QUENCHING OF EXCITED CHLOROPHYLL *A* IN VIVO BY NITROBENZENE

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ABSTRACT Nitrobenzene exerts a dual effect on the excitation of chlorophyll *a* (Chl *a*) in vivo. (*a*) A 3(3,4-dichlorophenyl)-1,1-dimethylurea-inhibited quenching that manifests as a partial inhibition of variable chloroplast fluorescence and of 2,6-dichlorophenol indophenol (DCPIP) photoreduction and saturates at ca. 5–10 μM. Since nitrobenzene is not a Hill oxidant, this effect is attributed to a catalyzed back flow of electrons from intersystem intermediates to pre-photosystem II oxidants. (*b*) A direct quenching of the excited Chl *a* in vivo. This effect has a threshold of ca. 100 μM nitrobenzene; at higher concentrations it leads to almost complete suppression of chloroplast fluorescence and DCPIP photoreduction. Tris-washed chloroplast enriched in the photosystem II reaction center species Z^+Q^- and ZQ^- are nearly four times more sensitive to nitrobenzene quenching than those enriched in Z^+Q. On the other hand, normal chloroplasts are about 10^4 times more sensitive. Hence, it is argued that the extreme sensitivity of normal chloroplast fluorescence is not due to a preferential association of nitrobenzene with a particular redox species of the reaction center.

INTRODUCTION

Nitroaromatics are known to quench the fluorescence of chlorophyll *a* (Chl *a*) and of other porphyrins in solution (1, 2). This property has been used to study the effects of metal cations on the hydrophobicity of the lamella (3, 4), as well as the migration of Chl *a* excitons in the photosynthetic apparatus (5–8). To forestall possible interference of the nitroaromatic with the photosynthetic electron transport chain, these studies were restricted to isolated chloroplasts, or algae, whose capacity to photoevolve oxygen had been inhibited with 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Recently, however, Etienne and Lavergne (9) reported that unpoisoned preparations are significantly more sensitive to quenching by *m*-dinitrobenzene, than preparations poisoned with DCMU. On the basis of this, and of other results, they suggested that nitrobenzene converts the photosystem II (PS II) reaction centers to permanent excitation traps by binding irreversibly to them. It was subsequently recognized by Etienne et al. (10), however, that it is not necessary to postulate a specific affinity for the PS II reaction center in order to account for the effects of *m*-dinitrobenzene on the fluorescence and the photochemical activities of photosynthetic preparations.

We have investigated the effects of a related quencher, nitrobenzene, on the fluorescence of normal chloroplasts, and of chloroplasts inhibited on the oxidizing and on the reducing side of the PS II reaction center; also, on the fluorescence of chloroplast preparations enriched in different redox combinations of the primary electron donor (Z) and the primary electron acceptor (Q) of this reaction center. Our results, analogous in some respects with those of Etienne and Lavergne (9) and Etienne et al. (10), do not support a special affinity of this nitroaromatic with the PS II reaction center. Nitrobenzene appears to quench the fluorescence of normal chloroplasts by means of two mechanisms: (*a*) by direct interactions with the excited chlorophylls; and (*b*) by mediating a back flow of electrons from an intersystem intermediate to free oxygen, or to a pre-PS II oxidant.

MATERIALS AND METHODS

Broken (stroma-free) chloroplasts were prepared from selected leaves of market spinach by a conventional technique (4). Fluorescence was measured with chloroplasts suspended in 50 mM potassium morpholine ethane sulfonate (MES · KOH), 300 mM sucrose, pH 6.4, at an absorbance difference ($A678$–$A740$) of 0.45. Oxygen evolution was measured by means of a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) with chloroplasts suspended in 50 mM tricine-NaOH, 250 mM sucrose, 10 mM NaCl, pH 7.8, at a density of 33 μg Chl/ml.

Photoreduction of 2,6-dichlorophenol indophenol (DCPIP) was carried out according to Vernon and Shaw (11) with chloroplasts isolated in 50 mM tris-hydroxymethyl aminomethane hydrochloride (tris · HCl), 175 mM NaCl, pH 7.8, and resuspended in 100 mM phosphate (K salts), pH 6.4, at a density of 36 μg Chl/ml. For the photoreduction of DCPIP in the presence of nitrobenzene, the chloroplasts were preincubated with the nitroaromatic for 7–9 min in the dark. The samples were then exposed to white saturating light for 15 s. Control runs have shown that the 590 nm absorbance decrease was linear with the time for up to 60 s exposure to the light.

