Biochimica et Biophysica Acta, 430 (1976) 165–181

BBA 47061

EVIDENCE AGAINST PROTON GRADIENT FORMATION BEING THE CAUSE OF CHLOROPHYLL FLUORESCENCE QUENCHING BY *N*-METHYLPHENAZONIUM METHOSULFATE

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(Received July 14th, 1975)

SUMMARY

In strong illumination, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts exhibit a high yield of chlorophyll fluorescence while *P*-700 turnover, proton uptake, and phosphorylation are inhibited and a pH gradient is undetectable. When 10 μM *N*-methylphenazonium methosulfate (PMS) is included, the fluorescence yield in light is substantially reduced, and when 100 μM ascorbate is also included, the yield is diminished approximately to the level in darkness. Only very slight increases in *P*-700 turnover and proton uptake (but no detectable pH gradient) accompany the fluorescence yield decline.

When 10 μM PMS and 15 mM ascorbate are added to poisoned chloroplasts (the oxygen concentration being greatly reduced), *P*-700 turnover, proton uptake, the pH gradient and phosphorylation all reach high levels. In this case, the yield of chlorophyll fluorescence is low and is the same in both light and dark. Further addition of an uncoupler eliminates proton uptake, the pH gradient and phosphorylation but does not significantly elevate the fluorescence yield. From these observations we suggest that, in DCMU-poisoned chloroplasts, the fluorescence quenching with PMS occurs by a mechanism unrelated to the generation of a phosphorylation potential.

With chloroplasts unpoisoned by DCMU, PMS quenches fluorescence and considerably stimulates proton uptake, the pH gradient and phosphorylation. However, in this case, PMS serves to restore net electron transport.

INTRODUCTION

At least two different factors have been proposed to regulate the yield of

Abbreviations: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; NADP, NADPH, nicotinamide adenine dinucleotide phosphate and it's reduced form; PMS, *N*-methylphenazonium methosulfate; *P*-700, reaction center pigment for Photosystem I.

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chlorophyll fluorescence in plant cells and chloroplasts. One factor, identified by Duysens and Sweers [1], is the redox state of Photosystem II trapping centers, or of the primary electron acceptor Q associated with these centers. Murata and Sugahara [2] showed that an additional mechanism may govern the fluorescence yield. They observed that the strong fluorescence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts is quenched in light by the addition of cofactors (e.g. *N*-methylphenazonium methosulfate (PMS) and ascorbate) known to support cyclic electron transport and phosphorylation. They also found that the quenching with PMS is partly relieved when a high concentration of methylamine, an uncoupler of phosphorylation, is added. It was previously established that (i) PMS and ascorbate support cyclic phosphorylation [3], (ii) an impressed pH gradient can drive phosphorylation [4], and (iii) uncouplers prevent the formation of a pH gradient [5] and abolish phosphorylation [6]. With this information, Murata and Sugahara [2] hypothesized that fluorescence quenching by PMS is the direct result of a "high energy state", the essential characteristic of which is a pH gradient.

Using the cofactor diaminodurene, Wraight and Crofts [7] and Cohen and Sherman [8] have supported these observations and adopted this hypothesis. Recently, Krause [9] and Barber et al. [10] have modified the proposed ion mechanism of quenching: they suggested that Mg^{2+} extrusion from the thylakoid membrane, rather than the accompanying proton accumulation [11], is directly responsible for fluorescence quenching.

In contrast to the support for a PMS-catalyzed ion gradient regulation of fluorescence, several studies have shown that much of the PMS quenching may be unrelated to proton uptake activity. Papageorgiou [12] found that more than 50 % of the fluorescence quenching could be attributed to a direct interaction between PMS and excited chlorophyll molecules. Mohanty et al. [13] reported that chloroplasts fixed with glutaraldehyde retain the ability to pump protons while the PMS quenching of fluorescence is absent.

In this investigation, we re-examined the effects of the concentrations of PMS, ascorbate and uncouplers on the fluorescence yield, *P*-700 turnover and proton transport phenomena for two reasons. First, there is a weakness in the evidence available for the relation between fluorescence quenching and the generation of a "high energy state" or phosphorylation potential. In previous studies, the ability of a cofactor to cause chlorophyll fluorescence quenching was demonstrated with DCMU poisoned chloroplasts, but the ability of these cofactors to promote proton uptake or phosphorylation was either not measured [2, 12], or shown only for unpoisoned chloroplasts [7, 8]. Hence direct and quantitative evidence that fluorescence quenching and phosphorylation potential actually occur under the same experimental conditions is lacking. Secondly, to attribute the fluorescence quenching to a Photosystem I-generated phosphorylation state presupposes the occurrence of cyclic electron flow through *P*-700. In light of this presumption, it is essential to establish the correlation between the cofactor stimulation of *P*-700 turnover, a phosphorylation potential and the quenched level of chlorophyll fluorescence.

This study will show that the yield of chlorophyll fluorescence is not controlled by the magnitude of PMS-mediated proton uptake, or proton gradient formation or by the rate of phosphorylation. On the other hand, proton translocation and phos-

phorylation potential are well correlated with the *P*-700 turnover induced by PMS in DCMU-poisoned chloroplasts.

METHODS AND MATERIALS

Chloroplasts were isolated from 2–3-week-old dwarf peas (Greater Progress). About 5–10 g of fresh chilled leaves were ground in 10 ml of medium containing 0.4 M sucrose, 10 mM NaCl, and 20 mM Tris · HCl (pH 7.6), with a mortar and pestle, at 4 °C. After filtration through two layers of nylon cloth and a glass wool plug, the suspension was centrifuged in a Clay Adams table top centrifuge at full speed for 1–1.5 min. The pellet was resuspended in 0.2–0.4 ml of the same medium. Chlorophyll was determined by the method of Arnon [14].

For *P*-700 turnover measurements chloroplasts were suspended in the isolation buffer. The reaction medium for chlorophyll or 9-aminoacridine fluorescence measurements was the same except that 40 mM Tris · HCl at pH 7.1 was used. For proton uptake measurements, the medium contained 0.4 M sucrose and 10 mM NaCl; reaction mixtures were titrated to pH 7.05 with HCl or NaOH (0.01 M). The reaction medium for phosphorylation contained 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4 and 40 mM Tris · HCl, pH 7.86. ADP was added separately to a concentration of 5 mM.

