Light-Dependent Interactions of Phenazine Methosulfate with 3-(3,4-Dichlorophenyl)-1,1-dimethylurea-Poisoned Chloroplasts *

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Chlorophyll Fluorescence, Chloroplasts, Fluorescence Quenching, Energized Membrane

The chlorophyll fluorescence of isolated chloroplasts in the presence of phenazine methosulfate (PMS) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) can be quenched in a light-dependent reaction. This phenomenan has been studied and the following observations were made:

1. Quenching occurs under non-phosphorylating conditions and is stimulated by Mg^{2+} ions.
2. Under the same conditions, a light-dependent, Mg^{2+} stimulated transient decrease of absorption at 388 nm is observed which shows the spectral characteristics of PMS.
3. PMS is reversibly bound to chloroplasts. Under the experimental conditions used, binding amounts to as much as 0.5 mol PMS/mol chlorophyll.
4. Some uncouplers of photophosphorylation such as carbonylcyanide-*m*-chlorophenylhydrazon (CCCP) and atebrin analog abolish quenching, transient absorption change and binding of PMS. Others, such as methylamine, ammonia, gramicidin and nigericin do not.

It is suggested that fluorescence quenching, transient absorption change and binding of PMS are causally related. The concept, postulated by others, that a high-energy state of the chloroplast membrane is involved in the fluorescence lowering is questioned.

Introduction

Changes in chlorophyll fluorescence of isolated chloroplasts have generally been interpreted in terms of alterations in the redox level of Q, the supposed trap of photosystem II. In its simplest form the hypothesis states that fluorescence and electron transport yields are inversely related [1]. Although many observations support this concept, some results suggested that other events may be involved. Thus, Homann [2] and Murata [3] among others, showed an increase in the fluorescence level due to divalent cations even when electron transport was completely inhibited by DCMU. Also, Rurainski and Hoch [4] reported that Mg^{2+} simultaneously increased both the fluorescence and the rate of electron transport measured as NADP reduction in isolated chloroplasts.

Under some conditions a light-dependent decrease of the fluorescence level may occur. As a number of authors have shown, such a quenching can be observed when PMS or DAD [5–8] together with DCMU are present in the reaction mixture. These reagents are cofactors of cyclic photophosphorylation. Therefore, the observations that the quenching could be abolished by some uncouplers of phosphorylation led to the conclusion that the quenching reflected the development of a high-energy state across the thylakoid membranes which is related to the formation of ATP.

Slovacek and Bannister [9] contradicted this interpretation for the results with PMS since they found no consistent correlation between the fluorescence lowering and the proton gradient which is thought to be a precursor of phosphorylation. Also, B. Schmidt [10] recently reported marked quenching of chlorophyll fluorescence by chemically or enzymatically reduced PMS. He implied that a similar mechanism may govern the light-induced fluorescence lowering.

In this report we will show that the light-induced fluorescence quenching in the presence of DCMU and PMS occurs under non-phosphorylating conditions and is inhibited only by some uncouplers. Moreover, the quenching is correlated with a light-induced transient absorption change of PMS and a binding of PMS to the chloroplasts. A causal relation is supposed between these activities and the fluorescence quenching.

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Abbreviations: PMS, phenazine methosulfate; CCCP, carbonylcyanid-*m*-chloro-phenylhydrazon; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DAD, diamino durene; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-2-hydroxy-ethyl-piperazine-N′-2-ethane sulfonic acid.

* This paper is dedicated to the memory of Prof. Günter Jacobi, who died June 25, 1976.

Materials and Methods

Chloroplasts were isolated as described elsewhere [4]. Prior to illumination, they were incubated in the reaction mixture for 2 min in the dark. The standard reaction mixture contained 100 mM sucrose, 10 mM NaCl, 20 mM Tricine, pH 8.0, 40 μM DCMU, 5 mM $MgCl_2$ and 20 μM PMS. Changes from this composition are indicated in the legends.