Tris-washed chloroplasts, prepared according to Vernon and Shaw (11), had less than 10% of the normal rate of DCPIP photoreduction with electrons derived from water. Maximum rate was attained with 0.5 mM of 1,5-diphenyl carbazide serving as the electron donor. Tris-washed chloroplasts enriched in selected oxidation species (ZQ^-, Z^+Q, and Z^+Q^-) of the PS II reaction center were prepared by the method of Mohanty et al. (12).

Fluorescence was measured with an instrument described elsewhere (4). Excitation was isolated from a power stabilized 150 W projector lamp by means of an interference filter (480 nm, $\Delta\lambda$ = 12 nm, Baird Atomic, Inc., Bedford, Mass.; I = 300 ergs · cm^{-2} · s^{-1}). The excitation intensity was measured at the plane of the cuvette with a calibrated Bi-Ag thermopile (The Eppley Laboratory, Inc., Newport, R.I.). Chl *a* fluorescence was detected at 680 nm ($\Delta\lambda$ = 20 nm) with guard filter C.S. 2–58 (Corning Glass Works, Corning, N.Y.) in front of the entrance slit of the measuring monochromator.

Reagent grade chemicals, without further purification, were used. Nitrobenzene, however, was recrystallized twice from 75% ethanol by freezing to −15° C. The concentration of the ethanolic stocks of nitrobenzene was corrected on the basis of the absorptivities given in the literature (13). Chlorophyll was determined by the method of Arnon (14). Final ethanol was always below 3%.

All experiments were carried out at room temperature.

RESULTS

Fig. 1 shows the chloroplast fluorescence rise in the absence of additions (normal), and in the presence of 10 μM DCMU and of 1 mM potassium ferricyanide. Prior to the recording of fluorescence, the samples were fully adapted to darkness. This figure characterizes the fluorescence properties of the samples employed in these experiments, and the responsiveness of our fluorimeter. The steady-state fluorescence of normal chloroplasts consists of about equal contributions of constant (zero to *I*) and variable yield fluorescence (*I* to *P*). In the presence of 10 μM DCMU, 30–40% of the total emission is constant yield fluorescence and 60–70% variable yield fluorescence. In the presence of 1 mM ferricyanide, on the other hand, nearly all the steady-state emission is constant yield fluorescence.

Nitrobenzene modifies the *IP* rise of normal chloroplast fluorescence substantially. Very low concentrations (0–5 μM) suppress the amplitude *IP*, but within the limits of the measurement, the time constant of the rise is not affected (Fig. 2, curves A–C; $t_{1/2} \simeq 3$ s). At about 5 μM nitrobenzene, nearly two-thirds of the variable fluorescence had vanished (curve C). In contrast to this, the constant yield fluorescence exhibits very remarkable resistance to quenching (curves D and E).

The extremely disparate responses of the constant yield fluorescence, and of the variable fluorescence of poisoned and unpoisoned chloroplasts, are epitomized in Fig.

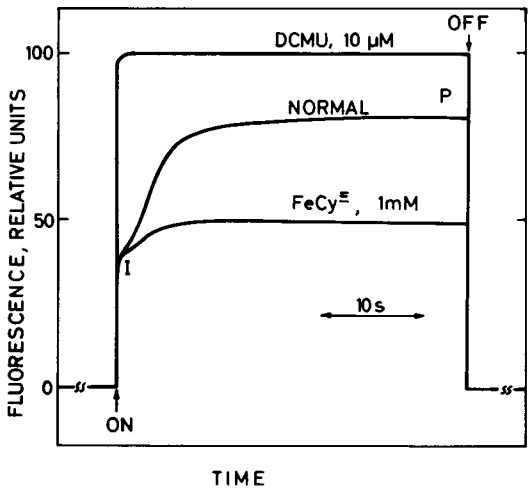

FIGURE 1

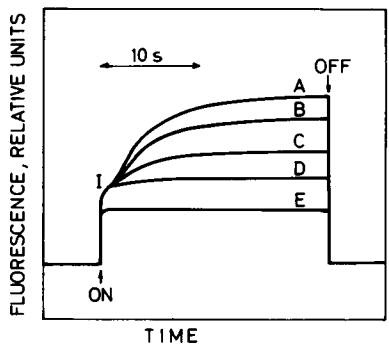

FIGURE 2

FIGURE 1 The fast rise of Chl *a* fluorescence on illumination of dark-adapted chloroplasts. DCMU (10 μM) and potassium ferricyanide (1 mM) were added during the dark incubation period. On and off arrows mark the onset and the end of the illumination.