Measurements of *P*-700 turnover were performed with the apparatus and methods of Hoch [15] and as previously described [16]. Chlorophyll fluorescence at 680 nm was measured with the same apparatus used in the fluorescope mode (see Hoch and Randles [17]).

9-Aminoacridine fluorescence was determined in the same manner except that the slow modulated exciting beam for 9-aminoacridine emission was 362 nm (monochrometer plus 362 nm interference filter and a Corning No. 5874 glass filter). 9-Aminoacridine emission occurring in the region 380–600 nm was isolated by Corning No. 3060 and No. 9782 glass filters. Transmitted exciting beam light, due to the minor overlap in this filter combination, was measured in samples without 9-aminoacridine and accounted for no more than 5 % of the total signal when 9-aminoacridine fluorescence was unquenched. This constant stray light component was automatically subtracted from the signal before calculating the quenching of 9-aminoacidine fluorescence. Quenching was calculated as: $Q = F^{D} - F^{L}/F^{D}$. Following Schuldiner et al. [18] internal pH was calculated as:

$$pH_{int} = -\log\left(H^{+}_{out} \cdot \frac{Q}{1-Q} \cdot \frac{1}{v}\right).$$

The value of v was taken to be 25 μl/mg chlorophyll $\times$ (mg chlorophyll per ml in the sample), based on estimates of osmotic volume by Dilley and Rothstein [19] and on work in this laboratory with chloroplast volumes. ΔpH was then determined as $pH_{ext} - pH_{int}$. 20 μM 9-aminoacridine was routinely used in this assay as it was determined to give maximum fluorescence quenching signals without apparent uncoupling. As a check, the inclusion of 20 μM 9-aminoacridine in a proton uptake measurement, described below, also had no net effect on the proton uptake although it did cause a transient overshoot and undershoot in the recorded pH change.

Proton uptake was calculated from the change in external pH, measured with

a combination pH microelectrode (Corning 476050), which was inserted into a magnetically stirred 4-ml sample chamber. The output of the pH meter (Instrumentation Laboratory Model 245) was recorded on a Mosley 7100 B recorder while illumination was obtained with a Kohler projection system. Proton uptake was calculated from the expression:

$$H^+(\mu\text{equiv./mg chlorophyll}) = \Delta\text{pH} \cdot \frac{K}{C}$$

where C was the total mg chlorophyll in the 4-ml sample, and K (μequiv./per ΔpH) was determined from the addition of 0.01 M NaOH to chloroplasts in the light. At pH 7.1, typical values for the chloroplasts in 0.4 M sucrose plus PMS were $C = 0.23$, $\Delta\text{pH} = 0.09$, $K = 0.55$ (μequiv. per unit pH): the calculated proton uptake was 0.215 μequiv. H^+/mg chlorophyll.

Phosphorylation experiments were performed in conical centrifuge tubes with approx. 0.1 mg of chlorophyll in 2 ml of the phosphorylation medium given above. All samples including those poisoned with DCMU were preilluminated for 1 min, after which, 5 mM ADP and other cofactors were added in darkness. The samples were then illuminated for 6 min at 14 °C. The reaction was stopped by the injection of 1 ml of 18 % $HClO_4$ and the samples placed on ice for 15 min. (Note, dark controls were stopped with acid immediately after the addition of ADP and cofactor. Also, those samples containing 15 mM ascorbate and PMS were allowed to incubate 6 min in dark before exposure to light.) The samples were centrifuged for 5 min and 2 ml of the supernatant were neutralized by the addition of 1 ml buffer containing 1.0 M morpholinopropane sulfonic acid and 2.2 M KOH. The samples were again placed on ice for 15 min, centrifuged and the supernatant withdrawn for ATP analysis. ATP was assayed by measuring the formation of NADPH coupled to the oxidation of glucose (see ref. 20).

The disappearance of dissolved oxygen in samples containing either methyl viologen or PMS was measured polarographically with a Clark type electrode (YSI model 5331).

With the exception of phosphorylation (14 °C) and net electron transport measurements (20 °C), all of the chloroplast experiments were carried out at 11–12 °C. At this temperature, chloroplast activity remained approximately constant for 1.5 h or more.

For the sake of comparison, all the above parameters with the exception of *P*-700 turnover were measured at or very close to saturating intensities of broad band (530–645 nm) actinic light. Despite the 10-fold difference in the intensities used for proton uptake and 9-aminoacridine fluorescence quenching (see Fig. 1), saturation curves revealed that greater than 75 % of proton uptake (measured in more dense samples) occurred with the same intensity used in the 9-aminoacridine and chlorophyll fluorescence measurements.

RESULTS

Qualitative effects of PMS, ascorbate, and uncouplers on chloroplast activities

The quantitative dependence of several activities on the concentrations of PMS, ascorbate and uncouplers will be presented in the following sections. The