Fluorescence time courses were measured in a non-commercial spectrophotometer. The actinic light, which also served to excite the fluorescence, came from a 150 W halogen lamp. Its beam was collected with lenses and passed through heat filters, a Compur shutter and an interference filter with maximum transmission at 595 nm and a half-band width of 10 nm before impinging upon the sample. The shutter required 3 ms for complete opening or closing.

Fluorescence was detected by a photomultiplier which was situated at a right angle to the actinic beam and which was protected against scattered light by a broad-band interference filter (peak transmission at 700 nm, half-band width 50 nm) and a narrow band interference filter (peak transmission at 687 nm, half-band width 10 nm).

The signals from the photomultiplier were amplified and stored in a computer with 1024 signal addresses per sweep. At the end of a measurement, the signals were recalled from storage and recorded on a x/y recorder.

PMS absorption changes were measured at 388 nm in the same instrument. The weak measuring beam came from a Bausch and Lomb 500 mm monochromator. For these measurements, the photomultiplier was shaded with a broad-band interference filter having maximum transmission at 390 nm and a half-band width of 50 nm.

Binding of PMS to the chloroplasts was studied with a centrifugation method. Samples of 1 ml were placed in a polyethylene cup and illuminated while sitting in a table-top centrifuge. The light was filtered through a broad band interference filter with peak transmission at 610 nm and was led by means of light guides to the top of the reaction vessels. After 1 min of illumination, the samples were centrifuged at approx. 12 000 rpm for 45 s in the light. One half ml of the supernatant was immediately withdrawn and the extinction was measured spectrophotometrically. The extinction coefficient used for calculating concentrations was 23.8 $(mM\,cm)^{-1}$ at 388 nm [11]. Dark controls were treated identically except that the light was turned off prior and during centrifugation.

Results

The experimental conditions chosen for the following experiments include red actinic light, which is not absorbed by PMS and therefore cannot cause photodestruction of the molecule, the presence of a high concentration of DCMU to eliminate electron transfer between the photosystems, aerobiosis and lack of ascorbate. Under these conditions, no appreciable proton gradient or photophosphorylation can be observed [11–13]. Thus, if the quenching of fluorescence reflected a high energy state related to photophosphorylation, *ad hoc* no quenching might be expected.

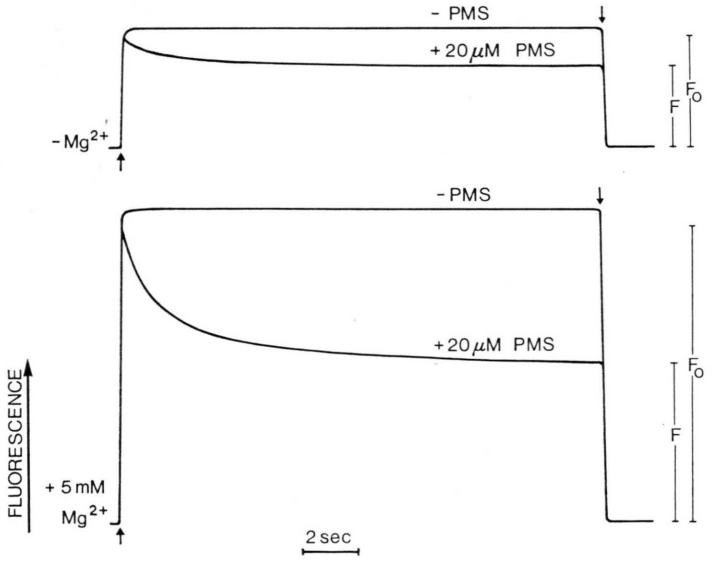

Fig. 1. Light-dependent fluorescence quenching by PMS in DCMU-poisoned chloroplasts in the absence and presence of Mg^{2+}. Reaction conditions are described under Methods. Chlorophyll concentration: 8 μg/ml. Light was turned on or off at the upward or downward pointing arrow, respectively.