FIGURE 2 The fast rise of Chl *a* fluorescence on illumination of dark-adapted chloroplasts containing increasing amounts of nitrobenzene. A, without nitrobenzene; B, with 0.4 μM; C. with 5.47 μM; D, with 2.42 mM; E, with 11.62 mM.

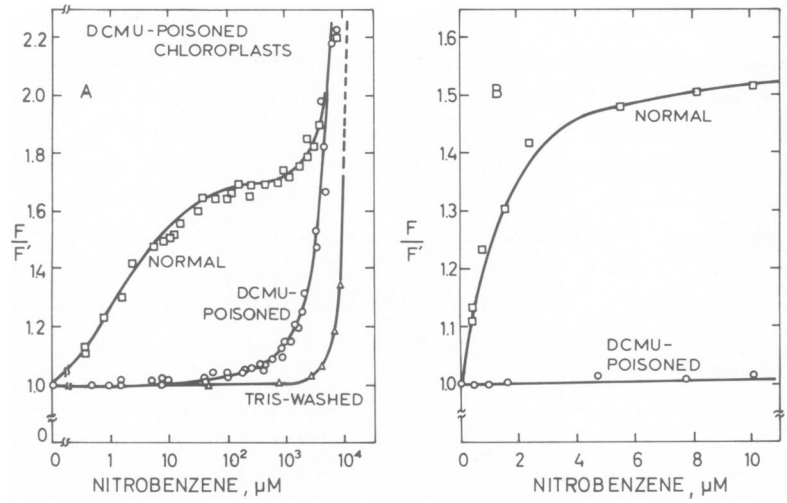

FIGURE 3 Quenching curves of the total (constant plus variable) Chl *a* fluorescence of normal, DCMU-poisoned, and tris-washed chloroplasts. The results are presented as the ratio of the unquenched (F) to the quenched (F') fluorescence, plotted against a logarithmic (A), and a linear (B) scale of nitrobenzene concentration.

3 A. This figure displays concentration curves (on a logarithmic scale) of the ratio of total fluorescence in the absence (F) and in the presence (F') of the quencher. In this particular experiment, F_I, F_P, and F_{DCMU} were 17, 40, and 55 chart units, respectively. In the case of normal chloroplasts, quenching is observable at very low concentrations of nitrobenzene. On the other hand, in the case of DCMU-poisoned chloroplasts measurable quenching is obtained only beyond a threshold of about 100 μM nitrobenzene. From about 100 μM to about 1–2 mM nitrobenzene, the quenching curves of the normal and of the DCMU-poisoned samples run roughly parallel; at higher concentrations, they converge to an asymptote parallel to the ordinate (a few points off the plot are not shown). After the elimination of a vestigial variable fluorescence by 10 μM nitrobenzene, the constant yield fluorescence of tris-washed chloroplasts is the most resistant to quenching. Appreciable effect is observed only beyond 4 mM nitrobenzene, but the quenching curve converges quickly to an asymptote parallel to the ordinate axis.

Fig. 3 B reproduces the early portions of the quenching curves of Fig. 3 A on a linear concentration scale. DCMU-poisoned chloroplasts appear to be completely insensitive, while normal chloroplasts become also resistant to quenching after a partial suppression of 55–60% of their variable fluorescence. Apparent quenching constants, $K_Q = (F - F')/F'C$, for normal chloroplasts, calculated from the initial slopes, are in the neighborhood of 3×10^5 M^{-1}, while even larger values ($7–8 \times 10^5$ M^{-1}) are obtained when only the variable fluorescence is considered. The reported quenching constants for nitrobenzene and Chl *a* in methanol range from 33 to 36 M^{-1} (1, 4).

