Eqn 1 is minimum (assuming no other parameter affects the yield). If all the Q is kept reduced, *e.g.* during illumination in the presence of DCMU, ϕ_f will be represented as:

$$\phi_{f_{DCMU}} = \frac{k_f}{k_r + k_t + k_f} \tag{2}$$

Eqn 2 can then be rearranged as follows:

$$k_H = \left[\frac{1}{\phi_{f_{DCMU}}} - 1\right], \text{ and } k_p = k_f\left[\frac{1}{\phi_f} - \frac{1}{\phi_{f_{DCMU}}}\right] \tag{3}$$

where $k_H = k_r + k_t$.

If one assumes that the spillover of quanta from Photosystem II to Photosystem I is negligible when both DCMU and Mg^{2+} are present, the rate constant of radiationless loss can be represented as:

$$k_r = k_f\left[\frac{1}{\phi_{f_{(DCMU+Mg^{2+})}}} - 1\right] \tag{4}$$

From the algaebraic difference of Eqn 4 from 3, one derives:

$$k_t = (k_H - k_r) = k_f\left[\frac{1}{\phi_{f_{DCMU}}} - \frac{1}{\phi_{f_{(DCMU+Mg^{2+})}}}\right] \tag{5}$$

From our measurements, the relative ratio of $\phi_{f_{(DCMU+Mg^{2+})}}/\phi_{f_{DCMU}}$ is approximately 1.60 (Table I). Substituting this value into Eqn 5, we obtain:

$$k_t = 0.375 \times k_f/\phi_{f_{DCMU}} \tag{6}$$

The value for K_f can be assumed to be the same as estimated by Brody and Rabinowitch[46] for chlorophyll *a in vitro*. From their computation of τ_0 for chlorophyll *a* in solution as $15.2 \cdot 10^{-9}$ s, the k_f is $6.57 \cdot 10^7$ s^{-1}. This assumption may not be accurate in view of the existence of various forms of chlorophyll *a* (see French[47]). However,

in the first approximation, k_f *in vivo* should be very close to that of k_f for chlorophyll *a* in solution (*i.e.* $6.57 \cdot 10^7$ s^{-1}), because the absorption spectra of chlorophyll *a* *in vivo* and *in vitro* are quite similar.

The $\phi_{f_{DCMU}}$ can also be estimated from the measurements of the ratio of variable to constant (F_0) fluorescence level, assuming that the lifetime of fluorescence at F_0 level is the same as measured by Müller *et al.*[48] in very weak intensity of excitation. We usually observe a value of 3.0 for $(F\infty - F_0)/F_0$ in isolated broken spinach (or oat) chloroplasts (Fig. 1). If the lifetime of F_0 is taken to be $0.3 \cdot 10^{-9}$ s, hence, that of $F\infty$ is $0.9 \cdot 10^{-9}$ s.

$$\phi_{f_{DCMU}} = \tau/\tau_0 = \frac{0.9 \cdot 10^{-9}\, s}{15.2 \cdot 10^{-9}\, s} = 0.059 \tag{7}$$

(If, however, we assume that the lifetime of chlorophyll *a* fluorescence at F_0 in chloroplasts is equal to that of the lifetime of fluorescence at the "0" level in *Chlorella*, as measured by Briantais *et al.*[29] to be $0.6 \cdot 10^{-9}$ s, then $\phi_{f_{DCMU}}$ could be as high as 0.118.)

Inserting the values of k_f ($6.57 \cdot 10^{-7}$) and $\phi_{f_{DCMU}}$ (0.059) into Eqn 6, $k_t = 4.2 \cdot 10^8$ s^{-1}. The efficiency of energy transfer from Photosystem II to Photosystem I in chloroplasts can be estimated from the following equation:

$$\phi_t = \frac{k_t}{k_f + k_H + k_p} \tag{8}$$

where ϕ_t represents the quantum yield of spillover energy from Photosystem II to Photosystem I. Inserting the values of k_t, k_f, k_H, and k_p we estimate ϕ_t to be 0.118. (The value of k_H and k_p were estimated, from Eqn 3, to be $10.5 \cdot 10^8$ s^{-1} and $23.4 \cdot 10^8$ s^{-1}, respectively.) Assuming that the spillover photons are utilized in driving photochemical reactions at the reaction centers of Photosystem I, one estimates the overall efficiency of photochemical reactions to be approximately 70–78%. (For a calculation of the theoretical limit of 70–80%, see refs. 49 and 50.)

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REFERENCES

1 Franck, J. (1949) in *Photosynthesis in Plants* (Franck, J. and Loomis, W. E., eds), pp. 293–340, Iowa State College Press, Ames, Iowa
2 Krey, A. and Govindjee (1966) *Biochim. Biophys. Acta* 120, 1–18
3 Bonaventura, C. and Myers, J. (1969) *Biochim. Biophys. Acta* 189, 366–383
4 Govindjee and Papageorgiou, G. (1971) in *Photophysiology. Current Topics in Photobiology and Photochemistry* (Giese, A. C., ed.), Vol. 6, pp. 2–46, Academic Press, New York
5 Duysens, L. N. M. and Sweers, H. E. (1963) in *Studies on Microalgae and Photosynthetic Bacteria* (Ashida, J., ed.), pp. 353–372, University of Tokyo Press, Tokyo
6 Papageorgiou, G. and Govindjee (1968) *Biophys. J.* 8, 1299–1315
7 Papageorgiou, G. and Govindjee (1968) *Biophys. J.* 8, 1316–1328
8 Bannister, T. T. and Rice, G. (1968) *Biochim. Biophys. Acta* 189, 171–181
9 Homann, P. (1969) *Plant Physiol.* 44, 932–936