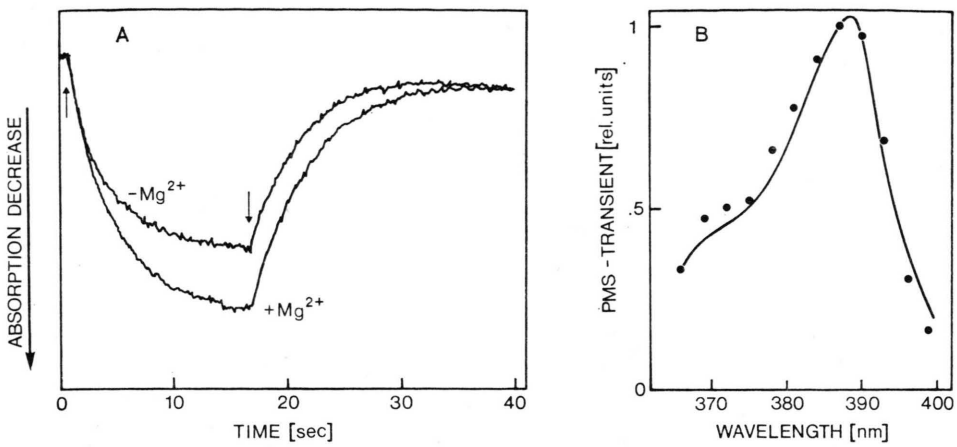

Fig. 2. Time course and wavelength dependence of transient absorption change. Reaction conditions described under Methods. Chlorophyll concentration 8 μg/ml. Actinic light intensity was 3.2×10^4 erg/cm^2 sec. A: effect of Mg^{2+} on the time course; B: wavelength dependence of the absorption change. The drawn out line is an oxidized *minus* reduced spectrum of PMS in aqueous solution; the points are amplitudes of the absorption change measured in the presence of DCMU, PMS and Mg^{2+}. The data were normalized at 388 nm.

Fig. 1 shows that this expection is not borne out. In the absence of PMS a fluorescence level is reached which does not change within the time of observation. When PMS is present, fluorescence rises to an initial peak (F_0) followed by a decrease which approaches a steady-state level (F). We express the extent of the quenching in percent as $100(F_0-F)/F_0$. Fig. 1 also shows that the fluorescence intensity as well as the extent of the quenching is stimulated by Mg^{2+}. With light intensities routinely used here the extent of the quenching was $20-30\%$ in the absence and $40-50\%$ in the presence of Mg^{2+}. By extrapolation to infinite intensity of reciprocal plots of a light intensity curve we determined that maximal quenching amounted to approx. 65%.

Under the same experimental conditions, we observed a light-induced absorption change at 388 nm of which a recorder tracing is shown in Fig. 2. Upon cessation of the light, the signal returns to its original level. Addition of Mg^{2+} stimulates its amplitude but has no effect on the initial rate of change. The magnitude of the stimulation by the cation is a function of actinic light intensity, being highest at saturating intensities. The wavelength dependence of this absorption change closely matches an oxidized *minus* reduced spectrum of PMS (Fig. 2). This spectral characteristic suggests that the signal is due to an interaction between PMS and the chloroplast. The maximum amplitude in the presence of Mg^{2+} and extrapolated to infinite light intensity corresponds to approximately 0.5 mol PMS per mol of chlorophyll.

We also observed that PMS is bound to the chloroplasts. This binding was measured by the disappearance of the co-factor from the supernatant after a 1 min illumination followed by rapid centrifugation of the reaction mixture in the light. As is shown in Table I, binding was largely reversed when a dark period preceeded the centrifugation. Also, Mg^{2+} increased the retention of PMS on the chloroplasts by a factor of approximately 2.