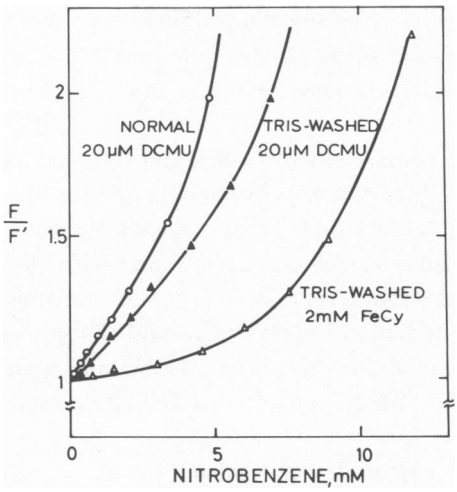

FIGURE 4 Quenching curves of the total Chl *a* fluorescence of normal and tris-washed chloroplasts. Normal chloroplasts with 20 μM DCMU; tris-washed chloroplasts with 20 μM DCMU; tris-washed chloroplasts with 2 mM potassium ferricyanide.

Fig. 4 shows the quenching to be more extensive when the fraction of variable fluoresence is larger. The figure displays quenching curves of three chloroplast preparations, which had identical chlorophyll contents and constant yield fluorescence, but different variable fluorescence. None of these samples could support sustained photosynthetic electron transport with water as the terminal donor. (*a*) Tris-washed chloroplasts in the presence of 2 mM potassium ferricyanide. This preparation emitted only constant yield fluorescence. The apparent quenching constant calculated from the initial slope, is $K_Q \simeq 21\ M^{-1}$. (*b*) Tris-washed chloroplasts in the presence of 20 μM DCMU. This preparation emitted equal fractions of constant and variable yield fluorescence; the corresponding quenching constant is $K_Q \simeq 110\ M^{-1}$. (*c*) Untreated with tris chloroplasts, poisoned with 20 μM DCMU. About 70% of their steady-state emission is variable fluorescence; the corresponding quenching constant is $K_Q \simeq 150\ M^{-1}$.

To investigate whether nitrobenzene associates specifically with the PS II reaction centers, we employed preparations enriched in various redox species of these centers. Since they draw on a readily exhaustible supply of pre-PS II electrons, tris-washed chloroplasts can be conveniently enriched in different redox combinations of Z and Q (12). At ordinary temperature, the reaction center chromophore ($P680$) does not accumulate in the oxidized state (15). We may assume, then, that the treatments to be described affect only the couple Z, Q. By adding first ferricyanide and then DCMU to illuminated tris-washed chloroplasts, the sample is enriched in the reaction center species Z^+Q. Such samples emit relatively weak fluorescence. Conversely, when DCMU is added in the darkness and then the light is turned on, the sample is enriched

in Z^+Q^- and emits relatively strong fluorescence. In the latter instance, when an artificial electron donor to Z, such as hydroxylamine (16, 17) is included in the incubation mixture, the chloroplasts become, on illumination, enriched in the reaction center species ZQ^-.

The fluorescence time courses of tris-washed chloroplasts, incubated with hydroxylamine, DCMU, and DCMU plus hydroxylamine, are illustrated in Fig. 5. All additions were made during the dark period that precedes the recording of the kinetics. In the interest of comparison, similar curves obtained with samples, having the same chlorophyll content and initial level fluorescence but not subjected to treatment with tris, are also shown. The largest fraction of variable fluorescence, in the case of tris-washed chloroplasts, is realized when both DCMU and hydroxylamine are present. However, this fraction is smaller than that of DCMU-poisoned, but unwashed with tris, chloroplasts.

Table I lists relative magnitudes of total fluorescence (level I = 1) of chloroplast preparations enriched in various redox combinations of the PS II reaction centers. The table lists, also, the nitrobenzene requirement for 10% quenching in each particular case. Tris-washed chloroplasts enriched with ZQ^- and Z^+Q^- appear to be equally fluorescent, and they resemble normal chloroplasts also enriched in ZQ^-. Reduced Q is associated with a higher sensitivity toward the quencher, but the redox state of Z appears to be completely irrelevant in this respect. It is noteworthy, that, on the basis of the nitrobenzene requirement, normal chloroplasts are three orders of magnitude more sensitive to quenching than corresponding DCMU-poisoned preparations, although both are enriched in the species ZQ^-. The irrelevance of the oxidation state of Z, in regard to the quenching sensitivity of chloroplasts, is further substantiated in