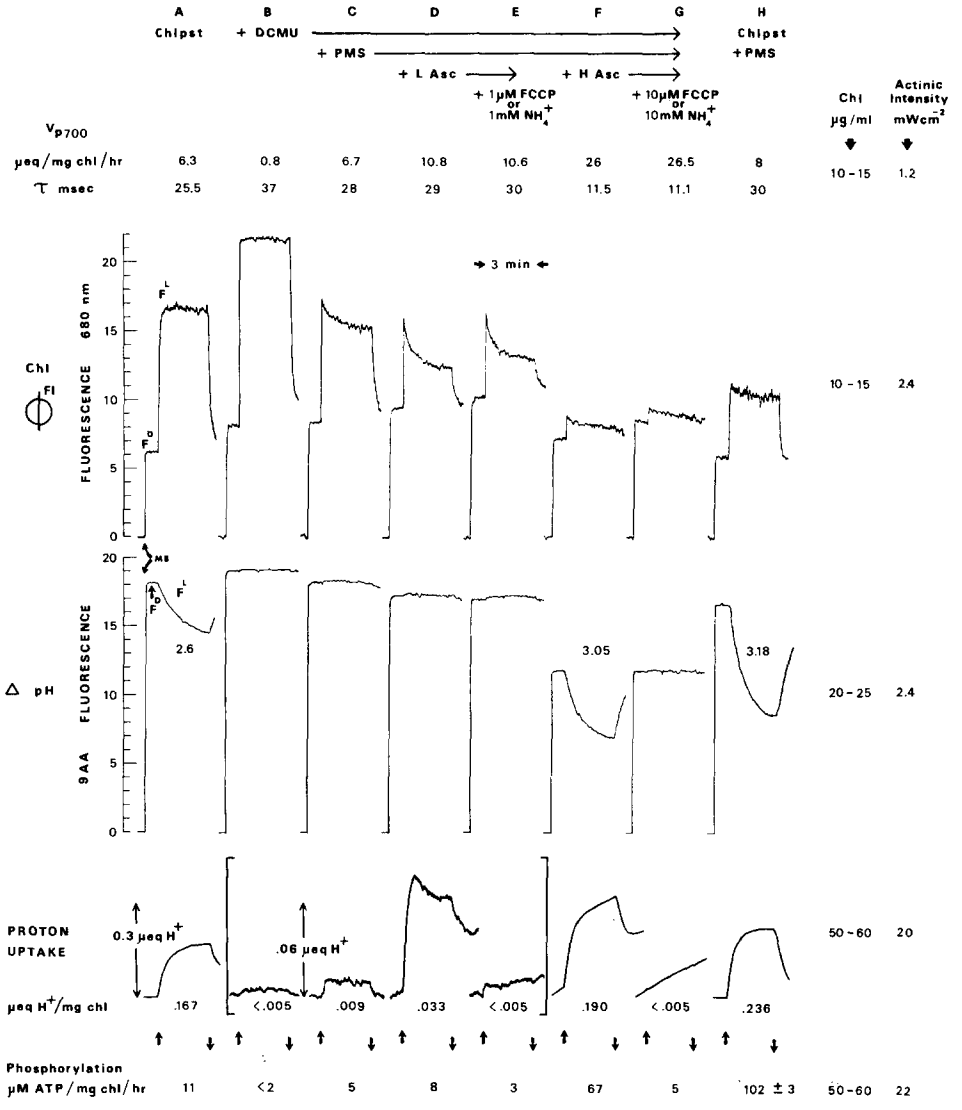

Fig. 1. Qualitative observations on chlorophyll fluorescence and photosystem I activity in the PMS system. Temperature, pH and reaction buffers as described in Methods and Materials. Additions as indicated in the figure were 10 μM DCMU, 10 μM PMS, 50 μM sodium ascorbate (L asc), and 15 mM sodium ascorbate (H asc). F^D and F^L indicate the fluorescence yield in dark (weak measuring beam MB only) and with strong actinic light, respectively. The weak (approx. 2 μW/cm^2) measuring beam to excite chlorophyll emission was 480 and 362 nm in the case of 9-aminoacridine emission. Arrows at the bottom of proton uptake traces indicate actinic light on (↑) and off (↓) for all traces. Note sensitivity change for proton uptake in brackets. The experimental range of chlorophyll concentrations and 530–645 nm actinic light intensities used in these experiments and throughout the paper are indicated in the last two columns on the right.

effects of one or two concentrations of each reagent are summarized in Fig. 1. The effects seen in this figure provide several arguments against proton uptake, a pH gradient, or the potential for phosphorylation (realized when ADP and P_i are present) being the cause of a lowered yield of chlorophyll fluorescence. (1) With unpoisoned chloroplasts, suspended in buffer without additions (Fig. 1A), there is a large proton uptake, a substantial pH gradient, and some limited capacity for phosphorylation; yet the fluorescence yield displays no evidence of a light-induced quenching effect. Neither proton uptake nor the pH gradient appreciably quench the fluorescence yield initially obtained in light. (2) When DCMU is added (Fig. 1B), proton uptake, the pH gradient and phosphorylation potential are abolished while the fluorescence yield is elevated somewhat. (3) The addition of PMS (Fig. 1C) leads to a decline of the fluorescence yield in light, but this substantial lowering is not accompanied by any restoration of proton uptake, a pH gradient, or phosphorylation potential. (4) When a low (50 μM) concentration of ascorbate is added (Fig. 1D), the fluorescence yield decreases further; there is a small proton uptake, but no pH gradient or phosphorylation. Evidently, the decrease in the fluorescence yield is not caused by the small proton uptake, since the latter can be abolished by an uncoupler (Fig. 1E) without affecting the fluorescence yield. (5) When a high (15 mM) concentration of ascorbate is added (Fig. 1F), there is an additional decline in both the light and dark fluorescence yields and very large increases in proton uptake, the pH gradient and phosphorylation potential. But again, an uncoupler (Fig. 1G) abolishes the latter three activities without significantly changing the fluorescence yield. (6) When PMS is added to unpoisoned chloroplasts (Fig. 1H), the fluorescence yield is quenched and proton uptake, the pH gradient and phosphorylation attain maximum levels. However, in this case, PMS may support non-cyclic electron transport (in a Mehler reaction [21]), and the fluorescence quenching could be due to a more oxidized state of Q, rather than to the modest increases in proton uptake and the pH gradient.

These results show that the fluorescence yield can be either high or low, whether proton uptake or a pH gradient occur or not; clearly, the fluorescence quenching with PMS is not caused by proton uptake, a pH gradient or phosphorylation potential.

The importance of oxygen in PMS-reducing systems

As previously suggested [3], we observed that PMS catalyzes the oxidation of ascorbate by dissolved oxygen in the absence of chloroplasts. Since photosynthetic activities were measured in a period of 1–5 min after the addition of PMS and ascorbate to our samples, it was conceivable that the concentrations of ascorbate and or oxygen had changed considerably. To assess these changes the dissolved oxygen concentration was measured with a Clark electrode.