Table I. Light-dependent binding of PMS to chloroplasts. Reaction mixture and experimental procedure as described under Methods. The chloroplast concentration was equivalent to 23.4 μg chlorophyll/ml. "Light" samples were illuminated through a broad-band interference filter transmitting between 570 and 630 nm for 1 min and centrifuged in the light. The "Dark" sample was illuminated for 1 min, held in darkness for 1 min and was then centrifuged in darkness.

Addition	μmole PMS bound/mg chlorophyll	
	Dark	Light
none	0.04	0.26
+5 mM Mg^{2+}	—	0.49

Because of a similar response toward Mg^{2+} of both the light-induced quenching reaction and the transient absorption change, we supposed a causal relationship between the two phenomena. This sup-

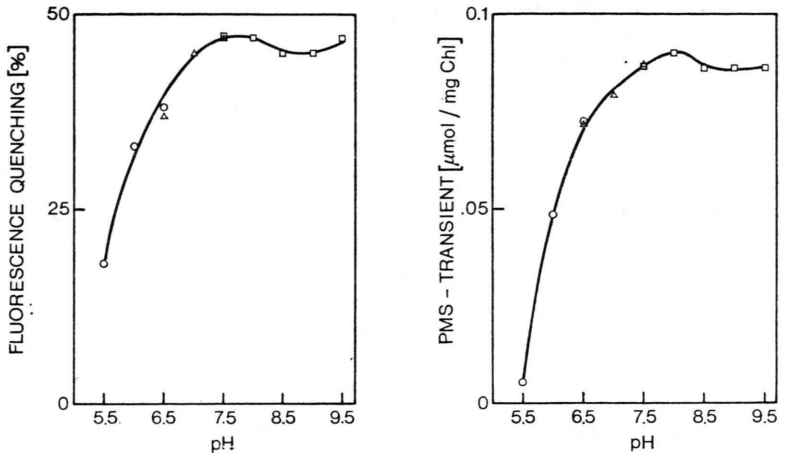

Fig. 3. pH profiles of absorption change at 388 nm and of fluorescence quenching. Reaction conditions described under Methods. Chlorophyll concentration 8 μg/ml. Actinic light intensity was 3.2×10^4 erg/cm^2 sec. The following buffers were used: ○ MES, △ HEPES, □ tricine, all 20 mM.

position is supported by the data of Fig. 3, which shows the extent of the fluorescence lowering and the absorption change expressed in μmol PMS as a function of pH. Both parameters increase in activity as the pH is raised, reaching a broad maximum at approximately pH 7.5. Except for values around pH 5.5, the profiles are closely correlated. The decreasing activity at low pH values may in part be due to a competition for binding sites between the cationic PMS and protons [14]. A similar curve for the fluorescence quenching has been reported by Murata and Sugahara [5].

A causal relationship between the fluorescence quenching and PMS absorption change is also suggested by the data of Fig. 4. Both parameters were measured in the same instrument under identical experimental conditions. The upper recorder trace shows the decrease of the absorption change in light and its return in darkness as a function of time; the lower trace depicts the time course of the fluorescence change. With the onset of the light, the emission decreases, and returns to its original level in darkness. In order to visualize this return the dark period was interrupted at regular intervals by test flashes of approximately 70 ms duration. These flashes were too short to cause an appreciable lowering of the fluorescence, yet they were long enough to indicate the fluorescence level at a particular point in time.

A semi-logarithmic plot of these curves (not shown) revealed that both the forward and back reactions of the transient absorption change follow a simple first-order rate law. The kinetics of the fluoresence lowering, on the other hand, were com-