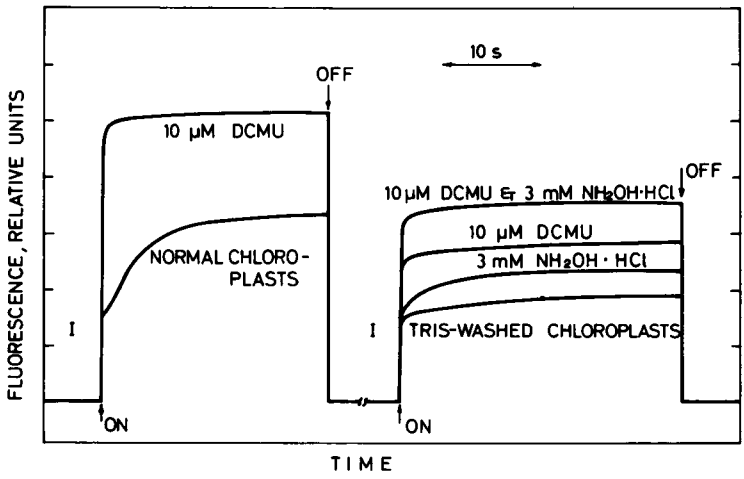

FIGURE 5 The fast rise of Chl *a* fluorescence of dark-adapted normal and tris-washed chloroplasts. The indicated additions were made during the dark incubation that preceded the light period. All preparations (normal and tris-washed) had the same chlorophyll content and the same constant yield fluorescence.

TABLE I

ENRICHED REACTION CENTER SPECIES, RELATIVE TOTAL FLUORESCENCE, AND NITROBENZENE REQUIREMENT FOR 10% QUENCHING*

Preparation and treatment	Reaction center species (PS II)	Total fluorescence	Nitrobenzene requirement for quenching
Normal chloroplasts	ZQ^-	2.21 ± 0.01	0.4 μM
Normal chloroplasts; 20 μM DCMU added	ZQ^-	3.49 ± 0.01	0.8 mM
Tris-washed chloroplasts; 2 mM $K_3Fe(CN)_6$ added	Z^+Q	1.18 ± 0.02	4.6 mM
Tris-washed chloroplasts; 10 μM DCMU added during dark adaptation	Z^+Q^-	2.28 ± 0.06	1.1 mM
Tris-washed chloroplasts; 10 μM DCMU and 3 mM $NH_2OH \cdot HCl$ added during dark adaptation	ZQ^-	2.36 ± 0.02	1.1 mM

*The reaction center species are those obtained by exposing the chloroplast preparations to continuous illumination (480 nm; 300 ergs·cm^{-2}·s^{-1}), which follows the treatments indicated in the first column. Total fluorescence in units of level I fluorescence; level I was identical and reproducible in all examined samples. Nitrobenzene concentrations are those of the suspension, not of the chloroplast microenvironment. At least three samples were tested in each particular case.

Fig. 6. This figure displays quenching curves for preparations enriched in Z^+Q^- and ZQ^-.

It has been reported that the suppression of chloroplast fluorescence by less than 100 μM *m*-dinitrobenzene, is not attended by an analogous inhibition of the photosynthetic electron transport (9, 10). Fig. 7 A presents a relevant experiment concerning the effect of nitrobenzene on the photoreduction of DCPIP by normal chloroplasts.

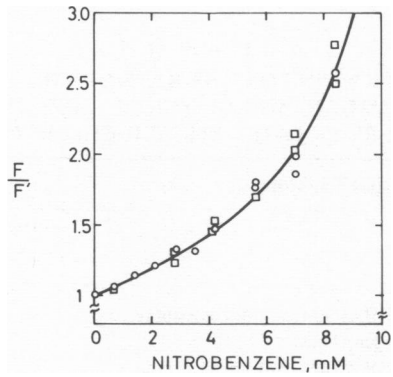

FIGURE 6 Quenching curves of total Chl *a* fluorescence of tris-washed chloroplasts enriched in the PS II reaction center species Z^+Q^- (○) and ZQ^- (□). Species Z^+Q^- was obtained by adding 10 μM DCMU to tris-washed chloroplasts during protracted dark incubation, followed by illumination of the sample. Species ZQ^- was obtained by including 3 mM $NH_2OH \cdot HCl$ in the dark incubation medium, in addition to DCMU.