10 Murata, N. (1969) *Biochim. Biophys. Acta* 172, 242–251
11 Duysens, L. N. M. (1972) *Biophys. J.* 12, 858–863
12 Govindjee and Yang, L. (1967) cited in Govindjee, Papageorgiou, G. and Rabinowitch, E., in *Fluorescence Theory, Instrumentation and Practice* (Guilbault, G. G., ed.), pp. 511–564, Marcel Dekker, New York
13 Murata, N. and Sugahara, K. (1969) *Biochim. Biophys. Acta* 189, 182–192
14 Wraight, C. A. and Crofts, A. R., (1970) *Eur. J. Biochem.* 17, 319–327
15 Braun, B. Z. and Govindjee (1972) *FEBS Lett.* 25, 143–146
16 Yamashita, T. and Butler, W. L. (1968) *Plant Physiol.* 43, 1978–1986
17 Huzisige, H., Usiyama, H., Kikuti, T. and Azi, T. (1969) *Plant Cell Physiol.* 10, 441–455
18 McCarty, R. E. (1971) in *Methods in Enzymology* (San Pietro, A. ed.), Vol. 23 (Part A), pp. 251–253, Academic Press, New York
19 Park, R. B. (1971) in *Methods in Enzymology* (San Pietro, A. ed.), Vol. 23 (Part A), pp. 248–250, Academic Press, New York
20 Bruinsma, J. (1963) *Photochem. Photobiol.* 2, 241–249
21 Arnon, D. I. (1949) *Plant Physiol.* 24, 1–15
22 Govindjee (1966) in *Currents in Photosynthesis* (Thomas, J. B. and Goedheer, J. C., eds), pp. 93–103, Ad Donker Publ., Rotterdam
23 Shimony, C., Spencer, J. and Govindjee (1967) *Photosynthetica* 1, 113–125
24 Munday, Jr, J. C. and Govindjee (1969) *Biophys. J.* 9, 1–21
25 Cho, F., Spencer, J. and Govindjee (1966) *Biochim. Biophys. Acta* 126, 174–176
26 Malkin, S. (1968) *Biochim. Biophys. Acta* 153, 188–196
27 Malkin, S. and Kok, B. (1966) *Biochim. Biophys. Acta* 126, 413–432
28 Tumerman, L. A. and Sorokin, E. M. (1967) *Mol. Biol.* 1, 628–638
29 Briantais, J-M., Merkelo, H. and Govindjee (1972) *Photosynthetica* 6, 133–141
30 Mohanty, P., Braun, B. Z. and Govindjee (1972) *FEBS Lett.* 20, 273–276
31 Murata, N. (1971) *Biochim. Biophys. Acta* 245, 365–372
32 Mar, T., Govindjee, Singhal, G. S. and Merkelo, H. (1972) *Biophys. J.* 12, 797–808
33 McCarty, R. E. and Racker, E. (1967) *J. Biol. Chem.* 242, 3435–3439
34 Molotkovsky, Yu. G. and Dzybenko, V. S. (1967) *Biochim. Biophys. Res. Commun.* 29, 298–302
35 Bennoun, A. (1971) *Nature New Biol.* 233, 5–8
36 Packer, L., Allen, J. M. and Stark, M. (1968) *Arch. Biochem. Biophys.* 128, 142–152
37 West, J. and Packer, L. (1970) *J. Bioenerg.* 1, 405–412
38 Molotkovsky, Yu. G. and Dzybenko, V. S. (1968) *Nature* 219, 496–498
39 Dilley, R. A. (1966) *Brookhaven Symp. Biol.* 19, 258–280
40 Scarpa, A. and Azzi, A. (1968) *Biochim. Biophys. Acta* 150, 473–481
41 Cohen, W. S. and Sherman, L. A. (1971) *FEBS Lett.* 16, 319–323
42 Murakami, S. and Packer, L. (1969) *Biochim. Biophys. Acta* 180, 420–324
43 Packer, L., Deamer, D. W. and Crofts, A. R. (1966) *Brookhaven Symp. Biol.* 19, 281–302
44 Sun, A. S. K. and Sauer, K. (1971) *Biochim. Biophys. Acta* 256, 409–427
45 Weber, G. (1960) in *Comparative Biochemistry of Photoreactive Systems* (Allen, M. B., ed.), pp. 395–411, Academic Press, New York
46 Brody, S. S. and Rabinowitch, E. (1957) *Science* 125, 555
47 French, C. S. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 2893–2897
48 Müller, A., Lumry, R. and Walker, M. S. (1969) *Photochem. Photobiol.* 9, 113–126
49 Duysens, L. N. M. (1958) *Brookhaven Symp.* Biol. 11, 10–25
50 Ross, R. T. and Calvin, M. (1967) *Biophys. J.* 7, 595–614