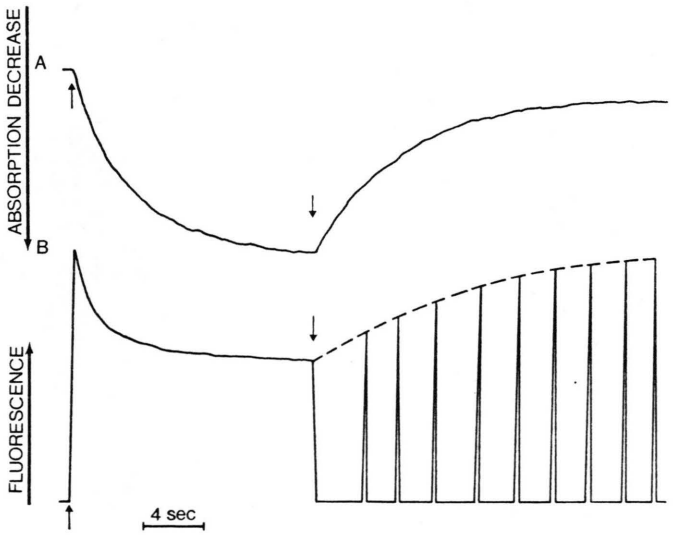

Fig. 4. Time courses of fluorescence quenching and PMS absorption change. Reaction conditions described under Methods. Chlorophyll concentration 8 μg/ml. Actinic light intensity was 3.2×10^4 erg/cm^2 sec. Light was turned on or off at the upward or downward pointing arrow, respectively.

plex. A reason for this apparent kinetic discrepancy is indicated in Fig. 5 which is a re-plot of the traces in Fig. 4. The ratio of the fluorescence level at time zero (F_0) and that at any time (F) was plotted against the square root of the amplitude of the absorption change at an equal time. The resulting graph shows that fluorescence quenching depends on the square root of the PMS activity reflected by the absorption change. We interpret the small lag at the beginning of the curve as being due to a temporal delay between the two parameters reflecting the diffusion of the quenching species after the onset of illumination to the site of quenching. From the initial slopes of Fig. 4 we estimate the delay to be approximately 200 msec. A lag in the same range of time has been described before [5].

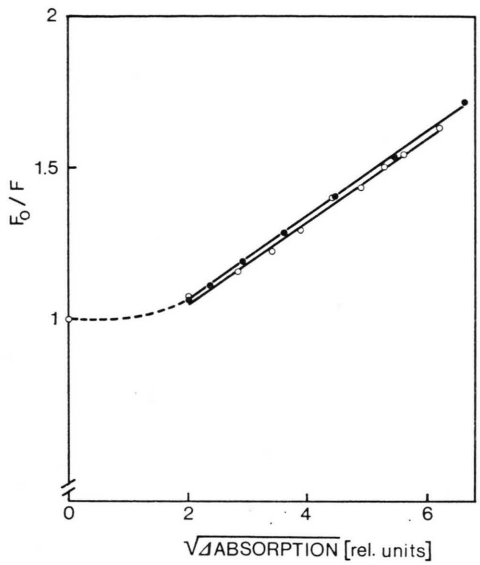

Fig. 5. Dependence of fluorescence intensity on square root of PMS absorption change measured at equal times. Data taken from traces in Fig. 4. ● Light reaction, ○ dark reaction.

The dependence of the fluorescence intensity on the square root of the PMS activity is also observed in the steady-state. Here it is to be expected that the temporal shift suggested for the time courses above would be abolished. Fig. 6 A shows steady-state values for the extent of the quenching and the amplitude of the absorption change as a function of light intensity. The data indicate that the former reaction is saturated at much lower intensities than the latter. In Fig. 6 B, the ratio F_0/F and the square root of the absorption change at equal intensities are plotted. The data follow a modified, classical Stern-Volmer equation [15]

$$F_0/F = 1 + \alpha[Q]^{1/2}$$

where α is a collection of parameters including the probability of effective collisions between fluorescence emitter and quencher as well as the fluorescence life time. [Q] is the concentration of the quencher.