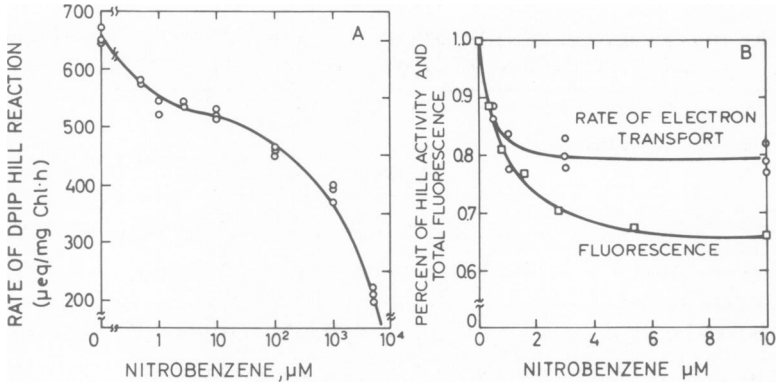

FIGURE 7 The inhibition of the DCPIP photoreduction (A), and the relative suppression of the DCPIP photoreduction and of the Chl *a* fluorescence (B) by nitrobenzene. The photoreduction of DCPIP was measured with saturating white light; the fluorescence of Chl *a* with weak blue excitation (480 nM; 300 ergs·cm^{-2}·s^{-1}; cf. Materials and Methods).

A 20% inhibition is already evident with as little as 3 μM nitrobenzene. Thereafter, and up to 100 μM, the chloroplasts are remarkably tolerant, and only at higher concentrations the photoreduction is inhibited further.

Qualitatively, the partial suppression of the DCPIP photoreduction by low concentrations of nitrobenzene is analogous to the quenching of the fluorescence of normal chloroplasts, although the light conditions were different in each case. This analogy is illustrated in Fig. 7 B (cf. also Figs. 7 A and 3 A) where the relative effects are plotted against a linear concentration scale. Both inhibitions saturate at about the same range of nitrobenzene concentration (3–10 μM).

We examined, also, the possibility whether nitrobenzene itself is a Hill oxidant, in which case it would compete with DCPIP for PS II electrons. This expectation is con-

TABLE II

EFFECT OF NITROBENZENE ON THE RATE OF THE PHOTOSYNTHETIC OXYGEN EVOLUTION, AND OF THE PS I-MEDIATED OXYGEN UPTAKE BY ISOLATED SPINACH CHLOROPLASTS*

Additions to the chloroplasts	Relative rate
None	9 ± 0.5
Nitrobenzene, 23 μM	5 ± 0.5
Nitrobenzene, 490 μM	3 ± 0.3
DCMU, 50 μM; DCPIP, 50 μM; sodium ascorbate, 2 mM; methyl viologen, 2 mM	250 ± 20
DCMU, 50 μM; DCPIP, 50 μM; sodium ascorbate, 2 mM; methyl viologen, 2 mM; nitrobenzene, 466 μM	295 ± 12
$K_3Fe(CN)_6$, 2 mM	100

*With the exception of the rate obtained with $K_3Fe(CN)_6$, all the other listed rates are averages of three independent experiments.

tradicted by Table II, which shows that nitrobenzene inhibits photosynthetic oxygen evolution both at catalytic, as well as at substrate concentrations. Table II shows, also, a stimulation of the PS I-mediated electron transport from $DCPIPH_2$ to oxygen by nitrobenzene. This result was not investigated further.

DISCUSSION

The stronger quenching of the variable fluorescence of DCMU-poisoned chloroplasts, relative to the constant yield fluorescence, can be rationalized on the basis of the longer lifetime of the former. In support of this, we find that limiting quenching constants of the total and of the constant yield fluorescence stand, approximately, in the same ratio (7; cf. Fig. 4) as the reported fluorescence life-times, measured in strong and in weak excitation (6; ref. 18). Also, contributing to the insensitivity of the constant yield fluorescence is the fact that it originates mostly from PS I chlorophylls (8, 10, 19). The very short lifetime of this fluorescence (τ = 50 ps; ref. 20) should make it highly resistant to quenching.

The extreme sensitivity of normal chloroplast fluorescence toward nitrobenzene is reduced sharply on poisoning with DCMU (Figs. 3 A and 3 B). According to Table I, 20 μM DCMU raises the nitrobenzene requirement for 10% quenching nearly 2,000 times. Since DCMU is an electron transport inhibitor, this effect suggests that nitrobenzene may quench by interacting with the intersystem electron transport chain, in addition to the direct quenching interactions with the excited chlorophylls. This notion must be reconciled with the inability of nitrobenzene to support photosynthetic oxygen evolution (Table II). If nitrobenzene could catalyze, however, a back flow from post-PS II intermediates to pre-PS II ones, or to free oxygen, this process would quench the variable fluorescence by oxidizing Q, and it would delay the photoreduction of DCPIP (Fig. 7 B) by providing an alternate path for PS II electrons.