Fig. 2 shows the time courses of oxygen concentration, in freshly prepared samples, for several different initial concentrations of ascorbate. The samples were initially air saturated (O_2 approx. 300 μM) and were kept in darkness. With the highest ascorbate concentration (15 mM), for which the reaction is the fastest, about 70 % of the oxygen is depleted in 5 min. In this case the ascorbate concentration is in excess and does not change significantly. (Related to this rapid uptake of oxygen were changes in photosynthetic activities which only reached steady-state levels after about 5 min.) With a low initial ascorbate concentration (e.g. 50 μM), it is the ascorbate rather than the oxygen concentration which can change significantly. How-

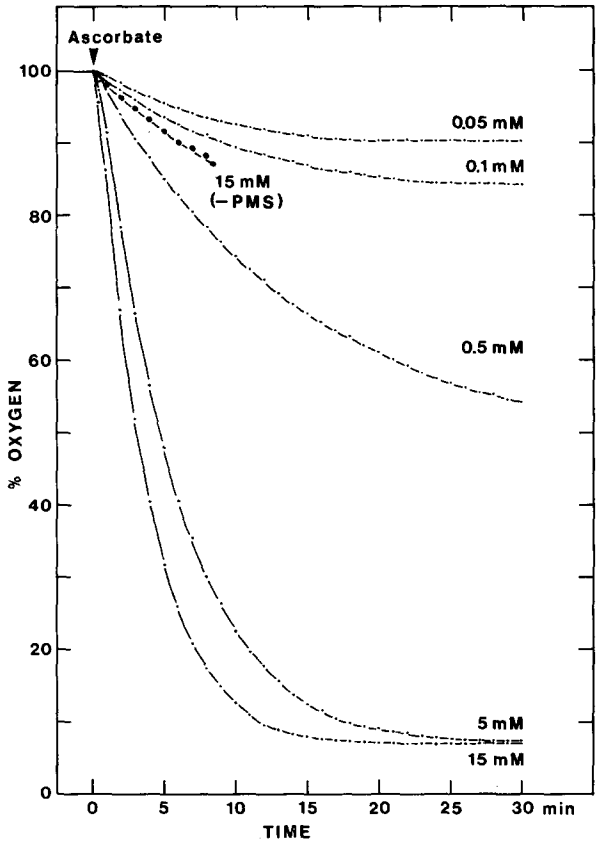

Fig. 2. Percent dissolved oxygen concentration versus time after the addition of ascorbate. Concentration of sodium ascorbate added at time zero as indicated. Reaction mixture included 10 μg chlorophyll per ml, 10 μM DCMU and 10 μM PMS (except where indicated) in the isolation buffer described in Methods and Materials. Oxygen recorded with a Clark electrode immersed in a plexiglass chamber containing 8 ml of magnetically stirred suspension at 14 °C and in the dark. Zero indicates the level recorded with excess dithionite.

ever, the rate of the reaction is much slower in this case, and it appears that not more than 50 % of the ascorbate is consumed in 5 min.

These measurements of oxygen concentration, together with the results shown in Fig. 1F and others presented later in Fig. 3, indicate that, cyclic Photosystem I activity is only restored to DCMU-poisoned chloroplasts when, besides PMS, enough ascorbate is added to achieve a low oxygen concentration. That it is low oxygen which is important, and not some other function of high ascorbate concentration, is shown by the fact that the removal of oxygen by glucose-glucose oxidase also restores *P*-700 turnover and the pH gradient to high values (Table I). These results agree with those of Jagendorf and Margulies [3] who showed that either aneerobic conditions or high ascorbate restore PMS-mediated phosphorylation in 3-(*p*-chlorophenyl)-1,1-dimethylurea(CMU)-treated chloroplasts.

The rapid removal of dissolved oxygen by ascorbate-reduced PMS suggests that PMS might also function as a Mehler reagent [21] in unpoisoned chloroplasts.

TABLE I

THE EQUIVALENCE BETWEEN HIGH ASCORBATE AND ANAEROBIC CONDITIONS IN THE DCMU-PMS SYSTEM

Measurement conditions as in Methods and Materials and Fig. 1 except all experiments were performed in isolation buffer at pH 7.6. Samples containing 10 mM glucose and 5 μg/ml glucose oxidase or 15 mM sodium ascorbate were allowed a 5-min incubation period before measurement.

	+10 μM DCMU →	+10 μM PMS →	+15 mM ascorbate + glucose + glucose oxidase
τ (ms)	36	28	10	16.2
v_{700} (μequiv./mg chlorophyll per h)	0.9	6.4	25	22
9-Aminoacridine Fluorescence Quenching	0	0	45 %	33 %
Δ pH	—	—	3.05	2.87

In Table II, the ability of PMS to support net electron transport from water to O_2 is compared with methyl viologen. Although about one half as effective as methyl viologen (column 1), PMS does support net electron transport at μM concentrations commonly used in phosphorylation experiments (column 5). Since this reaction is DCMU inhibitable (column 8), it is evident that substantial net electron transport occurs when PMS is added to unpoisoned chloroplasts. It follows that the large chlorophyll fluorescence quenching and maximal values of proton uptake, a pH gradient and phosphorylation in chloroplasts lacking DCMU (Fig. 1H) can be explained by the presence of net electron transport.

Surprisingly, the Mehler reaction supported by PMS is not uncoupled; rather it is inhibited by the addition of NH_4Cl in column 7. This anomaly may occur as a result of a chemical reaction between PMS and the amine, thereby rendering the cofactor inactive. Alternatively, the function of PMS as a Mehler reagent may be

TABLE II

A COMPARISON BETWEEN METHYL VIOLOGEN AND PMS AS MEHLER REAGENTS

Net electron transport rates measured as oxygen uptake in 8-ml samples with 17.8 μg chlorophyll per ml in "TN" buffer containing 10 mM NaCl and 20 mM Tris · HCl, pH 7.6. All samples contained 0.47 mM KCN. Concentrations of methyl viologen and PMS as indicated while DCMU and NH_4Cl concentrations were 10 μM and 2.3 mM, respectively. Actinic illumination of 20 mW/cm² broad band light was identical to that provided for proton uptake measurements (see Fig. 1 and Methods and Materials).

	11 μM methyl viologen	0.47 mM methyl viologen			11 μM PMS	0.47 mM PMS		
			NH_4Cl	DCMU			NH_4Cl	DCMU
Electron transport (μequiv./mg chlorophyll per h)	149	175	583	0	60	116	43	0

dependent on the coupling state of the chloroplast membrane. This data alone does not allow the distinction between the alternatives.