In contrast to the light-induced fluorescence lowering, the quenching of the emission by enzymatically reduced PMS (shown previously in detail by B. Schmidt [10]) follows a straight Stern-Volmer equation [15]

$$F_0/F = 1 + \alpha[Q].$$

The data, shown in Fig. 7 were obtained by incubating the reaction mixture containing the PMS concentration indicated with glucose/glucose oxidase and measuring the level of fluorescence after 5 min. In separate measurements it was determined that this time sufficed to reduce PMS completely. Com-

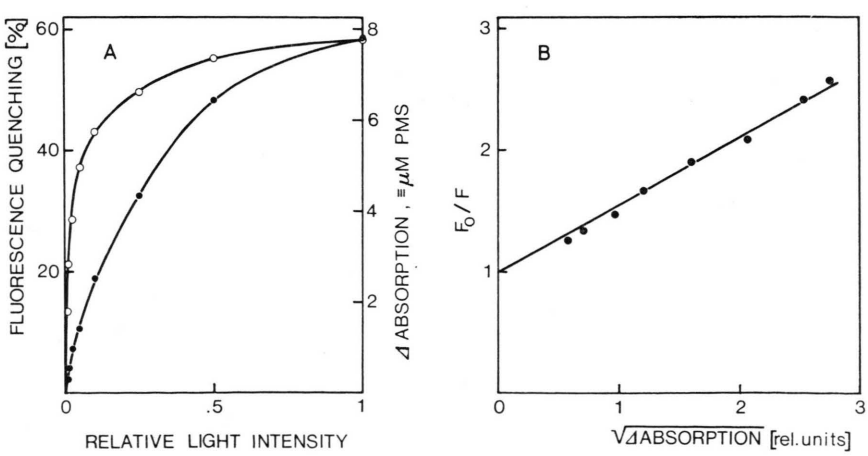

Fig. 6. Fluorescence quenching and transient absorption change as a function of light intensity. Experimental conditions are described under Methods. Chlorophyll concentration: 6.8 μg/ml. For actinic illumination a broad band interference filter was used with peak transmission at 610 nm. Relative intensity of 1.0 corresponded to approx. 5×10^5 erg/cm^2 sec. A: open symbols, extent of quenching; closed symbols, amplitude of absorption change expressed as μmol PMS/l. B: Modified Stern-Volmer plot. Fluorescence level and absorption change at equal light intensities were plotted.

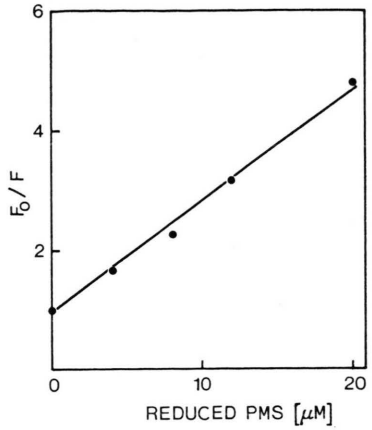

Fig. 7. Stern-Volmer plot of fluorescence quenching by reduced PMS. Experimental conditions were described elsewhere [10]. Chlorophyll concentration: 8 μg/ml. The reaction mixture contained also 10 mM glucose and about 1 unit/ml glucose oxidase. Fluorescence was excited by 595 nm light with an intensity of 3.2×10^4 erg/cm^2 sec.

parison of the light- and enzyme-induced quenching of the emission suggests that both are due to an interaction of a bleached form of PMS with the fluorescence emitter but that there exist mechanistic differences.

The principal argument for ascribing the light-induced fluorescence lowering in the presence of PMS to a high energy state of the thylakoid membrane was based on the observation that the quenching reaction was sensitive toward the uncouplers CCCP, atebrin and methyl amine [5]. Table II shows that CCCP and atebrin analog indeed largely abolished the quenching. However, a number of other uncouplers, as well as the inhibitor of electrical potential changes, gramicidin [16, 17] were without effect. Methyl amine in concentrations which uncouple phosphorylation had only a marginal influence. Thus, the involvement in the fluorescence lowering of an energized membrane state related to photophosphorylation is highly questionable. Rather. we like to suggest that CCCP and atebrin analog act as inhibitors of electron transport of photosystem II. This property has been ascribed to CCCP in several reports [18–20] and will be described in detail in a forthcoming report.