Whether a nitrobenzene oxidoreduction couple can operate at a potential that is compatible with the redox potential range of the intersystem electron transport remains an open question. The only evidence, in this respect, comes from the irreversible polarographic reduction of nitrobenzene to phenyl-hydroxylamine (21). The half-wave potential of this four-electron process is −530 mV. It is conceivable, however, that a reversible one-electron reduction of nitrobenzene could take place at a more positive potential, in which case it could mediate electron transport from the intersystem reductants to pre-PS II oxidants.

The incomplete inhibition of the DCPIP photoreduction and of the variable fluorescence (Fig. 7 B) suggests that the nitrobenzene-mediated back flow of electrons is an inefficient process compared with the ordinary photosynthetic electron transport. These inhibitions saturate at very low concentrations (ca. 3–10 μM; Figs. 3 B and 7 B); beyond that the chloroplasts are remarkably tolerant and only when a threshold of about 100 μM nitrobenzene is exceeded further inhibition can be observed. We consider the latter effect to arise from direct nitrobenzene-chlorophyll quenching interactions; because of them, less excitation is made available to the reaction centers.

The concentration curves of Fig. 3 A and 7 A emphasize the different nitrobenzene requirements of the two quenching processes. It is interesting to compare these data with those reported for *m*-dinitrobenzene (9, 10). This quencher has no effect on the fluorescence and the photochemical activities of chloroplasts and algae below about 100 μM; above that concentration, both types of phenomena are inhibited. The two nitroaromatics differ not only in their affinity for electrons (at pH 6.4, a four-electron reduction of *m*-dinitrobenzene occurs at -370 mV; ref. 21) but also in their solubility properties. Perhaps, nitrobenzene, as the more water soluble of the two, can penetrate to the neighborhood of electron transport intermediates, while *m*-dinitrobenzene cannot. On the other hand, its higher solubility in lipid phases would make *m*-dinitrobenzene a more drastic quencher of Chl *a* in vivo than nitrobenzene. Consistent with this notion are the apparent quenching constants of the fluoresence of DCMU-poisoned chloroplasts, for nitrobenzene and *m*-dinitrobenzene (4); these are 116 M^{-1} and 3,100 M^{-1}, respectively.

According to Table I (see also Fig. 6), tris-washed preparations enriched in the species Z^+Q, ZQ^-, and Z^+Q^- are about equally sensitive to quenching. This proves that nitrobenzene does not discriminate between different oxidation states of the PS II reaction center complex. Furthermore, untreated with tris chloroplasts have very different nitrobenzene requirements for 10% quenching, depending on whether they are poisoned, or not, with DCMU, although the enriched species is the same (ZQ^-). It is, therefore, unlikely that preferential attachment of nitrobenzene to the reaction center could be the cause of the extreme quenching sensitivity of normal chloroplasts. It should be noted, also, that according to Table I preparations enriched in Z^+Q^- and ZQ^- are equally fluorescent. This suggests that, in contrast to oxidized *P*680 (15), oxidized *Z* is not an intrinsic quencher of Chl *a* fluorescence.

The results of Table I allow for an analysis for the site of DCMU inhibition. Inhibition on the reducing side of *Q* has been supported by Duysens and Sweers (22) and by Butler (23), on the oxidizing side of *Z* by Rosenberg et al. (24), while Döring et al. (25) advocated an effect directly on *P*680. Illumination of dark-adapted and DCMU-poisoned preparations of tris-washed chloroplasts can effect an enrichment in reaction center species with reduced $Q(Z^+P680\ Q^-)$ only when the DCMU inhibition site is after *Q*. In all other possible cases, i.e. when the inhibition site is between *P*680 and *Q*, between *Z* and *P*680, and before *Z*, the enriched species will be, respectively, $Z\ P680\ Q$, $Z\ P680^+Q$, and Z^+P680^+Q. Actually, as shown in Table I, this particular treatment generates a significant amount of variable fluorescence (ca. 128%). Assuming that the variable fluorescence manifests a reduced *Q* (as well as a reduced *P*680; ref. 15), the enriched species is $Z^+P680\ Q^-$, i.e. DCMU inhibits after *Q*. Very recently, Etienne (26) obtained evidence for an inhibition site on the oxidizing site of the PS II reaction center. This inhibition manifests at unusually high concentrations of DCMU (500 μM).

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