Dependence of photosynthetic functions on ascorbate concentration

Fig. 3 shows how the dissolved oxygen concentration, *P*-700 turnover, chlorophyll fluorescence yield, proton uptake and the pH gradient change as successive

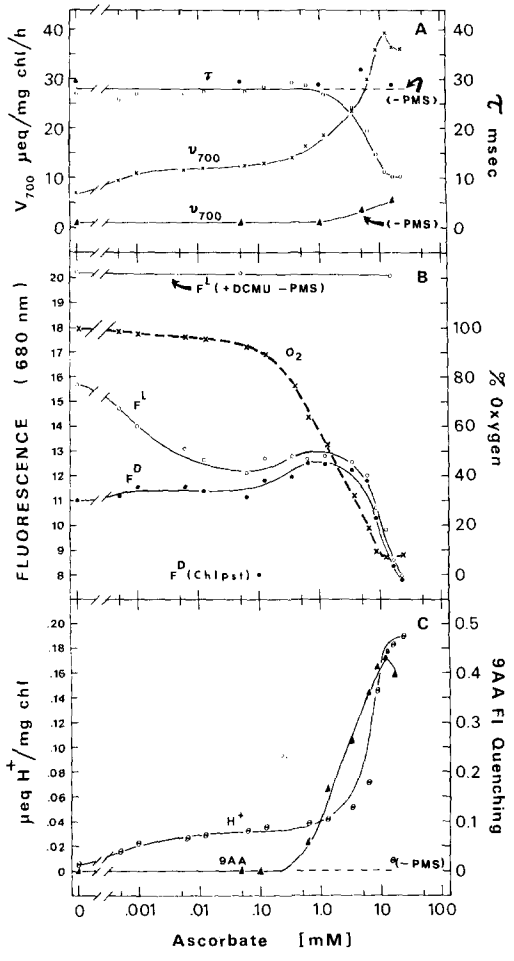

Fig. 3. Dissolved oxygen and photosystem I activity with PMS and as a function of successive additions of ascorbate. Ascorbate additions were spaced 5 min apart and the total ascorbate added is given on the abscissa. All samples included 10 μM DCMU and 10 μM PMS except when omitted in controls labelled (−PMS). In part A, the relaxation time (τ) is shown with PMS (○–○), and without PMS (●–●), and *P*-700 turnover with PMS (×–×), and without PMS (▲–▲). In part B, percent dissolved oxygen is given by the curve (×–×). Also in part B, chlorophyll fluorescence in light (F^L, ○–○) without PMS is given at the top and with PMS in the middle; the dark yield with PMS is given by (F^D, ●–●) and the dark yield of unpoisoned chloroplasts is indicated at the bottom. In part C, proton uptake (⊖–⊖) and 9-aminoacridine fluorescence quenching (▲–▲) is given with PMS; the dashed line is without PMS. See Figs. 1 and 2 and Methods and Materials for measurement conditions.

additions of ascorbate were introduced into a sample containing chloroplasts, 10 μM DCMU and 10 μM PMS. Ascorbate additions (totalling the amount shown) were made at fixed intervals of time, usually 5 min. The steady-state photosynthetic activities and dissolved oxygen were measured in the 4th to 5th min following each addition.

Over the lower range of ascorbate concentrations ($\leqslant$ 100 μM), the oxygen concentration is, at most, only a little reduced from that at air saturation (Fig. 3B). In this range, there is a decline in the fluorescence yield in the light and a small increase in the yield in the dark; at the upper end of this range, the light and dark yields become essentially equal. Correlated with these yield changes, there is a small increase in *P*-700 turnover and proton uptake, but in both cases the final values reached at 100 μM ascorbate are still quite low. No pH gradient, and no change in the relaxation time for *P*-700 turnover, is detected in this range. Clearly, all the light-induced quenching of chlorophyll fluorescence, with PMS, occurs over an ascorbate concentration range where little if any Photosystem I activity is restored.

In the upper range, from 1 to 20 mM ascorbate, the oxygen concentration at the time of measurements was substantially lowered (see Fig. 3B). In this range, the light and dark fluorescence yields drop sharply to the minimum level, that of chloroplasts alone in dark. Apparently, maximal quenching of the fluorescence with PMS occurs under highly reducing conditions (high ascorbate and low oxygen). This result agrees with Papageorgiou's finding [12] that semi-reduced PMS is a better chemical quencher than oxidized PMS. Also in this range, the time constant for *P*-700 turnover falls and *P*-700 turnover, proton uptake and the pH gradient rise to maximum values. As indicated previously, the ability of PMS to catalyze cyclic electron transport is strongly dependent on a lowered oxygen concentration. Similar concentration effects were seen when the reductant dithioerythritol was used in place of ascorbate.

Dependence of photosynthetic functions on PMS concentration

In Fig. 4 the light and dark fluorescence yields, proton uptake, *P*-700 turnover and the pH gradient are shown to vary with PMS concentration, in the presence of no (0), low (50 μM) and high (15 mM) ascorbate. The upper and lower arrows in Fig. 4A indicate the extreme range of fluorescence yield in these chloroplasts: the lower limit is that of chloroplasts alone in darkness and the upper limit that of DCMU-poisoned chloroplasts in light. In terms of these limits, it is seen that up to 10 μM PMS (in the absence of ascorbate) reduces the fluorescence yield in light by about 40 % of the maximum range. Alternatively, since both DCMU and PMS increase the fluorescence yield somewhat in darkness, one might prefer to define as the lower limit the yield in darkness in the presence of these substances. In this case, PMS causes the fluorescence yield in light to decline by about 60 % of this range. Viewed either way, PMS alone causes a considerable decline of the fluorescence yield in light, but, as shown in Figs. 4B and 4D, does not generate a detectable proton uptake or pH gradient. Addition of 10 μM PMS does cause a slight increase in *P*-700 turnover (Fig. 4C).

The inclusion of 50 μM ascorbate enhances the effects of PMS in Fig. 4A. With 10 μM PMS, the fluorescence yield in light is reduced by about 60 % of the extreme range, or by about 90 % of the range when the lower limit is taken as the yield in darkness in the presence of PMS and ascorbate. Thus, 50 μM ascorbate