Table II. The effect of uncouplers on fluorescence quenching, PMS absorbance change and PMS binding. Reaction mixture as described under Methods. Data are expressed as % inhibition relative to a control sample. Negative numbers indicate stimulation.

	PMS			DAD
	Quenching	Δ Absorption	Binding	Quenching
40 μM CCCP	71.4	91.4	90.1	82.8
160 μM atebrin analog	77.6	91.4	—	—
4 μM gramicidin	5.9	−5.0	6.0	100
10 mM NH_4Cl	0	−1.0	2.0	94.4
10 mM methylamine	2.3	3.2	—	—
1 μM nigericin	−1.3	9.0	—	—

A qualitatively similar result is observed for the inhibition of the absorption change at 388 nm and the binding of PMS to the chloroplasts. Only those reagents which affected the fluorescence lowering also influence the transient and binding. An exact correspondence cannot be expected because of the non-linear relationship between fluorescence lowering and absorption change described above. Nevertheless, the data provide additional evidence for a correlation of the three parameters.

A light-dependent quenching of the fluorescence in the presence of DCMU has also been reported with DAD as co-factor [8]. As shown in Table II, several uncouplers abolish the quenching in this system. Thus, the postulated involvement of a high-energy state of the membrane could be an acceptable interpretation in this case.

Discussion

Several authors [5–8] have interpreted the quenching of chlorophyll fluorescence in DCMU-poisoned chloroplasts in the presence of PMS or DAD in terms of the development of a high-energy state across the thylakoid membrane. Evidence for this interpretation was the sensitivity of the quenching reaction toward a number of uncouplers. As far as our results with DAD are concerned and in agreement with Wraight and Crofts [8], all uncouplers tested abolished the quenching. Moreover, since Cohen and Sherman [21] reported a parallel behavior of the fluorescence lowering and the proton gradient which is thought to be a precursor of photophosphorylation, the high energy state may be an acceptable conclusion in this case.

In the presence of PMS, however, we observed that only CCCP and atebrin analog had any influence on the quenching reaction whereas other uncouplers, including gramicidin, an inhibitor of the membrane potential [17], were without effect. Thus, on the basis of this criterion, we feel that the energized membrane concept needs to be amended.

This view is corroborated by several additional observations. Slovacek and Bannister [9] for example, found no consistent relationship between the quenching of the fluorescence and the proton gradient with PMS and questioned the high energy hypothesis. Also, phosphorylation in the presence of PMS is known to be saturated at exceptionally high light intensities [22] – quite in contrast to the quenching reaction as we show here. Finally, B. Schmidt [10] reported that the fluorescence may be quenched by chemically or enzymatically reduced PMS.

The latter results prompted us to search for a light-dependent reduction of the co-factor. The transient absorption change at 388 nm whose wavelength dependence follows closely the absorption spectrum of PMS could be interpreted that such a reaction occurs. Then the observed binding of PMS to the chloroplasts could also be understood since the reduced co-factor, according to Hauska [23], is quite insoluble in water and its disappearance from the medium could signal a binding to the lipid phase of the chloroplast membrane.

There are, however, several reasons why we doubt that PMS is actually reduced. The first one is the magnitude of the absorption change which in most experiments corresponded to 1 mol PMS/2 mol of chlorophyll (in a few experiments we calculated as much as 1 mol of PMS/mol of chlorophyll). To form such high concentrations of PMSH in the presence of DCMU would require a pool of reducing power that cannot be rationalized with current concepts of photosynthetic electron transport. More importantly, the semiquinoid and totally reduced forms of PMS show absorption peaks at 440 and 330 nm, respectively [24]. We failed to detect in these wavelength regions any absorption changes which would indicate a reduction. Finally, if PMS were reduced in the light and re-oxidized in darkness, we would expect the re-oxidation to be impaired under anaerobic conditions. This also was not observed.