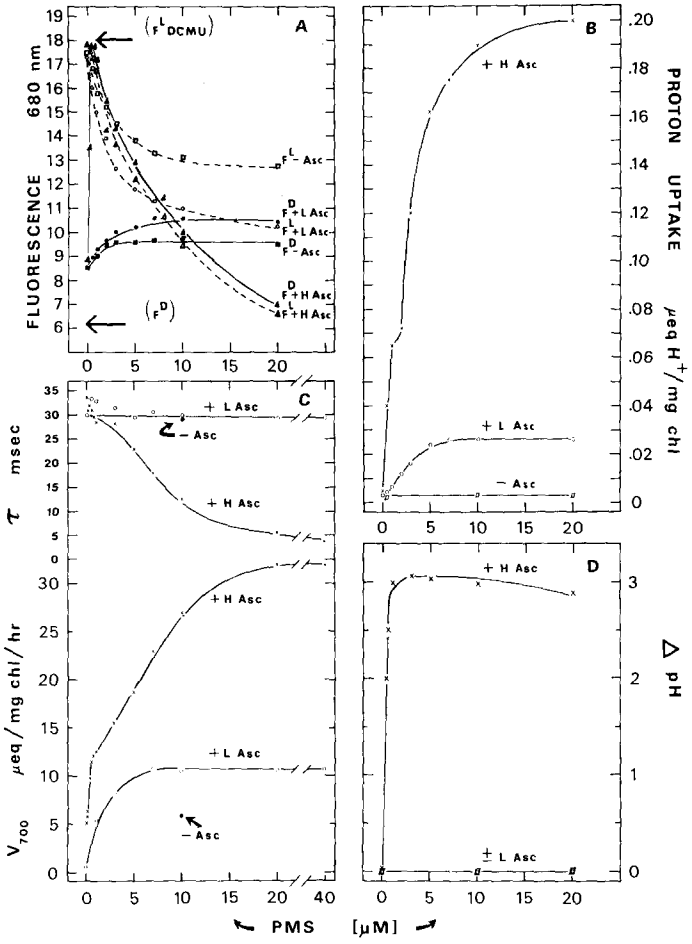

Fig. 4. PMS concentration curves for chlorophyll fluorescence and photosystem I activity in chloroplasts poisoned with 10 μM DCMU. Measurement conditions as described in Methods and Materials and Fig. 1. Results are presented without ascorbate (−Asc), with 50 μM ascorbate (+L Asc) and with 15 mM ascorbate (+H Asc). In part A, dashed lines indicate the fluorescence yield in light (F^L, open symbols) and solid lines indicate the fluorescence yield in dark (F^D, closed symbols). Extremes in the fluorescence yield are given by the arrows, DCMU-poisoned in light at the top and unpoisoned in dark at the bottom of part A. Fluorescence yield is given for the conditions (−Asc, (□, ■); +L Asc (○, ●); and +H Asc (△, ▲). In part B, proton uptake measured with −Asc (□), +L Asc (○), and +H Asc (×). In part C, *P*-700 turnover and (τ) are plotted for −Asc (●), +L Asc (○), and +H Asc (×). In part D, △ pH was obtained with −Asc (□), +L Asc (○), and +H Asc (×).

causes a further substantial decrease of the yield, beyond that produced by PMS alone. Figs. 4B–4D show that the inclusion of 50 μM ascorbate also causes (1) appearance of a small proton uptake, (2) a further modest increase in *P*-700 turnover, but (3) no detectable pH gradient.

With 15 mM ascorbate several remarkable features appear. (1) As little as 1 μM PMS elevates the fluorescence yield in dark almost to the extreme upper limit of

DCMU-poisoned chloroplasts in light. At progressively higher concentrations of PMS, the fluorescence yield, which is now always the same in light and dark, steadily falls, and, at 20 μM PMS, approaches the extreme lower limit, that of chloroplasts alone in darkness. (2) Figs. 4B and 4D show that proton uptake reaches a high value saturated by about 10 μM PMS while the pH gradient formed is saturated in the range of 1–2 μM PMS. That a relatively small proton uptake suffices for the development of a pH gradient is in accord with the findings of Schuldiner et al. [18]. (3) The time constant for *P*-700 turnover declines and *P*-700 turnover increases biphasically to a high rate, as the PMS concentration rises.

These observations do not point to a direct dependence of the fluorescence yield on the extent of proton uptake or the pH gradient. In the absence of ascorbate, PMS gives a sizeable quenching of the fluorescence without detectable proton uptake or a pH gradient. With low ascorbate (50 μM), PMS causes the fluorescence yield in light to be quenched nearly to the level in darkness, but there is no pH gradient and only a small proton uptake. With 15 mM ascorbate and concentrations of PMS greater than 1 μM, the fluorescence yield is the same in light and dark, and therefore, the light yield appears to be independent of the presence of a light-generated proton uptake or pH gradient. Moreover, whereas the pH gradient is maximized by 1 μM PMS, the fluorescence yield in light and dark steadily declines up to 20 μM PMS.

Dependence of the fluorescence yield and proton uptake on uncoupler concentration

Fig. 5 shows how the fluorescence yield and proton uptake change when the uncoupler concentration is gradually increased. With a low concentration of ascorbate (50 μM), Figs. 5A and 5B show that proton uptake is gradually abolished as the uncoupler concentration rises to 1 mM, in the case of NH_4Cl, and 1 μM, in the case of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Up to these concentrations, the fluorescence yield remains perfectly constant. With further augmentation of uncoupler concentration, small increases in the fluorescence yield occur, but these yield changes take place only after proton uptake has been totally eliminated. With high ascorbate concentration (15 mM), elimination of proton uptake requires about 10-fold higher concentrations of each uncoupler (Figs. 5C and 5D). With FCCP, the fluorescence yield remains constant. With NH_4Cl, a small increase in the yield accompanies the decline in proton uptake. However, since the fluorescence yield continues to increase gradually even after the total elimination of proton uptake, it appears that the yield increase and the decline in proton uptake must be independent phenomena.

These results confirm the findings of Murata and Sugahara [2] that high concentrations of uncoupler can, under some conditions, increase the fluorescence yield; but it is clear that such yield increases (which in any case are small when PMS is the cofactor) are not the result of inhibiting proton uptake.

Dependence of the fluorescence yield on magnesium and osmotic strength

Large increases in the fluorescence yield have been shown to result from additions of divalent cations, such as Mg^{2+}, to chloroplasts [22, 23]. This knowledge led Krause [9] and Barber et al. [10] to propose that Mg^{2+} extrusion and the subsequent reversal of the divalent cation effect [22–24] might be the direct cause of "high energy state"-dependent quenching of fluorescence. From the work of Krause

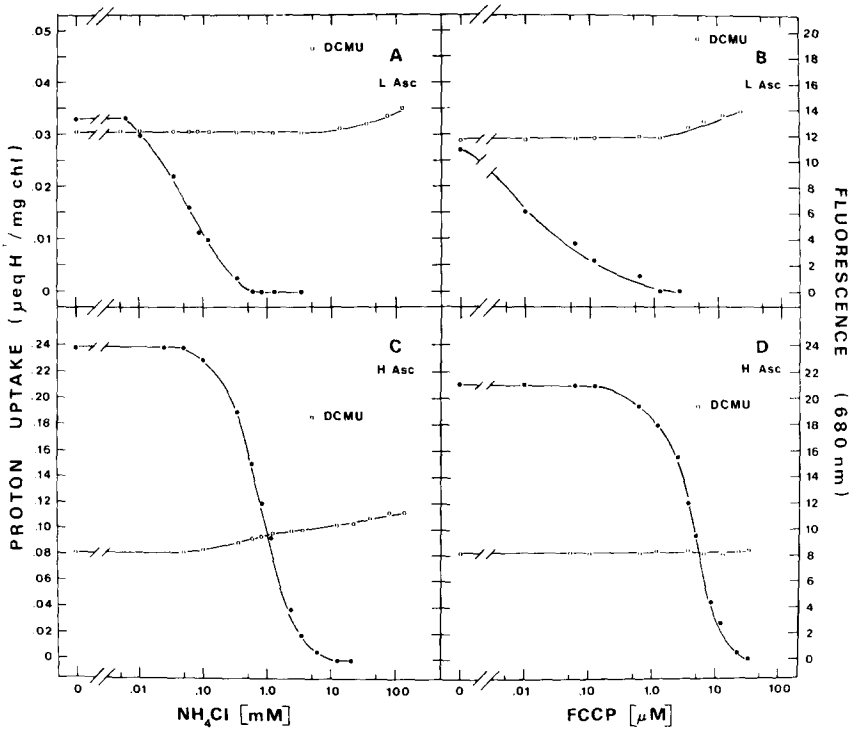

Fig. 5. Proton uptake and light-induced chlorophyll fluorescence in the DCMU-PMS system as a function of uncoupler concentration. The fluorescence yield in the presence of 10 μM DCMU is indicated in the top right hand corner of each graph. Otherwise, samples included 10 μM DCMU plus 10 μM PMS and 50 μM ascorbate (L Asc) or 15 mM ascorbate (H Asc). Log scales on the abscissa indicate the uncoupler concentration of either NH_4Cl or FCCP. Chlorophyll fluorescence curves given by (○–○) and proton uptake curves by (●–●).

[9] and Barber et al. [10] it is evident that fluorescence quenching can only be observed in intact chloroplasts or after the addition of Mg^{2+} to ruptured chloroplasts since the fluorescence yield is already low in broken chloroplasts lacking Mg^{2+}. Although our results with PMS indicate the fluorescence yield is not determined by proton uptake (and therefore not by the extrusion of Mg^{2+} accompanying proton movement [11]) we were concerned our results might be dependent on the presence of added Mg^{2+}. The conditions of our experiments and those of Krause [9] and Barber et al. [10] differ in two respects: in our experiments, Mg^{2+} was not added and we did not specifically try to isolate intact chloroplasts. Therefore, the effects of DCMU, PMS, ascorbate and uncouplers on the fluorescence yield of chloroplasts in the presence and absence of Mg^{2+} and osmotic shock treatment were examined.

Isolated chloroplasts were resuspended in medium either having or lacking sucrose, and Mg^{2+} was added at the time of reaction mixture preparation. Fig. 6 shows that the fluorescence yields in light are high whenever Mg^{2+} is present, low when Mg^{2+} and sucrose are both absent, and intermediate when Mg^{2+} is absent and sucrose is present. Apparently, chloroplasts in sucrose medium lacking added Mg^{2+} retain enough endogenous Mg^{2+} to support the intermediate level of fluorescence. More importantly, Fig. 6 shows that, although the absolute range of the

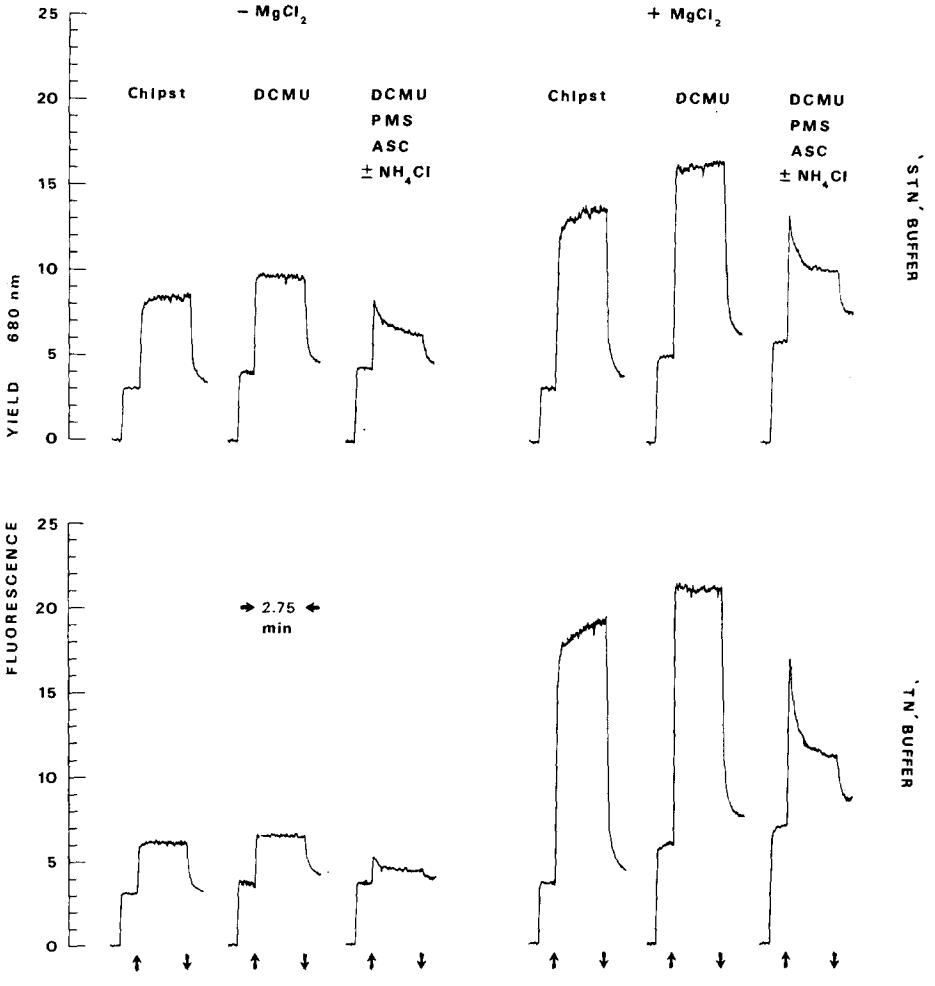

Fig. 6. Chlorophyll fluorescence traces obtained at high and low osmotic strength with and without added $MgCl_2$. "STN" buffer was the same as isolation buffer while "TN" buffer had the 0.4 M sucrose omitted. Concentrations of additions were as follows: 5 mM $MgCl_2$, 10 μM DCMU, 10 μM PMS, 50 μM sodium ascorbate and 5 mM NH_4Cl. Measurements as described in Methods and Materials and Fig. 1.