The observation, reported by others [25, 26], that the light-dependent binding of protons to chloroplasts approaches a similar magnitude (approx. 1 mol protons/mol of chlorophyll) as that of PMS suggests that the cationic PMS molecule may be bound in a similar way. This suggestion is supported by a report of Lynn [14] who demonstrated competition for chloroplast binding sites between H^+ and PMS^+. Also, Papageorgiou [7] showed that PMS^+ is capable of quenching chlorophyll fluorescence *in vitro* and in chloroplasts. Since during light dependent binding of PMS^+ the local concentration of the co-factor can be expected to increase greatly, this mechanism may also account for the light induced quenching.

Such a binding could also cause the observed decrease of the absorption band. As was shown by Ishizu *et al.* [27] for the interaction of PMS with native deoxyribonucleic acid and by several other authors [28, 29] for the interaction of various dyes with chloroplasts or mitochondria, binding may result in a decrease of absorption and/or a small red-shift of the absorption maximum. (For technical reasons, we would not be able to resolve a possible bathochromic shift of 2 – 3 nm.

On the basis of the experimental results thus far obtained, it is difficult to account mechanistically for the non-linear relationship between the light-dependent fluorescence lowering and the absorption change. A dependence of the former on the square not of the latter may be rationalized by speculating that there are two species of PMS formed in the light. A hypothetical model reaction may be

$$P^{II} + P \rightleftharpoons 2\,P^{I}\,.$$

Here, P is the concentration of PMS added and P^I and P^{II} the concentration of the hypothetical species. Assuming that the concentration of P is large compared to the other species and that the absorption change is due to P^{II}, we obtain from the equilibrium condition

$$[P^I]^2 = K'\,\Delta A$$

where $K' = K[P]$. The Stern-Volmer equation then becomes

$$F_0/F = 1 + \alpha[K'\,\Delta A]^{1/2}.$$

This result states that P^I is the quenching species. Alternatively, the coefficient α in the Stern-Volmer equation may be variable and depending on the concentration of PMS bound. A variable α can be interpreted with changes in the probability of effective collisions between the fluorescence emitter and the quencher. Further experiments will be necessary to distinguish between these and other possible mechanisms.

At present we believe that binding of PMS may be mediated by a photosystem II activity remaining in the presence of DCMU. It has been suggested [18–20, 30] that CCCP in the concentrations required for the experiments reported here inhibits

electron transport between water and the primary reductant of PS II. As several other uncouplers are ineffective, we believe that this inhibitory action of CCCP rather than its uncoupling property is responsible for the abolishment of PMS binding and fluorescence quenching. Mg^{2+}, which is thought to activate PS II (see *e. g.* [31]) stimulated both parameters and yields a further indication for this interpretation. Also, P_{700}, the supposed trap of photosystem I requires reduced PMS for efficient turnover. Under the conditions used here, we observed a rather sluggish activity of the pigment. In a separate publication [33], additional experiments on the interaction of PMS with photosystem II as measured by the restoration of fluorescence following a brief illumination will be reported.

After completion of the present work, a paper by Homann [32] came to our attention in which some experiments similar to ours were reported. In his interpretation Homann agrees with the notion that fluorescence lowering is not an expression of an energized membrane related to phosphorylation.

In contrast to us, the author sees no causal relationship between binding and fluorescence lowering. We feel that the dependence of the quenching on the square root of the absorption change which follows a modified Stern-Volmer equation is suggestive for such a causality. We are not sure if the author considered this relationship. Also in contrast to our speculation, Homann suggests that bleaching of PMS is dependent on photosystem I activity. We reproduced one of this experiments showing that K-ferricyanide inhibited the absorption change. In addition, we found that the sluggish turn-over of P_{700} ceased under this condition as was supposed by Homann. On the other hand, CCCP, while also abolishing PMS bleaching, did not inhibit P_{700}.

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