fluorescence yield (defined by the low light yield of unpoisoned chloroplasts and the high light yield of DCMU-poisoned chloroplasts) is strongly affected by Mg^{2+}, the relative quenching brought about by PMS and ascorbate is not influenced by either Mg^{2+} or sucrose. Moreover, the lack of effect on the fluorescence yield by uncoupler is true whether Mg^{2+} is present or not. These findings indicate that quenching by PMS and ascorbate is a general phenomena independent of the level of Mg^{2+} in our chloroplast preparation.

DISCUSSION

We have shown that ascorbate-reduced PMS is rapidly oxidized by dissolved oxygen and that, in the absence of DCMU, PMS will serve as a Mehler reagent [21] in chloroplasts. In this case, the quenching of chlorophyll fluorescence may be attributed to the oxidation of Q [1] during net electron transport. Only in the presence of DCMU is the PMS quenching independent of the oxidation of Q and a basic prerequisite (elimination of net electron transport capability) for measuring only Photosystem I activity established.

In the presence of DCMU, the requirement of a low oxygen concentration for rapid *P*-700 turnover is apparently related to "redox poising". Since PMS must serve as both the oxidant and reductant for *P*-700 in cyclic electron transport [25], maximum *P*-700 turnover will require an appropriate balance between the oxidized and reduced forms of PMS. Our data suggests that a high oxygen concentration facilitates a rapid auto-oxidation of reduced PMS and results in an inadequate level to support a good rate of *P*-700 turnover. Evidently, an excessive or high ($\geqslant$ 1 mM) ascorbate concentration is required to catalytically lower the dissolved oxygen with PMS and thereby minimize the competitive depletion of reduced PMS by oxygen. This explanation accounts for our results and is consistent with the observations of Jagendorf and Margulies [3] that cyclic phosphorylation with PMS requires either anaerobic conditions or the addition of excessive amounts of ascorbate.

According to the Mitchell theory [26], electron transport is coupled to proton translocation across a membrane. In a steady state, the magnitudes of net proton uptake and a pH gradient are determined by (i) the rate of inward proton translocation driven by electron transport, and (ii) the rate of passive outward diffusion of protons. When the pH gradient becomes sufficiently steep, phosphorylation driven by the passive outward movement of protons will occur. Our results on the rate of cyclic electron transport (i.e. *P*-700 turnover), net proton uptake, 9-aminoacridine fluorescence quenching and phosphorylation are consistent with the hierarchical order of events predicted by the Mitchell theory. Phosphorylation only occurred when 9-aminoacridine fluorescence quenching (or ΔpH [18]) was maximal, and the latter only occurred when net proton uptake was large and *P*-700 turnover rapid in the cyclic system.

In view of some controversy over 9-aminoacridine [18, 27] and *P*-700 [23, 28] measurements, several aspects of our results are open to alternative interpretations. Recently, Fiolet et al. [27] have questioned whether quenching of 9-aminoacridine fluorescence is due to a pH gradient [18] and expressed the alternative view that quenching occurs as a result of 9-aminoacridine binding to sites generated in the light. In this case, one would have to say that proton uptake activity or phosphorylation is in some manner related to the number of 9-aminoacridine binding sites generated in the light.

Haehnel et al. [28] and Haehnel and Witt [29] reported several fast ($\tau_{\frac{1}{2}} \approx$ 10 and 200 μs) dark reductions of *P*-700 in addition to the slower (20 ms) component measured by Rurainski and Hoch [23] in unpoisoned chloroplasts. Thus, we must acknowledge that a faster reduction of *P*-700 may have escaped our detection limits [15]. However, it is unlikely that a faster reduction of *P*-700 would be significant in our measurements for the following reasons: First, in DCMU-poisoned chloroplasts, only a portion

of the electron transport chain between *P*-700 and it's electron acceptor X is most likely operable when PMS is used as the cofactor [25]. In this case, cytochrome *f* and plastocyanin are by-passed and the faster relaxation times attributed to their involvement [29] would be non-functional in *P*-700 reduction with PMS. Secondly, the *P*-700 turnover, measured here, is well correlated with Photosystem I activity as it shows a large increase under the conditions for maximizing proton translocation activity (Figs. 3A and 3C, and Figs. 4B and 4C).

In our examination of the conditions for cyclic Photosystem I activity, several observations argue against proton gradient formation being the cause of chlorophyll fluorescence quenching in the presence of PMS. (1) The light-induced quenching of fluorescence, with PMS and low ascorbate, is not accompanied by a pH gradient or significant restoration of a phosphorylation potential. (2) In the presence of high ($\geqslant$ 1 mM) ascorbate and PMS, the fluorescence yield decline which does accompany the restoration of a pH gradient is both uncoupler insensitive and independent of the presence of illumination. These observations strongly support the original suggestion of Mohanty et al. [13] that PMS quenching of the chlorophyll fluorescence yield is unrelated to proton transport phenomena. They are also consistent with Papageorgiou's finding [12] that PMS will chemically quench the fluorescence yield, the reduced form being more effective than the oxidized form.

In view of our findings, the inability of the cofactor 2,6-dichlorophenol indophenol to quench fluorescence [13] and the inconclusive results obtained with diaminodurene (fluorescence and phosphorylation potential being measured under separate conditions [7, 8]), more critical evidence is clearly required before the role of the "high energy state" in quenching the fluorescence of DCMU-poisoned chloroplasts is established.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. George E. Hoch for his helpful discussion and assistance with the *P*-700 investigation. This work was supported by P.H.S. Training Grant No. GM 00658 from the National Institute of General Medical Sciences.

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